



# Utilisation de traceurs isotopiques stables pour la spéciation du mercure et des butylétains application à l'étude de la bio méthylation du mercure dans les environnements aquatiques

Mathilde Monperrus

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## **THESE**

présentée à

**I'Université de Pau et des Pays de l'Adour**  
Ecole Doctorale des Sciences Exactes et de leurs Applications

par

**Mathilde MONPERRUS**

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**DOCTEUR**

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**UTILISATION DE TRACEURS ISOTOPIQUES STABLES POUR LA  
SPECIATION DU MERCURE ET DES BUTYLETAINS APPLICATION  
A L'ETUDE DE LA BIOMETHYLATION DU MERCURE DANS LES  
ENVIRONNEMENTS AQUATIQUES**

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**Soutenue le 14 décembre 2004**

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## SOMMAIRE

|                                                                                                                                    |                  |
|------------------------------------------------------------------------------------------------------------------------------------|------------------|
| <b>Chapitre A – PROBLEMATIQUES SCIENTIFIQUES .....</b>                                                                             | <b>11</b>        |
| <b><u>A-I- Problématiques du mercure et des organo-étains dans les environnements côtiers .....</u></b>                            | <b><u>13</u></b> |
| <b><u>A-II- Biogéochimie du mercure et des butylétains .....</u></b>                                                               | <b><u>16</u></b> |
| <b>A-II-1- Cycle biogéochimique du mercure dans les environnements aquatiques .....</b>                                            | <b>16</b>        |
| <u>A-II-1-1- Sources et spéciation du mercure dans l'environnement .....</u>                                                       | <u>16</u>        |
| <u>A-II-1-2- Le méthylmercure et sa toxicité .....</u>                                                                             | <u>17</u>        |
| <u>A-II-1-3- Le devenir du MMHg dans les environnements côtiers .....</u>                                                          | <u>18</u>        |
| <b>A-II-2- Cycle biogéochimique des butyl-étains .....</b>                                                                         | <b>19</b>        |
| <u>A-II-2-1- Sources et spéciation des butyl-étains .....</u>                                                                      | <u>19</u>        |
| <u>A-II-2-2- Les butyl-étains et leur toxicité .....</u>                                                                           | <u>20</u>        |
| <u>A-II-2-3- Le devenir du TBT dans les environnements côtiers .....</u>                                                           | <u>21</u>        |
| <b><u>A-III- Processus contrôlant les concentrations et le devenir du MMHg et du TBT dans les environnements côtiers .....</u></b> | <b><u>23</u></b> |
| <b>A-III-1- Transformations dans les environnements aquatiques .....</b>                                                           | <b>23</b>        |
| <u>A-III-1-1- Cas du MMHg .....</u>                                                                                                | <u>24</u>        |
| A-III-1-1-1- Méthylation .....                                                                                                     | 25               |
| A-III-1-1-2- Déméthylation .....                                                                                                   | 29               |
| A-III-1-1-3- Réduction .....                                                                                                       | 29               |
| <u>A-III-1-2- Cas du TBT .....</u>                                                                                                 | <u>31</u>        |
| A-III-1-2-1- Debutylation .....                                                                                                    | 31               |
| A-III-1-2-2- Méthylation .....                                                                                                     | 31               |
| <b>A-III-2- Transferts aux interfaces des environnements aquatiques .....</b>                                                      | <b>32</b>        |
| <u>A-III-2-1- Cas du MMHg .....</u>                                                                                                | <u>33</u>        |
| A-III-2-1-1- Transferts eau/atmosphère .....                                                                                       | 34               |
| A-III-2-1-2- Transferts eau/sédiment .....                                                                                         | 34               |
| A-III-2-1-3- Bioaccumulation .....                                                                                                 | 35               |
| <u>A-III-2-2- Cas du TBT .....</u>                                                                                                 | <u>36</u>        |
| A-III-2-2-1- Transferts eau/atmosphère .....                                                                                       | 36               |
| A-III-2-2-2- Transferts eau/sédiment .....                                                                                         | 37               |
| A-III-2-2-3- Bioaccumulation .....                                                                                                 | 37               |
| <b><u>A-IV- Spéciation chimique et isotopes stables .....</u></b>                                                                  | <b><u>39</u></b> |
| <b>A-IV-1- Les défis analytiques pour la spéciation du mercure et des organo-étains .....</b>                                      | <b>39</b>        |
| <u>A-IV-1-1- Les techniques analytiques classiques .....</u>                                                                       | <u>40</u>        |
| <u>A-IV-1-2- La dilution isotopique .....</u>                                                                                      | <u>40</u>        |
| A-IV-1-2-1- Principe .....                                                                                                         | 40               |
| A-IV-1-2-2- Avantages .....                                                                                                        | 42               |
| A-IV-1-2-3- Instrumentation .....                                                                                                  | 43               |
| <u>A-IV-1-3- Applications aux analyses de spéciation .....</u>                                                                     | <u>43</u>        |



|                                                                                                                                                                                                                                                                                       |                                |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------|
| A-IV-1-3-1- Les étapes critiques de la dilution isotopique .....                                                                                                                                                                                                                      | 43                             |
| A-IV-1-3-2- Les applications de la dilution isotopique .....                                                                                                                                                                                                                          | 47                             |
| A-IV-1-3-3- Le suivi des transformations pendant l'analyse .....                                                                                                                                                                                                                      | 47                             |
| <b>A-IV-2- Utilisation des isotopes stables comme traceurs des processus environnementaux .....</b>                                                                                                                                                                                   | <b>48</b>                      |
| A-IV-2-1- Principe et mise en œuvre .....                                                                                                                                                                                                                                             | 49                             |
| A-IV-2-2- Applications environnementales .....                                                                                                                                                                                                                                        | 49                             |
| <b>A-V- Présentation du travail .....</b>                                                                                                                                                                                                                                             | <b>53</b>                      |
| <b>A-V-1- Développement de techniques analytiques pour la spéciation du mercure et des butyl-étains</b><br>.....                                                                                                                                                                      | <b>53</b>                      |
| <b>A-V-2- Transferts et transformations du mercure et des butyl-étains dans les environnements</b><br>côtiers.....                                                                                                                                                                    | <b>54</b>                      |
| <b>REFERENCES BIBLIOGRAPHIQUES.....</b>                                                                                                                                                                                                                                               | <b>57</b>                      |
| <br><b>Chapitre B - DEVELOPPEMENT DE TECHNIQUES ANALYTIQUES POUR LA SPECIATION DU MERCURE</b><br><b>ET DES BUTYL-ETAINS .....</b>                                                                                                                                                     | <br><b>71</b>                  |
| <u>B-I- Analyse simultanée du MMHg et du TBT par dilution isotopique pour un échantillon certifié de</u><br><u>tissu d'huître. ....</u>                                                                                                                                               | <u><a href="#">71</a></u>      |
| <u>B-II- Analyse simultanée des espèces de mercure et butyl-étains par dilution isotopique pour des</u><br><u>échantillons naturels d'eau et de neige. ....</u>                                                                                                                       | <u><a href="#">81</a></u>      |
| <u>B-III- Détermination des espèces de mercure et de la formation accidentelle de MMHg par dilution</u><br><u>isotopique pour des sédiments certifiés. ....</u>                                                                                                                       | <u><a href="#">103</a></u>     |
| <br><b>Chapitre C - TRANSFERTS ET TRANSFORMATIONS DANS LES ENVIRONNEMENTS AQUATIQUES.....</b>                                                                                                                                                                                         | <br><b><a href="#">115</a></b> |
| <u>C-I- Détermination de la bioaccumulation des métaux et organo-métaux par les groupes trophiques de</u><br><u>la macrofaune benthique de l'Estuaire de l'Adour (Golfe de Gascogne).....</u>                                                                                         | <u><a href="#">115</a></u>     |
| <u>C-II- Biogéochimie du mercure à l'interface eau-sédiment de l'étang de Thau – Mesures des potentiels</u><br><u>de méthylation du mercure dans les sédiments et la colonne d'eau et devenir du méthylmercure ....</u>                                                               | <u><a href="#">139</a></u>     |
| <u>C-III- Méthylation, déméthylation et réduction du mercure dans des eaux de surface côtières et</u><br><u>marines de la Méditerranée.....</u>                                                                                                                                       | <u><a href="#">167</a></u>     |
| <u>C-IV- Etude de la méthylation du mercure et de la séquestration par des souches spécifiques de</u><br><u>bactéries sulfato-réductrices isolées d'un sédiment estuarien en utilisant les traceurs isotopiques</u><br><u>stables (<sup>199</sup>Hg(II) <sup>201</sup>MeHg) .....</u> | <u><a href="#">195</a></u>     |

|                                                                                                                                                                                                       |                     |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------|
| Chapitre D- SYNTHESE GENERALE.....                                                                                                                                                                    | <a href="#">219</a> |
| Chapitre E- ANNEXES.....                                                                                                                                                                              | <a href="#">227</a> |
| <u>E-I- Revue sur les potentiels et limites de l'analyse de spéciation par dilution isotopique pour la métrologie et l'évaluation de la réactivité environnementale .....</u>                         | <a href="#">227</a> |
| <u>E-II- Détermination du TBT dans des sédiments et tissus biologiques par l'analyse de spéciation en dilution isotopique, extraction micro-ondes et chromatographie en phase gazeuse-ICPMS. ....</u> | <a href="#">241</a> |
| <u>E-III- Détermination du TBT dans un sédiment marin par dilution isotopique – Exercice d'intercomparaison du Comité Consultatif pour la Quantité de matière (CCQM) .....</u>                        | <a href="#">251</a> |
| <u>E-IV- Flux benthiques de métaux (Cu, Cd, Pb, Mn, U, iHg) et organométaux (MMHg, TBT, DBT, MBT) dans l'étang de Thau – Rôle de l'activité biologique. ....</u>                                      | <a href="#">261</a> |



### Liste des figures

|                                                                                                                                             |                    |
|---------------------------------------------------------------------------------------------------------------------------------------------|--------------------|
| Figure 1 : Cycle biogéochimique du mercure ( <a href="http://www.ec.gc.ca/">http://www.ec.gc.ca/</a> ).....                                 | <a href="#">18</a> |
| Figure 2 : Gammes de concentrations en mercure pour les différents compartiments des environnements aquatiques.....                         | 19                 |
| Figure 3 : Cycle biogéochimique des butylétains (d'après Hoch 2001) .....                                                                   | 21                 |
| Figure 4 : Gammes de concentrations en TBT pour les différents compartiments des environnements aquatiques (d'après Veidenhaupt 1997) ..... | 22                 |
| Figure 5 : Mécanismes de formation et de dégradation des composés organométalliques. ....                                                   | 23                 |
| Figure 6 : Mécanismes majeurs de la méthylation/déméthylation du mercure. ....                                                              | 24                 |
| Figure 7 : Mécanismes de réduction du mercure par les bactéries (d'après Hobman et al. 2000).....                                           | 30                 |
| Figure 8 : Transferts aux interfaces des environnements aquatiques .....                                                                    | 33                 |
| Figure 9 : Les transferts et les transformations des formes chimiques du mercure dans l'environnement (d'après Stein et al. 1996). ....     | 33                 |
| Figure 10 : Principe de la dilution isotopique (cas du mercure). ....                                                                       | 42                 |
| Figure 11: Etapes critiques de la dilution isotopique pour les analyses de spéciation.....                                                  | 44                 |
| Figure 12 : Principe de l'utilisation des traceurs isotopiques stables pour la méthylation/déméthylation du mercure.....                    | 50                 |

**Liste des tableaux**

|                                                                                                                                        |    |
|----------------------------------------------------------------------------------------------------------------------------------------|----|
| Tableau 1 : Comparaison des potentiels de méthylation et de déméthylation du mercure dans les sédiments. .                             | 25 |
| Tableau 2 : Bactéries sulfato-réductrices méthylant le mercure et taux de méthylation. ....                                            | 27 |
| Tableau 3 : Techniques analytiques pour la spéciation du mercure et des organoétains dans différentes matrices environnementales. .... | 41 |
| Tableau 4 : Applications de la dilution isotopique aux analyses de spéciation. ....                                                    | 46 |
| Tableau 5 : Etudes sur les transformations des composés du mercure par l'utilisation de traceurs isotopiques.                          | 51 |

### Liste des articles

#### Chapitre B - DEVELOPPEMENT DE TECHNIQUES ANALYTIQUES POUR LA SPECIATION DU MERCURE ET DES BUTYETAINS

- B-I- Simultaneous sample preparation and species specific isotope dilution mass spectrometry analysis of monomethylmercury and tributyltin in a certified oyster tissue.  
M. Monperrus, R.C. Rodriguez, J. Scancar, D. Amouroux, O.F.X. Donard  
*Analytical Chemistry*, 75, 4095, 2003.....71
- B-II- Simultaneous speciation of mercury and butyltin compounds in natural waters and snow by Propylation and Species Specific Isotope Dilution Mass Spectrometry Analysis.  
M. Monperrus, E. Tessier, S. Veschambre, D. Amouroux, O.F.X. Donard  
*Soumis à Analytical Bioanalytical Chemistry*, 2004.....81
- B-III- Using speciated isotope dilution with GC-ICPMS to determine and unravel the artificial formation of monomethylmercury in certified reference sediments.  
 R.C. Rodriguez, M. Monperrus, E. Krupp, D. Amouroux, O.F.X. Donard  
*Analytical Chemistry*, 75, 3202, 2003.....103

#### Chapitre C - TRANSFERTS ET TRANSFORMATIONS DANS LES ENVIRONNEMENTS AQUATIQUES

- C-I- Determination of metal and organometal trophic bioaccumulation in the benthic macrofauna of the Adour estuary coastal zone (SW France, Bay of Biscay).  
M. Monperrus, D. Point, J. Grall, L. Chauvaud, D. Amouroux, G. Bareille, O.F.X. Donard  
*Soumis à Journal of Environmental Monitoring*, 2004.....115
- C-II- Biogeochemistry of mercury at the sediment water interface in the Thau lagoon. Measurements of mercury methylation potential in sediment and water and fate of methylmercury.  
M. Monperrus, E. Tessier, D. Point, K. Vidimova, D. Amouroux, R. Guyoneaud, A. Leynaert, J. Grall, L. Chauvaud, G. Thouzeau, O.F.X. Donard  
*Soumis à Environmental Coastal and Shelf Science*, 2004.....139
- C-III- Mercury methylation, demethylation and reduction in coastal and marine surface waters of the Mediterranean Sea.  
M. Monperrus, E. Tessier, D. Amouroux, A. Leynaert, O.F.X. Donard  
*Soumis à Environmental Science and Technology*, 2004.....167
- C-IV- Methylation and sequestration of labeled mercury species ( $^{199}\text{Hg}(\text{II})$ ,  $\text{Me}^{201}\text{Hg}$ ) by specific sulfate reducing bacteria strains isolated from an estuarine sediment.  
M. Monperrus, M. Dias, R. Guyoneaud, D. Amouroux, J.C. Salvado, M. Goni, R. Duran, P. Caumette, O.F.X. Donard  
*Soumis à Environmental Science and Technology*, 2004.....195

Chapitre E- ANNEXES

E-I- Potentials and limits of speciated isotope dilution analysis for metrology and assessing environmental reactivity.

M. Monperrus, E. Krupp, D. Amouroux, O.F.X. Donard, R.C. Rodriguez

*Trends in Analytical Chemistry*, 23, 261, 2003.....229

E-II- Rapid, accurate and precise determination of Tributyltin in Sediments and Biological Samples by Species Specific Isotope Dilution – Microwave Extraction- Gas Chromatography - ICP Mass Spectrometry.

M. Monperrus, O. Zuloaga, E. Krupp, D. Amouroux, R. Wahlen, B. Fairman, O.F.X. Donard

*Journal of Analytical Atomic Spectrometry*, 18, 247, 2003.....243

E-III- Determination of tributyltin in marine sediment : Comité Consultatif pour la Quantité de Matière (CCQM) pilot study P-18 international comparison.

R.E. Sturgeon, R. Walhen, T. Brandsch, B. Fairman, C. Wolf-Briche, J.I. Garcia Alonso, P. Rodriguez, J. Ruiz, A. Sanz-Medel, K. Inagaki, A. Takatsu, B. Lalère, M. Monperrus, O. Zuloaga, E. Krupp, D. Amouroux, O.F.X. Donard, H. Schimmel, B. Olsen, P. Konieczka, P. Schultze, P. Taylor, R. Hearn, L. Mackay, R. Myers, T. Win, A. Liebich, R. Philipp, L. Yang, S. Willie

*Analytical Bioanalytical Chemistry*, 376, 780, 2003.....253

E-IV- Measurement of metals and organometals fluxes at the sediment water interface in the Thau Lagoon using benthic chambers.

D. Point, M. Monperrus, E. Tessier, D. Amouroux, L. Chauvaud, G. Thouzeau, F. Jean, J. Grall, A. Leynaert, J. Clavier, O.F.X. Donard

*Soumis à Environmental Coastal and Shelf Science*, 2004.....263

**Chapitre A**  
**Problématiques scientifiques**





## Chapitre A – PROBLEMATIQUES SCIENTIFIQUES

### A-I- Problématiques du mercure et des organo-étains dans les environnements côtiers

Le mercure (Hg) et l'étain (Sn) sont des éléments chimiques appartenant à la famille des métaux lourds qui sont reconnus comme les polluants métalliques les plus dangereux pour l'environnement du fait de leur toxicité et de leur impact écologique sur le milieu aquatique. Les métaux sont présents dans tous les compartiments de l'environnement, mais en général en quantités très faibles. On dit que les métaux sont présents à l'état de traces. Ces éléments chimiques sont présents dans l'environnement sous différentes formes chimiques, chacune avec un comportement biogéochimique spécifique et une toxicité différente. Dans ce contexte, l'analyse consistant à déterminer la concentration totale de l'élément n'est plus suffisante afin de comprendre quels sont les effets de ces métaux dans l'environnement. L'analyse de spéciation, c'est à dire l'analyse des différentes formes chimiques d'un élément, permet de savoir quelles sont les formes de cet élément qui peuvent générer l'impact le plus préjudiciable pour l'environnement et, par conséquent pour la santé.

Les analyses de spéciation sont à l'heure actuelle de plus en plus utilisées et deviennent même des analyses de routine pour certains composés. Néanmoins, leurs résultats restent limités à la connaissance de la répartition des différentes espèces chimiques dans les différentes matrices environnementales et ne donnent pas accès à la réactivité et la mobilité de ces espèces chimiques dans les écosystèmes naturels. Le développement d'outils analytiques et expérimentaux est donc nécessaire afin de déterminer la spéciation biogéochimique, qui comprend en plus de la spéciation chimique, la réactivité et la mobilité de chaque espèce, et de ce fait permet d'appréhender le réel impact de ces polluants sur l'environnement et sur la santé.

Les composés du mercure et de l'étain jouent un rôle important au niveau de la pollution des écosystèmes aquatiques et notamment des zones côtières. Les écosystèmes côtiers présentent des intérêts importants en termes écologiques et socio-économiques. Ils sont le lieu privilégié pour le développement et la reproduction de nombreuses espèces animales ou végétales. Ils représentent aussi des enjeux économiques importants du fait des activités ostréicoles, touristiques, industrielles et portuaires qui leur sont associées. Les zones côtières sont très sensibles et sont néanmoins soumises à de nombreuses pressions anthropiques pouvant représenter un risque écologique fort. La connaissance des apports en mercure et butylétains est donc nécessaire ainsi que leur comportement et leur impact dans l'écosystème.

Les composés du mercure et les butylétains peuvent soit être introduits dans l'environnement par des sources anthropiques soit être naturellement présents puis soumis à des transformations dans l'environnement. Par exemple le tributylétain (TBT) a été très largement utilisé depuis les années 1970 dans les peintures antisalissures des bateaux et cause de graves dommages sur les organismes marins en anéantissant leur

système de reproduction (Imposex). Dans le cas du mercure, c'est la catastrophe de Minamata dans les années 1960, qui a déclenché la prise de conscience sur l'importance du cycle biogéochimique du mercure. Plus de 150 personnes sont décédées et plus de 1000 se sont retrouvées handicapées à vie, principalement par la consommation de poissons contaminés par du monométhylmercure (MMHg).

En plus de leur caractère persistant et ubiquiste, le mercure et les butyl-étains présentent la spécificité d'être particulièrement disponibles aux réactions chimiques et biologiques ayant lieu dans l'environnement. Ces composés métalliques peuvent être transformés en composés plus ou moins toxiques ou inertes sous l'effet de modifications des conditions physico-chimiques (pH, conditions rédox, force ionique, ...), de l'activité microbienne ou du fonctionnement écologique des organismes benthiques. Dans certaines conditions, le mercure inorganique (IHg) peut par exemple être transformé par une réaction de méthylation en un composé beaucoup plus toxique le MMHg. Cette forme du mercure est fortement bioaccumulable par les organismes et sa bioamplification le long de la chaîne trophique peut engendrer de graves conséquences pour le consommateur. Concernant le TBT, deux réactions majeures ont été observées dans l'environnement : la débutylation et la méthylation. Les facteurs contrôlant ces réactions d'alkylation/de-alkylation peuvent être soit abiotiques, ce sont alors des réactions chimiques ou photochimiques, soit biotiques par des réactions enzymatiques ou non enzymatiques régulées par l'activité d'organismes biologiques tels que les bactéries ou le phytoplancton.

Les transferts et transformations dans les différents compartiments d'un écosystème aquatique jouent donc un rôle important en terme de biodisponibilité et de toxicité des formes chimiques de ces éléments. Le cycle biogéochimique du mercure et des butylétains dépend des conditions physico-chimiques du milieu, des formes chimiques sous lesquelles ils s'introduisent, ainsi que de l'activité biologique. En effet, les cinétiques des réactions de transfert et de transformations dépendent non seulement des concentrations environnementales mais aussi de la disponibilité (ou labilité) des composés. La spéciation biogéochimique des différentes formes présentes dans l'environnement et leur réactivité est donc indispensable afin d'appréhender correctement le devenir de ces polluants et d'évaluer le risque éco-toxicologique associé. De plus, l'amplitude des mécanismes biotiques et abiotiques dépend d'un ensemble de paramètres environnementaux difficilement reproductibles lors d'expériences en laboratoire d'où la nécessité d'expériences in situ afin de se placer dans des conditions naturelles. Le couplage de techniques analytiques de pointe pour les analyses de spéciation et les méthodes expérimentales avec une caractérisation des paramètres environnementaux, aussi bien physico-chimiques que biologiques, est donc nécessaire afin de mieux caractériser ces processus.

Dans ce travail, deux parties majeures sont exposées. La première porte sur le développement de nouvelles techniques analytiques de spéciation pour les composés du mercure et des butylétains basées sur la quantification par dilution isotopique. Les techniques développées sont axées principalement sur la détermination simultanée des composés de mercure et des butylétains ce qui comprend la préparation d'échantillon simultanée ainsi que l'analyse simultanée. Le développement porte sur les différentes matrices composant les environnements aquatiques : l'eau, les tissus biologiques et les sédiments. Dans la deuxième partie de ce travail, les méthodes analytiques développées ont été appliquées à la recherche environnementale sur les cycles biogéochimiques du mercure et des butylétains dans les environnements côtiers. Par ailleurs, des méthodes expérimentales en utilisant des espèces enrichies isotopiquement comme traceurs, permettent d'évaluer les transformations du mercure (méthylation/déméthylation) dans les différents compartiments des environnements côtiers que sont les sédiments et la colonne d'eau. Les facteurs environnementaux et notamment les paramètres biologiques sont aussi contrôlés afin de déterminer le lien entre les transformations du mercure et les activités des organismes tels que les bactéries et le phytoplancton.

Ce travail a été effectué dans le cadre des programmes GIS-ECOBAG (Groupement d'intérêt Scientifique- Environnement ECOlogie et ECONomie du Bassin Adour-Garonne), PNEC (Programme National Environnements Côtiers) et ELOISE (European Land Ocean Interaction Studies). Dans le cadre du GIS-ECOBAG, le projet « Biogéochimie des contaminants métalliques à l'interface eau-sédiment dans les environnements côtiers (estuaire de l'Adour) » a été financé par le Ministère de l'environnement, la DIREN aquitaine, l'Agence de l'eau Adour-Garonne et la région aquitaine. Le but de ce projet est d'aborder de manière transdisciplinaire (géochimie-chimie-microbiologie-biologie) une partie importante du cycle biogéochimique des contaminants métalliques dans les environnements côtiers. Son objectif est d'étudier le transfert des contaminants métalliques entre la colonne d'eau, les sédiments et les organismes vivants dans l'estuaire de l'Adour et plus particulièrement dans cette thèse, leur bioaccumulation par les organismes benthiques. Le projet MICROBENT rentre dans le Cadre du Programme National de l'Environnement Côtier (PNEC, Ifremer) et plus particulièrement dans le chantier « Lagunes Méditerranéennes » financé par le Ministère de l'Environnement et l'Ifremer. L'objectif principal du volet « contaminants » est d'évaluer le rôle des sédiments en tant que source ou puit de contaminants métalliques et organiques à l'échelle de l'étang de Thau. Il requiert aussi la détermination des processus clefs dans la colonne d'eau et les sédiments qui contribuent à la dynamique et à la biodisponibilité de ces contaminants. Enfin dans le programme ELOISE, le projet MERCYMS, financé par la Communauté Européenne, a pour objectif de modéliser le cycle biogéochimique du mercure en Méditerranée. La contribution de cette thèse dans ce projet consiste principalement au développement de techniques analytiques pour la spéciation du mercure à très faibles concentrations et la détermination des potentiels de méthylation/déméthylation du mercure dans la colonne d'eau et les sédiments.

## **A-II- Biogéochimie du mercure et des butylétains**

### **A-II-1- Cycle biogéochimique du mercure dans les environnements aquatiques**

#### **A-II-1-1- Sources et spéciation du mercure dans l'environnement**

Le mercure est omniprésent dans le milieu naturel et il est donc nécessaire de considérer les sources de mercure dans tous les compartiments environnementaux. La majorité du mercure présent dans l'environnement est sous forme de vapeurs de mercure élémentaire ( $\text{Hg}^0$ ). Il est émis dans l'atmosphère soit par des sources naturelles soit par des sources anthropiques. Les activités volcaniques sont les sources majeures de mercure naturel avec une production annuelle estimée à  $3 \cdot 10^4$  kg pour les éruptions passives et  $8 \cdot 10^5$  kg pour les émissions actives, les autres sources géothermales représentant  $6 \cdot 10^4$  kg ([Varekamp and Buseck, 1981 et 1986](#)).

Cependant la majeure partie du mercure a été introduite de façon anthropique dans l'environnement. Ces émissions anthropiques ont fortement perturbé le cycle naturel du mercure depuis l'avènement de l'ère industrielle. Les émissions et les concentrations sont en moyennes trois fois supérieures à celles de l'époque préindustrielle ([Mason et al. 1994](#)). A l'heure actuelle, les mesures de réglementation sur l'utilisation et les émissions directes du mercure ont permis de réduire les sources. La principale source actuelle est la réémission du mercure provenant des différents compartiments de la surface du globe qui sont en fait des sources secondaires recyclant le mercure principalement anthropique introduit via l'atmosphère. L'émission de mercure élémentaire depuis les océans vers l'atmosphère est estimée à  $8 \cdot 10^5$  kg par an et la volatilisation depuis les continents représente  $10^6$  kg par an ([Lamborg et al. 2002](#)).

Les mesures de réglementation ont permis de réduire les utilisations du mercure qui sont en déclin avec une production annuelle mondiale de mercure de l'ordre de 3.000 tonnes ([U.S. EPA 1997](#)). En ce qui concerne les composés organomercuriels, leurs utilisations comme insecticide et fongicide ont été abandonnées dans les pays industrialisés. 80% du mercure anthropique, est rejeté dans l'atmosphère sur forme élémentaire via la combustion du charbon et du bois, l'incinération des ordures ménagères, les exploitations minières et fonderies ([Pacyna 2002 et 2003](#)). 15% des émissions anthropiques du mercure sont rejetées dans les sols par l'épandage de boues de station d'épuration et de composts. Enfin, 5% des émissions anthropiques sont directement rejetés dans les rivières et estuaires par des rejets industriels. ([Stein et al. 1996](#))

D'un point de vue physico-chimique, le mercure est un métal qui change facilement d'état physique et de propriétés. Très volatil, il passe aisément de l'état liquide à l'état gazeux à température ambiante. Le mercure

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s'oxyde très facilement passant de l'état métallique ( $\text{Hg}^0$ ), liquide ou gazeux, à l'état ionisé ( $\text{Hg}^{2+}$ ). Dans l'environnement, plusieurs composés du mercure inorganique sont naturellement présents. Le mercure inorganique peut en effet former des composés soit avec des ligands inorganiques, tels que les chlorures, les sulfures ou les hydroxydes, soit avec des ligands organiques tels que les acides humiques ou fulviques (Stumm et Morgan 1996, Meili 1997). Les formes les plus communément rencontrées dans l'environnement côtiers sont les sulfures et chlorures de mercure. La présence de formes complexées avec la matière organique joue aussi un rôle important mais leur détection souffre encore de lacunes analytiques.

En présence de composés organiques naturels spécifiques ou de microorganismes, le mercure peut former facilement des liaisons covalentes avec le carbone, principalement sous forme de groupements alkyles. Les formes les plus courantes sont le méthylmercure (MMHg) et le diméthylmercure (DMHg) volatil qui sont les formes les plus toxiques et les plus bioaccumulables du mercure pour les organismes aquatiques.

#### A-II-1-2- Le méthylmercure et sa toxicité

Le méthylmercure a une grande solubilité dans les lipides et les solvants, mais surtout une grande affinité pour les groupes thiols ( $-\text{SH}$ ), particulièrement abondant dans les protéines (cystéine). Le méthylmercure est un neurotoxique très actif qui peut passer dans le sang et à travers la membrane des cellules. Ces propriétés lui confèrent une grande stabilité avec un temps de demie vie long : 70 jours dans le sang, et 270 jours dans le cerveau (Picot et al 1998). Les cibles principales du MMHg sont le cerveau où il attaque le système nerveux en inhibant la synthèse de protéines et d'ARN. Il génère aussi des effets tératogènes sur l'embryon pouvant provoquer des malformations. Les effets toxiques ne sont pas aussi importants pour les formes inorganiques mais peuvent aussi endommager les reins et le système nerveux. La particularité des organométalliques et notamment le MMHg est qu'ils sont toxiques même à très faibles concentrations avec des doses létales pour le MMHg généralement comprises entre 10 et 100  $\mu\text{g.L}^{-1}$  pour des organismes aquatiques (Craig et al. 1986).

En plus de sa toxicité directe, les formes organomercurielles sont fortement bio-accumulables et faiblement dégradables. C'est pourquoi elles sont capables de se bioamplifier dans la chaîne trophique. Le MMHg peut ainsi s'accumuler dans la chaîne alimentaire générant des problèmes importants pour la santé à la fois des organismes aquatiques mais aussi des populations humaines exposées.

### A-II-1-3- Le devenir du MMHg dans les environnements côtiers

Le mercure est naturellement présent dans tous les compartiments environnementaux et son devenir dans les environnements aquatiques est dépendant des échanges entre les différents compartiments et des transformations que peuvent subir les différentes espèces (Figure 1).

Le cycle biogéochimique du mercure est tout d'abord dominé par le transport atmosphérique et les échanges entre l'atmosphère et la surface des continents et des océans. Dans les eaux de surface, le mercure inorganique ( $\text{IHg}$ ) peut être réduit en mercure métallique ( $\text{Hg}^0$ ) et ensuite volatilisé pour être transféré vers l'atmosphère. Dans l'atmosphère, le mercure gazeux va lui-même subir des réactions d'oxydo-réduction à la fois en phase gazeuse et en phase aqueuse. Il peut aussi être transféré dans les environnements côtiers par les retombées humides ou sèches. Une fois introduit dans les environnements aquatiques, les diverses formes du mercure sont susceptibles d'évoluer. L'une des principales particularités du mercure est de subir, dans les sédiments, l'eau et les organismes biologiques, des réactions de méthylation / déméthylation et de réduction / oxydation. Dans les sédiments par exemple, le mercure inorganique peut être transformé en méthylmercure selon divers mécanismes et être ensuite transféré vers la colonne d'eau. Il peut être ensuite bioaccumulé dans les réseaux trophiques aquatiques, le MMHg étant la forme du mercure capable de se bioamplifier.

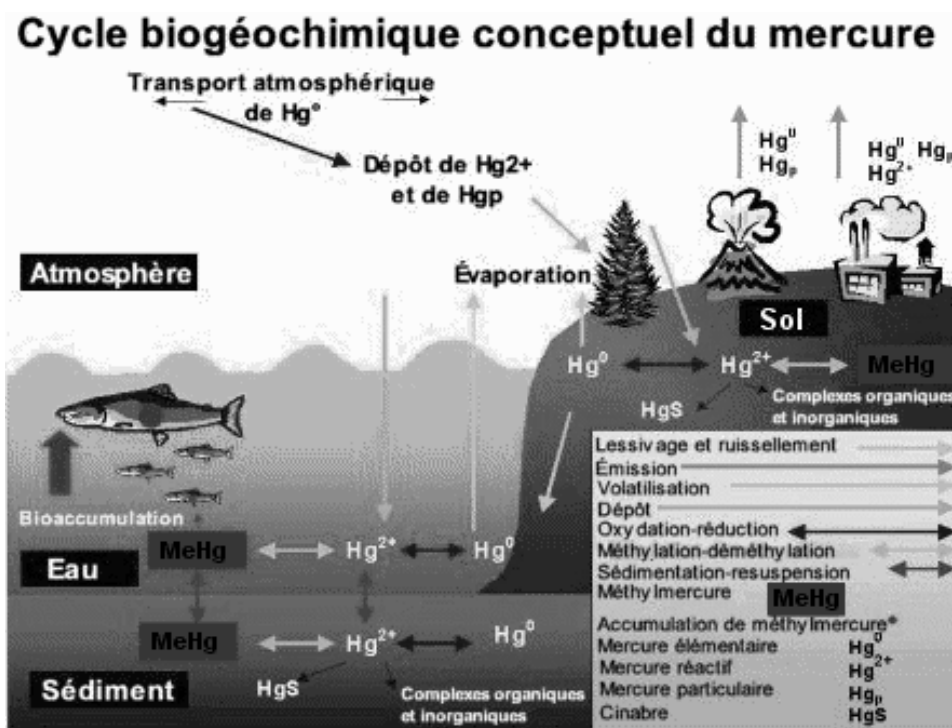
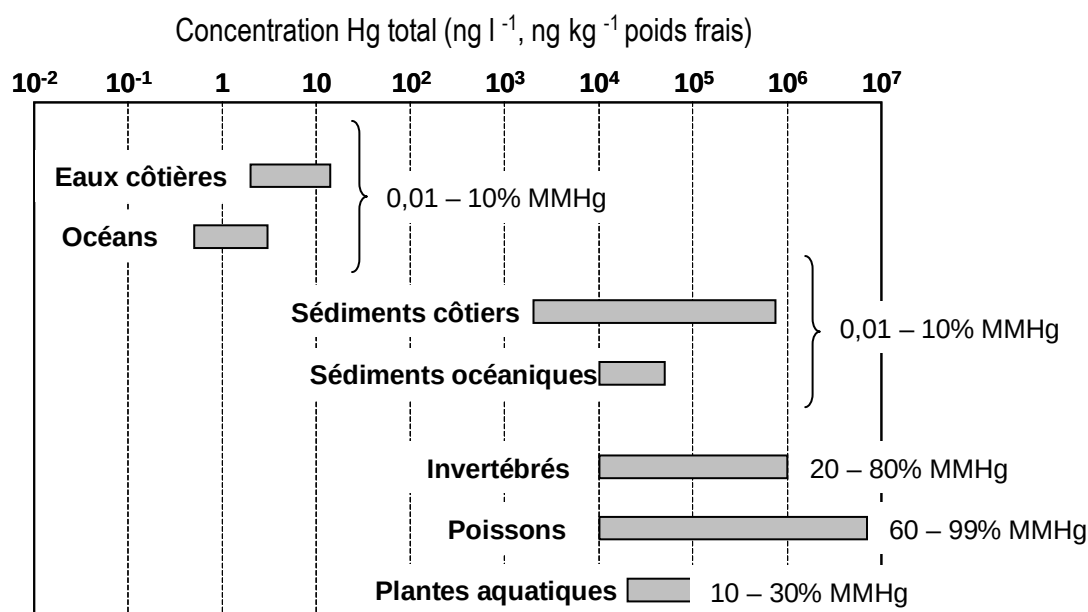


Figure 1 : Cycle biogéochimique du mercure (<http://www.ec.gc.ca/>)

En résumé, dans les milieux aquatiques, les niveaux de mercure inorganique et de méthylmercure sont principalement gouvernés d'une part par un équilibre entre la réduction et l'oxydation et d'autre part par les taux de méthylation et déméthylation. Les concentrations en MMHg dans l'environnement représentent donc la production nette résultant à la fois du taux de méthylation et du taux de déméthylation. Il semble que ces processus combinés de production et de dégradation du MMHg permettent d'arriver à un état d'équilibre dans les sédiments avec des concentrations en MMHg qui n'excèdent généralement pas 2% du mercure total pour des systèmes non perturbés (Mikac et al. 1999, Hines et al. 2000). Par contre, pour les organismes aquatiques, la proportion de MMHg est nettement plus forte avec 20 à 40% pour les invertébrés (Cossa et al. 1999, stoickev et al. 2002) et atteignant 80% voire plus du mercure total pour des poissons prédateurs (Cossa et al. 1999, Horvat et al. 1999). La figure 2 présente les gammes de concentrations en mercure total pour les différents compartiments des environnements aquatiques ainsi que les proportions de MMHg.



**Figure 2 :** Gammes de concentrations en mercure pour les différents compartiments des environnements aquatiques

## A-II-2- Cycle biogéochimique des butyl-étains

### A-II-2-1- Sources et spéciation des butylétains

Les butylétains sont des composés organiques contenant au moins une liaison entre un carbone et le métal étain. Le plus connu est de loin le tributylétain (TBT). Toutefois, plusieurs autres butylétains sont d'usage courant comme le mono- et dibutylétain (MBT, DBT).



La principale cause de l'abondance des butylétains dans les environnements côtiers est d'ordre anthropique. L'impact le plus important sur la vie aquatique est relatif à l'usage depuis les années 1970 du TBT comme agent biocide dans les peintures antisalissures pour carènes de bateaux. Les premiers effets secondaires de l'utilisation de ces peintures ont été observés en France à partir de 1975 dans la zone ostréicole du Bassin D'Arcachon. Son utilisation sur les embarcations de petite taille (<25m) a été interdite en France depuis 1982, suite aux impacts dévastateurs sur les populations d'huîtres et d'autres mollusques marins.

Bien que les peintures antisalissures aient représenté l'utilisation majoritaire de TBT, ce composé est également utilisé comme agent antifongique dans certains produits de consommation, comprenant tapis, textiles et les revêtements de sol vinyliques (PVC) (Rudel 2003, Donard et al. 2001). Le MBT et le DBT sont, toutefois, bien plus abondants dans les produits de consommation où ils sont utilisés comme stabilisateurs de chaleur dans les produits en PVC ainsi que dans certaines applications de revêtements en verre (Sadiki and Williams 1999).

Enfin, les butylétains s'affirment comme des polluants globaux persistants, surtout dans les sédiments où les concentrations rendent compte des apports passés. Des profils sédimentaires ont permis de mettre en évidence le stockage du TBT et la diminution des concentrations en réponse aux mesures de restriction (Evans 1999, Champ 2000).

#### A-II-2-2- Les butyl-étains et leur toxicité

Les organoétains sont réputés pour leur toxicité à des niveaux d'exposition relativement faibles non seulement pour les invertébrés marins mais aussi pour les mammifères. A des concentrations inférieures à 1 ng.l<sup>-1</sup> d'eau, le TBT perturbe la reproduction des gastéropodes. Des concentrations de 2 à 3 ng.l<sup>-1</sup> provoquent des anomalies de calcification chez les huîtres et 20 ng.l<sup>-1</sup> mettent en péril leur reproduction (Alzieu 1986, Bryan et al. 1986).

Pour les invertébrés marins, le TBT est généralement plus toxique que le DBT, lui-même plus toxique que le MBT (Cima et al. 1996) car la toxicité globale de ces composés sur les organismes biologiques augmente généralement avec le nombre et la longueur des groupements organiques liés à l'atome d'étain. Toutefois, ce n'est pas toujours le cas, puisque le DBT est plus toxique que le TBT pour certains systèmes enzymatiques (Bouchard et al. 1999, Al-Ghais et al. 2000). Pour les poissons, le DBT est fréquemment une toxine plus puissante que le TBT (O'Halloran et al. 1998), avec le système immunitaire comme cible principale.

### A-II-2-3- Le devenir du TBT dans les environnements côtiers

Les mesures législatives prises sur l'utilisation du TBT ont permis une baisse de la concentration du TBT dans l'eau, mais l'utilisation intensive du TBT avant la réglementation a permis à celui-ci de s'accumuler grandement dans les sédiments côtiers et estuariens. En plus de sa persistance, son affinité pour les tissus biologiques a conduit à une vaste contamination des organismes aquatiques ([Iwata 1995](#)). Des études réalisées en Méditerranée dans les eaux du large ont permis de détecter la présence de TBT dans les eaux superficielles à plusieurs dizaines de kilomètres des côtes ([Michel et al. 1999](#), [Borghiand et Porte 2002](#)). Des profils verticaux ont permis leur détection dans les eaux profondes jusqu'à 2500 mètres. Ceci met en évidence une dégradation du TBT très ralentie dans les eaux du large et donc une persistance importante du TBT avec une durée de vie pouvant atteindre plusieurs années.

Les organo-étains sont donc largement répandus dans l'environnement mais leur biodisponibilité est fortement liée aux échanges entre la colonne d'eau et les sédiments ainsi qu'à l'activité biologique des organismes aquatiques (Figure 3).

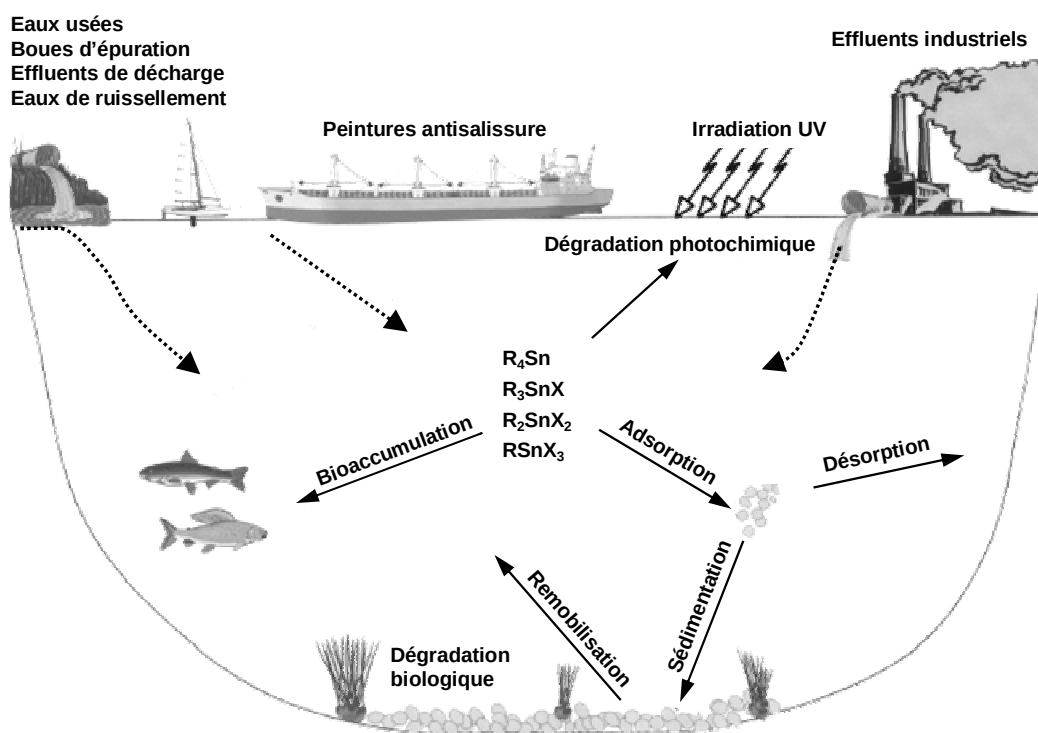
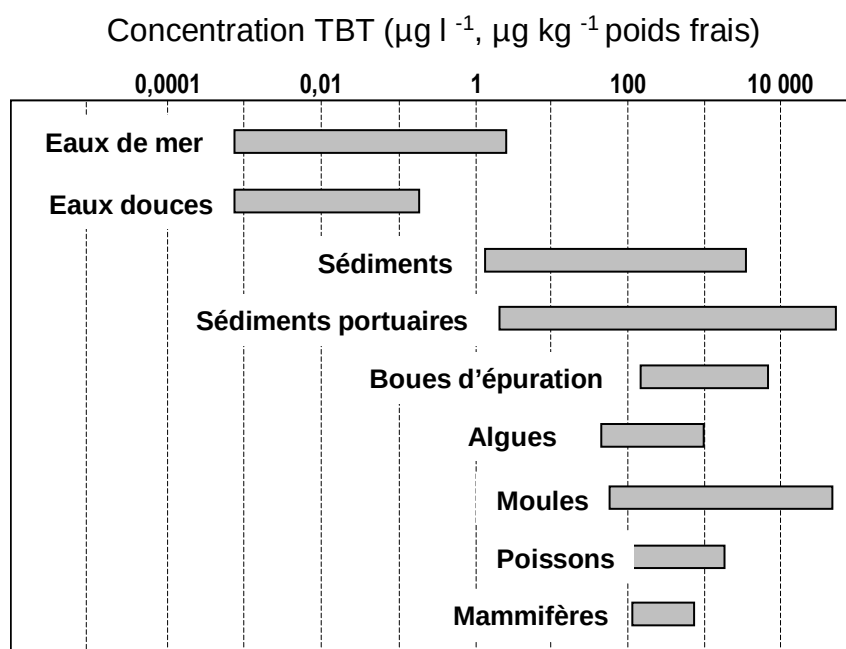


Figure 3 : Cycle biogéochimique des butylétains (d'après [Hoch 2001](#))

La persistance des butylétains dans l'environnement va être fonction de mécanismes physiques (adsorption par les particules en suspension et les sédiments), chimiques (dégradation chimique ou photochimique) et biologiques (dégradation biologique et accumulation). Dans les systèmes aquatiques, le pH, la salinité et la lumière déterminent la réactivité des organo-étains (Fent 1995, Donard et al. 2001). Des réactions thermiques, chimiques ou biologiques sont capables de dégrader le TBT en DBT, MBT et étain inorganique. D'autres processus, comme les interactions des butylétains avec la matière organique, ont aussi des effets significatifs sur la réactivité et la biodisponibilité dans les systèmes naturels (Fent 1996, Hoch 2001).

En résumé, le devenir du TBT dans les environnements aquatiques est régi par des mécanismes de transfert et de transformations qui sont à l'heure actuelle peu ou pas décrits. Néanmoins la contamination des systèmes aquatiques est bien documentée par des quantités importantes de données sur les concentrations en TBT dans les différents compartiments. Les gammes de concentrations pour les différentes matrices sont données dans la figure 4. Le problème actuel du TBT réside principalement de la contamination des sédiments et les implications éco-toxicologiques liées à sa remobilisation ou son transfert vers les autres compartiments environnementaux.



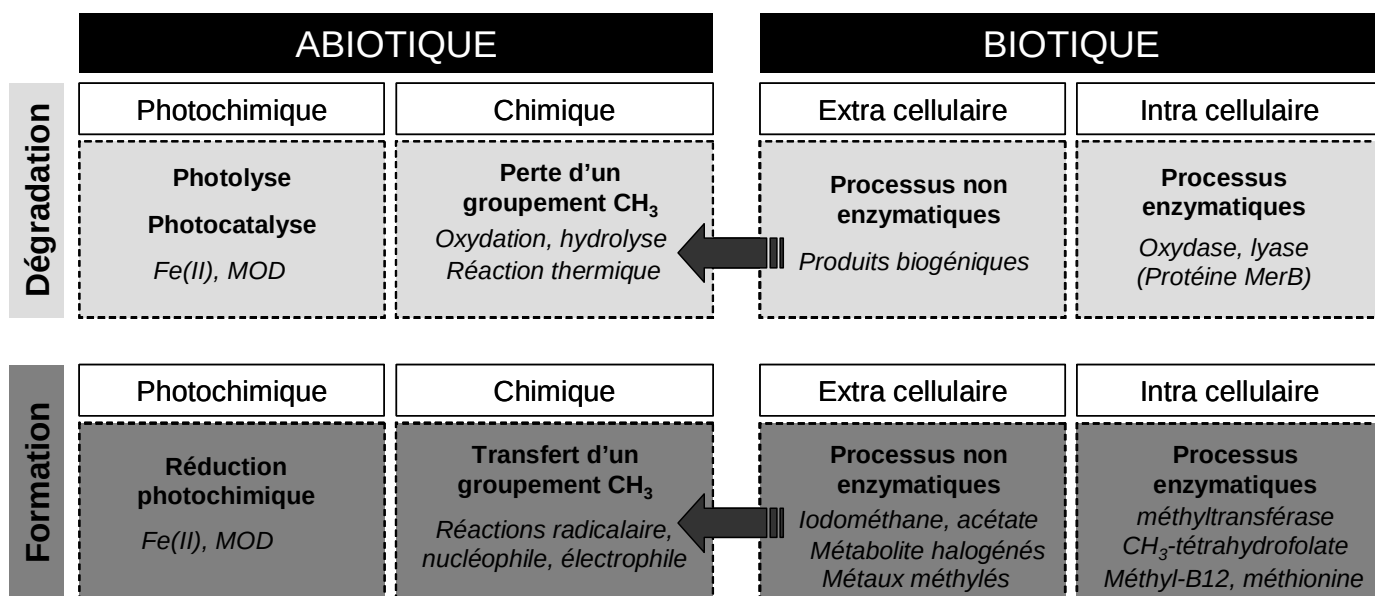
**Figure 4** : Gammes de concentrations en TBT pour les différents compartiments des environnements aquatiques (d'après Veidenhaupt 1997)

### A-III- Processus contrôlant les concentrations et le devenir du MMHg et du TBT dans les environnements côtiers

#### A-III-1- Transformations dans les environnements aquatiques

Une fois dans l'environnement, les composés organométalliques peuvent être soit alkylés soit de-alkylés. Ces réactions d'alkylation/de-alkylation sont gouvernées par des processus biotiques ou abiotiques (Figure 5) qui sont parfois dépendants. Les processus abiotiques sont généralement des réactions chimiques ou photochimiques liées à la présence de certains composés qui peuvent aussi avoir une origine biogénique (Craig 1986). Concernant les processus biotiques, les réactions sont soit extra soit intra cellulaires.

La méthylation est la réaction la plus courante dans l'environnement, elle peut se poursuivre jusqu'à la substitution complète du composé (Ridley et al. 1977). La réaction peut être purement chimique ou liée à l'activité biologique de micro-organismes. Des processus enzymatiques ont été mis en évidence par l'action de méthyltransférases et de leur substrats (methylcobalamine, méthionine, acide méthyltetrahydrofolique) (Wood, 1974). Enfin des produits biogéniques méthylants peuvent être synthétisés et excrétés par les microorganismes, tels que le iodométhane, les produits de dégradation du glucose (acétate, propionate), des métabolites halogénés et les métaux méthylés (Glocking 1987). Une synergie entre les processus biotiques et abiotiques existe du fait que les composés biogéniques peuvent ensuite se retrouver dans l'environnement et réagir chimiquement avec les métaux présents.



**Figure 5 :** Mécanismes de formation et de dégradation des composés organométalliques.

## A-III-1-1- Cas du méthylmercure

Les concentrations en MMHg dans les environnements côtiers sont régulées par deux processus antagonistes que sont la méthylation et la déméthylation. Ces mécanismes sont simultanés et se produisent aussi bien dans les sédiments que dans la colonne d'eau (Gilmour et al. 1991). Le tableau 1 résume les potentiels de méthylation et de déméthylation qui ont été trouvés pour des sédiments lacustres, fluviaux et côtiers.

Les mécanismes mis en jeu sont soit chimiques ou photochimiques (abiotiques) soit réalisés par des micro-organismes tels que les bactéries (biotique). Les mécanismes majeurs impliqués dans la méthylation et déméthylation du mercure sont décrits dans la figure 6.

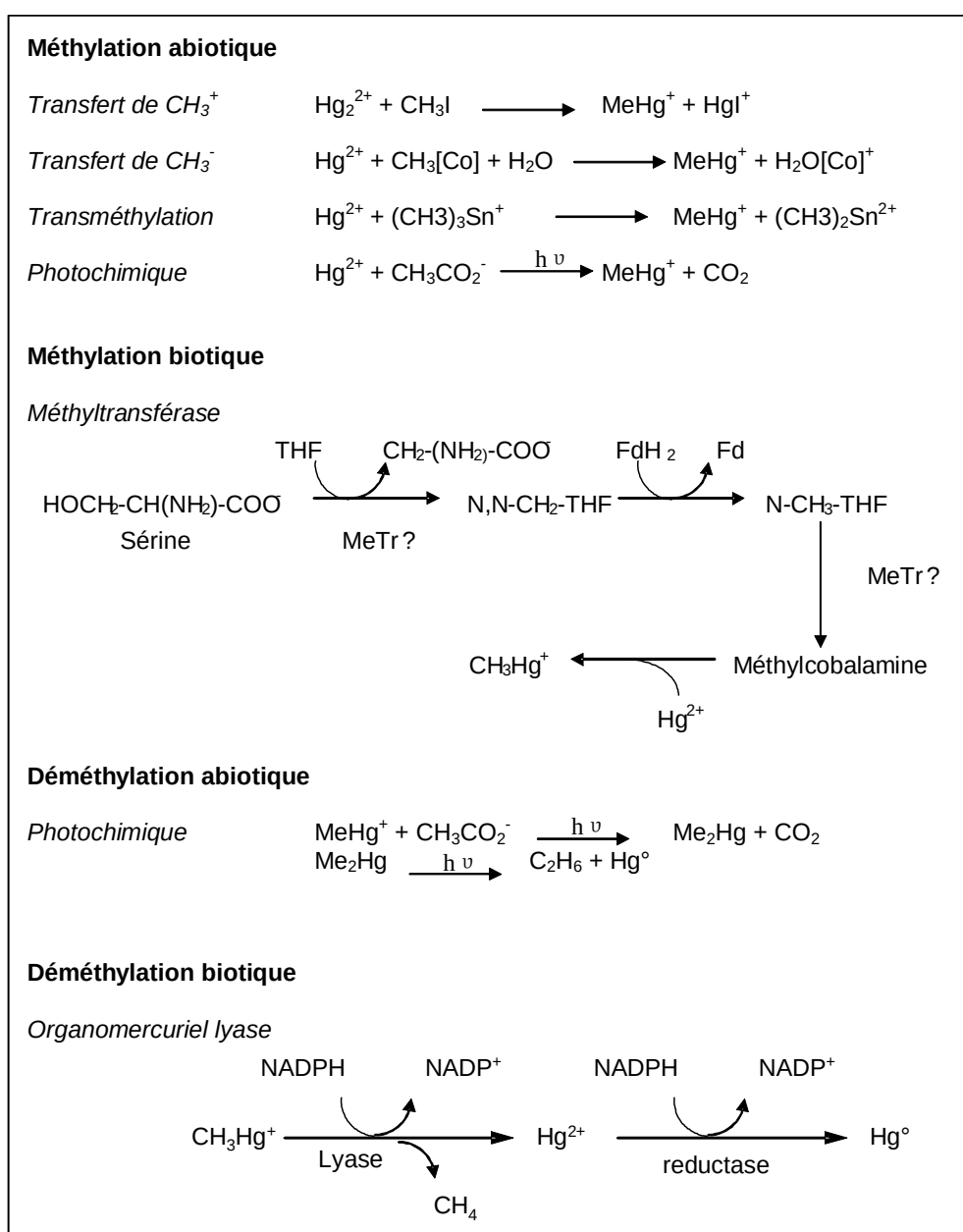


Figure 6 : Mécanismes majeurs de la méthylation/déméthylation du mercure.

## A-III-1-1-1- Méthylation

Des processus chimiques et biologiques peuvent transformer le mercure inorganique en espèces méthylées (MMHg et DMHg) qui sont les formes les plus toxiques du mercure et sont surtout très facilement bioaccumulées et bioamplifiées par la chaîne trophique dans les environnements aquatiques. La méthylation a été identifiée comme un processus qui peut être à la fois biotique et abiotique et qui a lieu aussi bien dans les sédiments que la colonne d'eau. L'un des mécanismes le plus étudié de méthylation du IHg en MMHg et/ou en DMHg par les bactéries, est celui du transfert du carbanion  $\text{CH}_3^-$  depuis la méthylcobalamine jusqu'au mercure inorganique (Wood, 1974). Ce mécanisme a été démontré chez une espèce sulfato-réductrice *Desulfovibrio desulfuricans*, où le groupement méthyle est transféré depuis le  $\text{CH}_3$ -tétrahydrofolate via la méthylcobalamine jusqu'au mercure inorganique. (Choi et Bartha 1994, Choi et al. 1994).

| Site                              | Méthode                                           | Quantités ajoutées                                       |                                                           | Potentiel de méthylation (% day <sup>-1</sup> ) | Potentiel de déméthylation (% day <sup>-1</sup> ) | Référence                        |
|-----------------------------------|---------------------------------------------------|----------------------------------------------------------|-----------------------------------------------------------|-------------------------------------------------|---------------------------------------------------|----------------------------------|
|                                   |                                                   | IHg<br>( $\mu\text{g g}^{-1}$ ou $\mu\text{g ml}^{-1}$ ) | MMHg<br>( $\mu\text{g g}^{-1}$ ou $\mu\text{g ml}^{-1}$ ) |                                                 |                                                   |                                  |
| Cheese quake (New jersey)         | slurry                                            | 100                                                      | 1                                                         | 0,035                                           | 0,25                                              | Compeau et Bartha 1984           |
| Lac Ontario (Canada)              | Slurry ( <sup>203</sup> IHg)                      | 0,25                                                     |                                                           | 0,001-0,22                                      | 0,02-0,27                                         | Ramlal et al. 1986               |
| Cadwell creek                     | slurry                                            | 0,25                                                     |                                                           | 0,08                                            |                                                   | Gilmour et al. 1987              |
| Pine Barrens (New Jersey)         | slurry                                            | 1                                                        | 0,1                                                       | 0,4                                             | 20                                                | Pak et Bartha 1998               |
| Rivière Minamata (japon)          | aquarium                                          | 1                                                        |                                                           | 0,6                                             |                                                   | Ikingura et Akagi 1999           |
| Clear lake (Californie)           | slurry                                            | 125                                                      |                                                           | 0,11                                            |                                                   | Macalady et al. 2000             |
| Lac Ontario (Canada)              | slurry                                            | 3                                                        | 0,3                                                       | 1,6                                             | 0,5                                               | Hintelmann et al. 2000           |
| Estuaire de l'Adour (France)      | Slurry ( <sup>199</sup> IHg, <sup>201</sup> MMHg) | 0,1                                                      | 0,02                                                      | 2,4                                             | 9                                                 | Rodriguez et al. 2004            |
| réservoir Quabbin (Massachusetts) | carotte                                           | 0,5                                                      |                                                           | 12                                              |                                                   | Gilmour et al. 1992              |
| Everglades                        | Carotte ( <sup>203</sup> IHg)                     | 0,001                                                    |                                                           | 0,12                                            |                                                   | Gilmour et al. 1998              |
| Skidaway river                    | Carottes                                          | 1                                                        |                                                           | 0,012                                           |                                                   | King et al. 2001                 |
| Etang de Thau (France)            | Carottes ( <sup>199</sup> IHg)                    | 0,1                                                      |                                                           | 0,75-0,90                                       |                                                   | Monperrus et al. 2003            |
| Long Island Sound (New York)      | Carotte ( <sup>200</sup> IHg)                     |                                                          |                                                           | 0,5-7                                           |                                                   | Hammerschmidt et Fitzgerald 2004 |

**Tableau 1 :** Comparaison des potentiels de méthylation et de déméthylation du mercure dans les sédiments.

### *Méthylation biotique*

Plusieurs organismes ont été caractérisés comme responsables de cette biotransformation comme des bactéries (Pak et Bartha 1998, Pontgratz et Heumann 1999, Cleckner et al. 1999, King et al. 2001 et 2002), des levures (Yannai et al. 1987), des algues (Pontgratz et Heumann 1998) ou même encore des macrophytes (Mauro et al. 2002). Dans les années 1980, des expériences sur la méthylation du mercure dans les sédiments ont montré le rôle important des bactéries sulfato-réductrices (BSR) pour cette transformation (Blum et Bartha 1980, Compeau et Bartha 1985, Gilmour et Capone 1987). D'autres travaux ont ensuite conforté cette hypothèse et ont montré que les bactéries sulfato-réductrices étaient la source majeure de formation du MMHg dans les sédiments (Gilmour et al. 1992, Pak et Bartha 1998a, Macalady et al. 2000, King et al. 2000 et 2001). Des études complémentaires sur des souches de bactéries isolées ont mis en évidence la méthylation générée par les BSR (Benoit et al. 2001, Jay et al. 2002, Ekstrom et al. 2003). A l'heure actuelle aucune autre famille de bactéries méthylantes n'a été trouvée mais d'autres bactéries anaérobies sont sûrement capables de méthyler le mercure (Pak et Bartha 1998b).

### *Bactéries sulfato-réductrices*

La méthylation biotique est donc fortement liée à l'activité des bactéries sulfato-réductrices et devient nulle avec l'utilisation de molybdate ( $\text{MoO}_4^{2-}$ ) qui est un inhibiteur spécifique de l'activité des BSR (Compeau and Bartha 1985, King et al. 1999, Gilmour et al. 1998). De plus quand les ions  $\text{SO}_4^{2-}$  sont absents, la méthylation du mercure par les BSR ne se fait plus (King et al. 2000). Les sulfates stimulent la production de MMHg en augmentant l'activité des BSR (Gilmour et al. 1992) mais peut aussi, à de trop fortes concentrations en sulfures produits par la sulfato réduction, limiter la production de MMHg (Compeau et Bartha 1985, Gilmour et al. 1992, Choi et Bartha 1994, Gilmour et al. 1998). Pour expliquer cette relation complexe entre sulfures, sulfates et production de MMHg, Benoit et al. (1999a, 1999b, 2001) et Jay et al. (2002) ont émis l'hypothèse que le MMHg est formé quand les BSR absorbent un complexe mercure-sulfure neutre de forme  $[\text{Hg}_x\text{S}_y]^\circ$ . En présence d'un excès de sulfures, la production de MMHg serait alors retardée en favorisant la formation d'un complexe chargé négativement ( $[\text{Hg}_x\text{S}_y]^{z-}$ ) pouvant précipiter avec le fer.

En revanche, le métabolisme mis jeu par les BSR pour la méthylation est à l'heure actuelle peu connu. King et al. (2000) ont montré que la méthylation dépend de la physiologie et de la phylogénie des BSR présentes dans l'échantillon ainsi que leur mode de métabolisation du carbone. Le tableau 2 présente les différentes souches bactériennes testées pour leur capacité à méthyler le mercure. Ces bactéries appartiennent à différents genres phylogénétiques et présentent des potentiels de méthylation différents. Ainsi les bactéries utilisant l'acétate comme source de carbone sont plus performantes probablement en raison de l'utilisation de l'enzyme méthyltransférase (King et al. 2000). Choi et al. (1994) ont montré que la souche *Desulfuvibrio desulfuricans*

était capable de méthyler le mercure par une réaction secondaire avec l'acétyl-CoA via une enzyme méthyltransférase (voir figure 6). Une étude récente a néanmoins prouvé que les BSR sont capables de méthyler par une autre voie que l'acétyl-CoA (Ekstrom et al. 2003). Enfin, il est aussi possible d'avoir une synergie entre les BSR et les bactéries méthanogènes pour avoir méthylation du mercure (Pak et al. 1998b). Néanmoins, ces résultats mettent en avant la difficulté à comprendre les mécanismes mis en jeu ainsi que les types de bactéries qui méthylent réellement le mercure dans les écosystèmes naturels.

| Genre                   | Espèce               | Potentiel de méthylation                | Référence           |
|-------------------------|----------------------|-----------------------------------------|---------------------|
| <b>Desulfovibrio</b>    | <i>desulfuricans</i> | 100 ng ml <sup>-1</sup> d <sup>-1</sup> | Pak et Bartha 1998a |
|                         | <i>desulfuricans</i> | 108 pg ml <sup>-1</sup> d <sup>-1</sup> | King et al. 2000    |
|                         | <i>desulfuricans</i> | 200 pg ml <sup>-1</sup> d <sup>-1</sup> | Jay et al. 2002     |
|                         | <i>africanus</i>     | 311 ng ml <sup>-1</sup> d <sup>-1</sup> | Ekstrom et al. 2003 |
|                         | <i>vulgaris</i>      | <LD                                     | Ekstrom et al. 2003 |
| <b>Desulfobulbus</b>    | <i>propionicus</i>   | 25 pg ml <sup>-1</sup> d <sup>-1</sup>  | King et al. 2000    |
|                         | <i>propionicus</i>   | 5 ng ml <sup>-1</sup> d <sup>-1</sup>   | Benoit et al. 2001  |
|                         | <i>propionicus</i>   | 255 ng ml <sup>-1</sup> d <sup>-1</sup> | Ekstrom et al. 2003 |
| <b>Desulfococcus</b>    | <i>multivorans</i>   | 110 pg ml <sup>-1</sup> d <sup>-1</sup> | King et al. 2000    |
|                         | <i>multivorans</i>   | 214 ng ml <sup>-1</sup> d <sup>-1</sup> | Ekstrom et al. 2003 |
| <b>Desulfobacter</b>    | <i>sp</i>            | 37 pg ml <sup>-1</sup> d <sup>-1</sup>  | King et al. 2000    |
|                         | <i>curvatus</i>      | <LD                                     | Ekstrom et al. 2003 |
| <b>Desulfobacterium</b> | <i>sp</i>            | 181 pg ml <sup>-1</sup> d <sup>-1</sup> | King et al. 2000    |

**Tableau 2** : Bactéries sulfato-réductrices méthylant le mercure et taux de méthylation.

Dans les autres compartiments des environnements aquatiques tels que la colonne d'eau, la méthylation biotique est nettement moins étudiée. Des expériences en région polaire ont montré que des macro algues (Pontgratz et Heumann 1998) ainsi que des bactéries marines (Pontgratz et Heumann 1999) étaient capables de méthyler le mercure. Ces travaux restent néanmoins incertains sur l'identification des bactéries et les mécanismes mis en jeu pour méthyler. A l'heure actuelle, la méthylation du mercure dans la colonne d'eau par des micro-organismes reste à démontrer.



### *Méthylation abiotique*

Le MMHg peut être aussi formé par des mécanismes abiotiques dans les sédiments et la colonne d'eau. Deux mécanismes importants ont été décrits précédemment : la méthylation induite photo-chimiquement et la méthylation par des réactions chimiques (Weber 1993). La méthylation photochimique s'effectue en présence de groupes donneurs de méthyle tels que l'acide acétique, le méthanol et l'éthanol (Gardfeldt et al. 2003).

D'autre part, de nombreux agents méthylants, naturellement présents dans l'environnement, sont susceptibles d'alkyler le mercure inorganique par le transfert d'un carbanion méthyle ( $\text{CH}_3^-$ ). La méthylation abiotique a été démontrée par Jewett et al. (1975) comme le résultat de réactions de trans-méthylation entre le mercure inorganique et les composés alkylés du plomb et de l'étain. Le triméthyl plomb a été trouvé comme un agent méthylant efficace du mercure (Jewett et al. 1975, Rosenkranz et al. 1997). Néanmoins, ces agents méthylants organométalliques ne sont présents qu'à l'état de traces dans les systèmes non perturbés, limitant en conséquence l'importance des mécanismes de transméthylation. Il existe d'autres agents méthylants très répandus dans l'environnement, ce sont les substances humiques (Weber 1993) et la formation de MMHg par ces composés a aussi été démontrée par Hamasaki et al. (1995). La capacité de formation du MMHg augmente généralement avec la température et la quantité de mercure inorganique, mais en fait reste très faible dans les conditions environnementales. Falter (1999) a montré que la méthylation du Hg atteignait 0,01% dans un sédiment à 40°C et était nulle en dessous de 40°C.

Baldi et al. (1995) ont montré que le MMHg pouvait aussi réagir avec le sulfure biogénique pour former du  $(\text{MMHg})_2\text{S}$  qui va rapidement se décomposer en DMHg et HgS. Le DMHg va lui-même se dégrader rapidement en MMHg. Du DMHg a aussi été détecté en mer Méditerranée par Cossa et al. (1994) et sa formation serait maximale pour la zone de déplétion de l'oxygène et liée à l'activité microbienne.

En résumé, la méthylation du mercure abiotique reste donc liée à plusieurs facteurs physico-chimiques qui souvent ne représentent pas les conditions réelles environnementales. Dans les systèmes naturels, la contribution de la méthylation abiotique semble donc être minime alors que la méthylation biotique apparaît comme prédominante. Berman et Bartha (1986) ont montré que la concentration de MMHg dans un sédiment anoxique résultant de la méthylation abiotique était dix fois plus faible que celui provenant de la méthylation biotique. Ebinghaus et al. (1994) ont montré que les composés organométalliques étaient des agents méthylants beaucoup plus efficaces que les agents méthylants biogéniques comme la méthylcobalamine. Néanmoins, dans des conditions environnementales, non seulement ces composés sont à l'état d'ultra traces mais aussi la méthylation enzymatique par la méthylcobalamine est beaucoup plus rapide que la transméthylation (Choi et al. 1994).

### A-III-1-1-2- Déméthylation

Les composés organométalliques présents dans l'environnement peuvent être transformés en composés moins toxiques par des mécanismes soit physico-chimiques, soit biologiques. Les principales réactions abiotiques de dégradation sont la photodégradation (photolyse UV), l'hydrolyse et la transméthylation (Marvin-Dipasqual et al. 2000). La déméthylation, dont les mécanismes sont eux bien connus, est possible chez les bactéries possédant la lyase organomercurielle.

La déméthylation, tout comme la méthylation, peut aussi être un processus biotique ou abiotique qui a lieu à la fois dans les sédiments et dans la colonne d'eau. Néanmoins la dégradation du MMHg semble être principalement attribuée à l'activité microbiologique et dépend des communautés bactériennes présentes (Oremland et al. 1991, Pak et al. 1998a). Le MMHg est dégradé dans les sédiments anoxiques soit par réduction soit par oxydation. La voie réductrice permet la formation de mercure élémentaire alors que la déméthylation oxydative génère du mercure inorganique qui peut être à son tour méthylé. Plusieurs études suggèrent que la déméthylation réductrice est majeure pour des environnements oxiques (Oremland et al. 1991, Hines et al. 2000) alors que la déméthylation oxydative est prépondérante en milieu anoxique (Barkay et al. 2003). Le mécanisme microbien de déméthylation par réduction implique l'action d'une enzyme organomercurielle lyase qui est codée par le gène *merB* (Barkay et al. 2003). Cette enzyme va casser la liaison Hg-carbone puis une enzyme réductase va réduire  $\text{IHg}$  en  $\text{Hg}^0$  (Barkay et al. 2003). Ce processus génère ainsi du méthane, du  $\text{IHg}$  et du  $\text{Hg}^0$ . Le mécanisme de déméthylation par oxydation va quand à lui générer du  $\text{CO}_2$  et du  $\text{IHg}$  (Barkay et al. 2003).

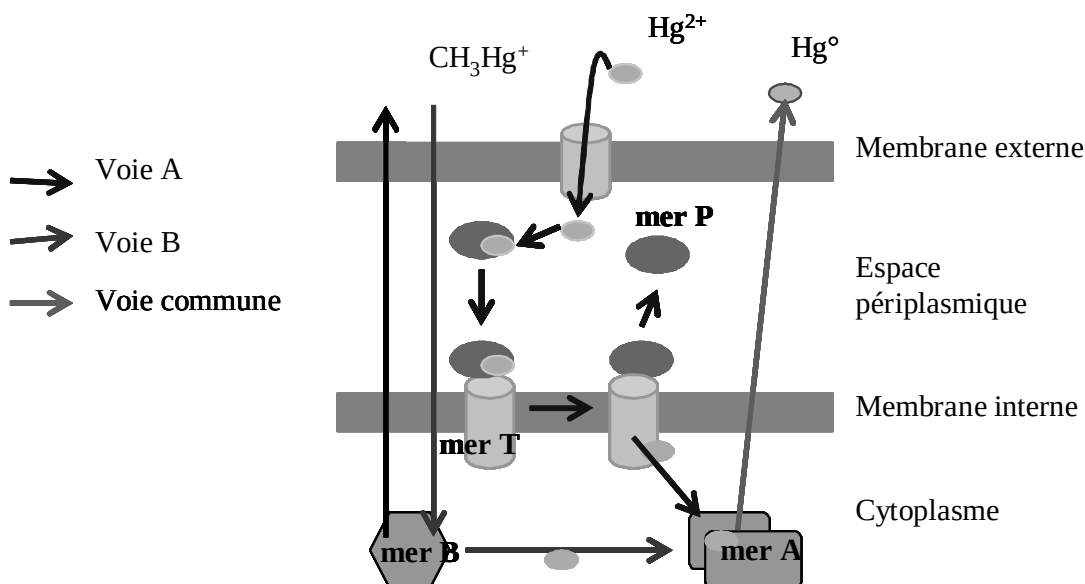
La déméthylation a été également identifiée comme un processus abiotique qui a lieu dans la colonne d'eau et qui est fortement contrôlée par la dégradation photochimique (Sellers et al. 1996). Ainsi pour des environnements très exposés à la lumière comme les eaux de surface des océans et des environnements côtiers, la photodégradation apparaît comme un processus majeur de dégradation du MMHg (Sellers et al. 1996). Cette photodégradation induit une importante source de mercure pour l'atmosphère qui varie donc en fonction des saisons et du cycle diurne (Sommar et al. 2001, Gardfeldt et al. 2001).

### A-III-1-1-3- Réduction

En plus de la déméthylation du MMHg, le  $\text{IHg}$  peut aussi être réduit en  $\text{Hg}^0$  et transféré vers l'atmosphère. Cette transformation permet de réduire la quantité de mercure inorganique dans les environnements aquatiques et donc limiter le substrat pour la méthylation (Fitzgerald et al. 1991). La réduction

de IHg en Hg° dans les eaux de surface est induite par les radiations UV (Amyot et al. 1994 et 1997, O'Driscoll et al. 2003).

Mais des observations de fortes concentrations de mercure gazeux dans des eaux profondes montrent que les réactions photochimiques ne sont pas les seules impliquées pour la réduction du IHg (Mason et al. 1995, Amyot et al. 1997). Des activités biologiques peuvent également être impliquées dans la réduction du IHg comme l'ont montré Mason et Fitzgerald (1993) dans l'océan Pacifique. Poulain et al. (2004) ont montré que des organismes phototrophes comme des algues étaient capables de réduire le mercure et que ce phénomène est étroitement lié à l'activité photosynthétique. Des expériences en laboratoire ou in situ, ont montré que certaines bactéries étaient capables de réduire le mercure par une voie enzymatique codée génétiquement par le gène *merA* (Barkay et al. 2003). Ce mécanisme intervient chez les organismes possédant les opérons à spectre étroit et à spectre large (figure 7). Les ions mercuriques entrent à l'intérieur de la cellule par l'intermédiaire de deux protéines de transport (Wilson et al. 2000) : la protéine périplasmique MerP et la protéine merT codées respectivement par les gènes *merP* et *merT* (Morby et al. 1995). Les ions mercuriques sont alors transférés jusqu'au cytoplasme, où la réduction de Hg<sup>2+</sup> en Hg° volatil, est catalysée par la protéine MerA qui est codée par le gène *merA*. Cette protéine est une réductase mercurique flavinique cytoplasmique NADPH dépendante spécifique du mercure inorganique (Bogdanova et al. 1998).



**Figure 7** : Mécanismes de réduction du mercure par les bactéries (d'après Hobman et al. 2000).

### A-III-1-2- Cas du tributylétain

#### A-III-1-2-1- Débutylation

Les mécanismes de transformation du TBT consistent essentiellement en sa dégradation qui peut être induite chimiquement, photochimiquement ou biologiquement. La dégradation du TBT implique généralement la rupture des liaisons Sn-C et donc la perte progressive d'un groupement butyl (DBT, MBT) pour arriver jusqu'à l'étain sous forme inorganique.

La dégradation du TBT dans l'eau est principalement due à la photolyse par les radiations UV. L'énergie de dissociation de la liaison Sn-C est comprise entre 190 et 220 kJ/mol ce qui permet la rupture de la liaison Sn-C par les radiations UV. La photolyse apparaît comme un processus important pour les eaux de surface mais probablement inexistante pour les eaux profondes et les sédiments où les radiations UV ne pénètrent pas ([Hoch 2001](#)). La dégradation chimique est aussi possible par un clivage de la liaison Sn-C par des composés nucléophiles et électrophiles comme par exemple les acides minéraux, les acide carboxyliques, les métaux alkylés,... ([Blunden et Chapman 1982 et 1986](#); [Hoch 2001](#)).

Enfin certains micro-organismes sont capables de dégrader le TBT comme les bactéries et les micro-algues. Des expériences sur des souches pures de bactéries ont montré leur capacité à dégrader le TBT en DBT et MBT ([Cooney 1987](#)). [Tsang et al. \(1999\)](#) ont montré que des algues (*Chlorella*) étaient aussi capables de métaboliser le TBT et de le transformer en DBT. Néanmoins ces mécanismes restent peu étudiés et apparaissent mineurs car le TBT semble être peu dégradé par les micro-organismes dans le milieu naturel.

Pour les sédiments, les taux de dégradation sont très faibles avec des temps de demie vie pour le TBT de 1 à 5 ans ([Sarradin 1995](#)). Cette capacité des sédiments à accumuler le TBT pose donc un risque permanent pour la contamination de l'eau par des processus de désorption et des organismes benthiques du fait de sa toxicité.

#### A-III-1-2-2- Méthylation

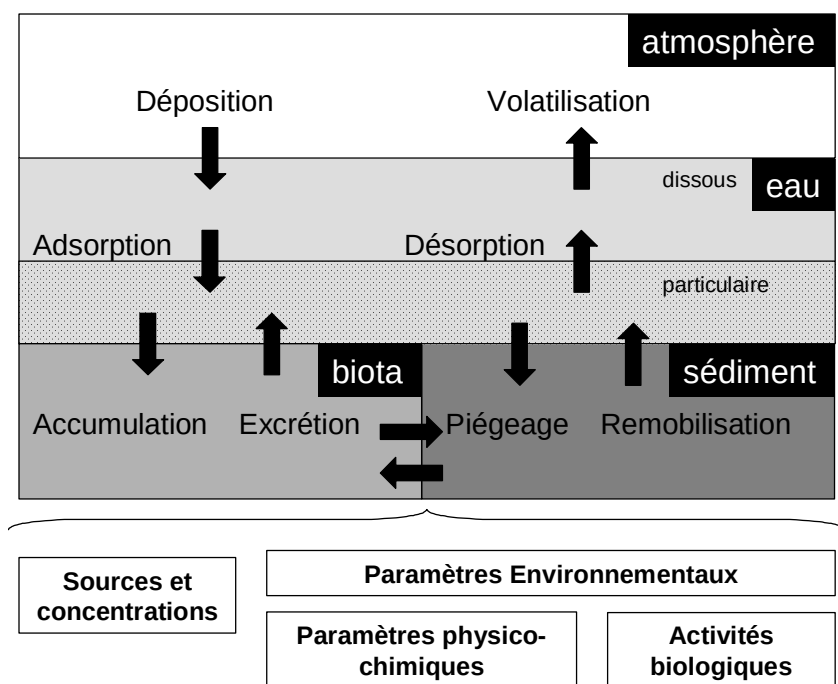
De même que le mercure, l'étain peut être méthylé dans l'environnement pour former des composés totalement substitués susceptibles d'être volatiles ([Weber 1999](#), [Amouroux et al. 1998](#)). La preuve de l'existence de ces espèces chimiques dans les environnements aquatiques n'a été que très récemment rapportée dans la littérature. [Maguire et al. \(1984 et 1996\)](#) ont rapporté la présence d'espèces mixtes méthylées des butylétains telles que le méthyltributylétain ( $\text{MeSnBu}_3$ ) et le diméthylidibutylétain ( $\text{Me}_2\text{SnBu}_2$ ) pour des sédiments de surface contaminés. Plus récemment, [Amouroux et al. \(2000\)](#) et [Tessier et al. \(2002\)](#) ont démontré la présence de ces dérivés méthylés dans les eaux et les sédiments côtiers.

Diverses études en laboratoire ont démontré que les bactéries sulfato-réductrices, présentes dans des sédiments anoxiques naturels, peuvent promouvoir activement la méthylation de l'étain organique et inorganique (Gilmour et al. 1985, Yozenawa et al. 1994). La formation d'hydrures et de méthylhydrures d'étain à partir d'étain inorganique a également pu être mise en évidence, durant l'incubation de cultures de micro-organismes et d'algues en laboratoire (Donard et al. 1987, Donard et Weber 1988). Bien que les mécanismes de formation de ces espèces ne soient pas totalement connus, ils sont fortement suspectés d'être d'origine biogénique et sont donc susceptibles de se produire dans les environnements naturels (Craig et Rapsomanikis 1985). Outre les mécanismes biologiques, Craig et Rapsomanikis (1985) suggèrent également que la méthylation abiotique peut avoir lieu dans les environnements naturels. Des agents méthylants naturels comme le iodométhane, la méthylcobalamine ou les substances humiques sont en effet capables de former, à partir des dérivés de l'étain, des composés méthylés (Weber 1999).

Ces processus de méthylation affectent donc la biodisponibilité des butylétains en formant des composés volatiles disponibles pour la bioaccumulation par les organismes marins ainsi que pour les échanges avec l'atmosphère. Ils constituent ainsi un processus non négligeable dans le cycle biogéochimique du TBT et de son devenir dans les environnements côtiers.

### A-III-2- Transferts aux interfaces des environnements aquatiques

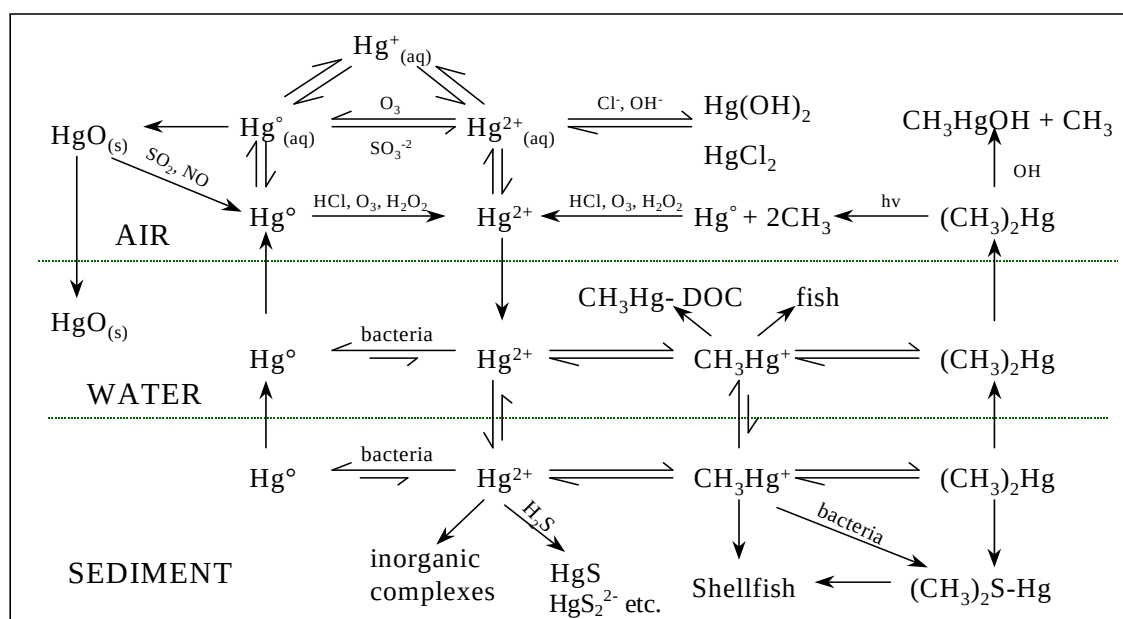
La figure 8 schématise les principaux processus de transferts des polluants entre les différents compartiments des environnements aquatiques. Les échanges entre l'atmosphère et l'eau sont principalement régulés par les phénomènes de déposition et de volatilisation. Ces transferts sont régis par des mécanismes de diffusion passive des composés vers l'eau ou l'atmosphère et de dégazage fonction du dynamisme hydrologique du milieu (vent, courant, houle). Dans l'eau, les composés organométalliques peuvent passer de la phase dissoute à la phase particulaire par des processus réversibles d'adsorption et de désorption sur des particules minérales, organiques ou biogéniques. Les sédiments et la biomasse peuvent constituer soit une source soit un piège pour ces polluants. Dans le cas des interactions avec la biota, les composés peuvent être assimilés en fonction du mode d'alimentation puis stockés dans les organismes ou évacués par excrétion. Dans les sédiments, les principaux mécanismes contrôlant les transferts vers la colonne d'eau sont la diffusion passive, la bioturbation générée par les organismes benthiques ou la remobilisation mécanique liée à l'hydrodynamisme. Enfin tous ces processus vont être régulés par différents facteurs environnementaux que sont les apports de ces polluants dans l'environnement, les paramètres physico-chimiques déterminant la réactivité et la mobilité des contaminants et les activités biologiques des organismes et micro-organismes aquatiques.



**Figure 8** : Transferts aux interfaces des environnements aquatiques

#### A-III-2-1- Cas du méthylmercure

Les transferts et les échanges des différents composés du mercure entre les différents compartiments des environnements aquatiques sont décrits dans la figure 9. On retrouve les échanges possibles entre les quatre grands compartiments de l'environnement qui sont l'atmosphère, l'eau, les sédiments et la biomasse.



**Figure 9** : Les transferts et les transformations des formes chimiques du mercure dans l'environnement (d'après Stein et al. 1996).

### A-III-2-1-1- Transferts eau/atmosphère

Les échanges entre l'eau et l'atmosphère sont des phénomènes qui régulent en grande partie la concentration de mercure dans les environnements aquatiques. Ces échanges sont de deux types : les dépositions atmosphériques et la volatilisation.

Les dépositions atmosphériques peuvent être des retombées humides ou sèches et constituent une source en mercure inorganique. La proportion de MMHg dans les dépôts atmosphériques n'excède généralement pas 0,5% du mercure total ([Mason et al. 1997](#), [Lamborg et al. 1999](#)) ce qui représente une quantité trop faible pour être considérée comme une source significative de MMHg. Les flux de mercure par dépôts humides, que sont les pluies et les neiges, sont généralement plus importants que les flux de mercure par dépositions sèches ([Guentzel et al. 1995](#)). Leur contribution dépend de la spéciation du mercure, de la température ambiante et de la quantité de retombées humides ([Stein et al. 1996](#)). Les dépôts atmosphériques secs (particules) sont généralement affectés par différents facteurs. Les facteurs les plus importants sont les conditions météorologiques comme la force et la direction du vent.

Le mercure n'est pas uniquement déposé dans les environnements aquatiques mais il peut aussi être émis vers l'atmosphère par dégazage et volatilisation du mercure élémentaire. Du fait de sa faible solubilité et de sa grande volatilité,  $\text{Hg}^0$  est facilement transféré de l'eau vers l'atmosphère. Cette propriété du mercure a conduit notamment à une dispersion globale de ce contaminant dans les zones très éloignées des sources de pollution ([Lindqvist 1991](#), [Fitzgerald et al. 1998](#)). Les flux de volatilisation de  $\text{Hg}^0$  généralement observés pour les zones côtières sont compris entre 2 et 20  $\text{pg m}^{-2} \text{ jour}^{-1}$  ([Coquery et al. 1997](#), [Baeyens et al. 1998](#), [Mason et al. 1993](#), [Coquery et al. 1995](#), [Cossa et al. 1996](#)). La lumière et les fortes températures augmentent généralement les émissions en favorisant l'accroissement des activités microbiennes capables de réduire le IHg en  $\text{Hg}^0$  ([Barkay et al. 2003](#)) et la réduction photochimique ([Stein et al. 1996](#)).

### A-III-2-1-2- Transferts eau/sédiment

Dans les environnements aquatiques, le mercure est efficacement lié aux matières en suspension dans l'eau du fait de sa grande affinité pour la matière organique ([Anderson et al. 1990](#), [Gilmour et al 1992](#), [Zillioux et al. 1993](#)). La sédimentation de ces particules permet donc le piégeage du mercure dans les sédiments. La sédimentation dans les zones côtières peut permettre le piégeage de 75% ([Mason et al. 1999a](#)) à 90% ([Horvat et al. 1999](#), [Coquery et al. 1995](#)) du mercure total introduit par la phase particulaire de la colonne d'eau, l'autre partie étant exportée vers le milieu marin. Les zones côtières représentent donc des pièges efficaces pour le

mercure mais peuvent aussi sous certaines conditions devenir des sources pour la colonne d'eau (Hines et al. 2000).

Plusieurs études ont montré que 75% du mercure reste enfoui dans les sédiments alors que 25% est recyclé vers la colonne d'eau par diffusion (Covelli et al. 1999, Horvat et al. 1999). Le mercure dans le sédiment est associé aux composés organiques (acides humiques) mais aussi aux composés inorganiques comme les sulfures ou les oxydes de fer et de manganèse. Les flux de mercure du sédiment vers la colonne d'eau sont donc directement liés aux processus géochimiques du sédiment de surface et sont différents pour le IHg et le MMHg (Bloom et al. 1999, Tseng et al. 2001). Les mécanismes de solubilisation varient d'un site à l'autre et peuvent être régis par la solubilisation de la matière organique (Mikac et al. 1999, Lacerda et al. 2001), la dissolution des hydroxydes de fer et de manganèse (Covelli et al. 1999) ou encore l'activité biologique (Hammerschmidt et Fitzgerald 2004).

Malheureusement, la détermination de ces flux entre le sédiment et la colonne d'eau demeure difficile. Le transfert du mercure au travers de l'interface eau-sédiment peut être déterminé de deux manières. La première consiste à évaluer un flux diffusif à partir des concentrations dans les eaux interstitielles. La deuxième est de déterminer directement les flux benthiques à l'aide de chambres benthiques qui permettent la mesure des flux diffusifs plus advectifs. Les flux par advection sont dus généralement aux courants et aux activités biologiques qui génèrent des phénomènes de bioirrigation et de bioturbation (Gill et al. 1999). Une étude comparative des flux diffusifs et des flux benthiques mesurés dans la baie de Trieste (Covelli et al. 1999) a montré d'importantes différences avec des flux benthiques beaucoup plus grands que les flux diffusifs. De plus les flux de mercure ne sont pas constants et subissent des variations saisonnières (Bloom et al. 1999, Gill et al. 1999) voire diurnes (Gill et al. 1999, Point et al. Annexe n°3) ce qui ne peut être intégré par la mesure de flux diffusifs. Enfin plusieurs auteurs appuient sur l'importance de l'activité des organismes benthiques et de la bioturbation sur les flux à l'interface eau-sédiment (Hammerschmidt et Fitzgerald 2004, Gill et al. 1999, Point et al. Annexe n°3).

#### A-III-2-1-3- Bioaccumulation

Le méthylmercure a la particularité d'être un composé organo-métallique non seulement très fortement bioaccumulable par les organismes aquatiques mais aussi bio-amplifiable le long de la chaîne trophique. En milieu aquatique, les premiers niveaux trophiques comme le plancton (phyto et zoo) absorbent du mercure selon un processus d'absorption passive en surface ou lors d'absorption de nourriture. Dans le cas des organismes autotrophes, l'absorption passive constitue la seule voie d'exposition (Mason et al. 1996). En revanche, les hétérotrophes peuvent être exposés à des niveaux de concentration dangereux par voie indirecte (Abreu et al. 2000). Le méthylmercure s'accumule dans la chaîne alimentaire et les prédateurs absorbent ce contaminant



contenu dans leur source d'alimentation. Par conséquent, les prédateurs de niveau trophique supérieur possèdent une charge corporelle de mercure plus élevée que celle des organismes qu'ils consomment (Horvat et al. 1999, Gilmour et al. 2000, Cossa 1999, Cossa et al. 1997).

Chez le plancton, le facteur de bioconcentration (BCF) du MMHg est de  $10^4$  par rapport à sa concentration dans l'eau. Chez les poissons carnivores il peut atteindre  $10^7$  (Horvat et al. 1999, Stein et al. 1996). Ce processus de bioamplification du MMHg s'explique par la vitesse d'entrée et de sortie dans un organisme. L'entrée d'un polluant, à partir du milieu ambiant ou de la nourriture, est réglée par des mécanismes de pénétration cellulaire. La sortie est globalement la fonction inverse de la stabilité des associations de l'élément avec les constituants de la cellule. C'est la conjonction de la stabilité des espèces chimiques dans le milieu aquatique externe et de l'affinité des espèces intracellulaires pour les protéines qui provoque sa bioaccumulation intense. La première facilite la pénétration, alors que la seconde favorise la rétention (Gagnon et al. 1997).

De plus, la méthylation du mercure étant plus importante en conditions anoxiques (Gilmour et al. 1991), les organismes benthiques vivant dans les sédiments sont directement exposés à de concentrations significatives en MMHg. La macrofaune benthique constitue un élément important des écosystèmes aquatiques et représente une source de nourriture pour les espèces pélagiques. C'est ainsi que plus de 80% du mercure total est sous forme méthylée dans les organismes supérieurs des chaînes trophiques (Andersson et al. 1990, Horvat et al. 1999)

#### A-III-2-2- Cas du tributylétain

De la même manière que les composés du mercure, les butylétains sont ubiquistes et vont être soumis à des échanges entre les différents compartiments. Ces transferts dans les environnements aquatiques sont décrits dans la figure 3.

##### A-III-2-2-1- Transferts eau/atmosphère

La perte de TBT par volatilisation dans les systèmes aquatiques semble être très limitée, même si pour des écosystèmes modèles elle a été démontrée comme significative (Adelman et al. 1990). L'hydrolyse est négligeable et le TBT est chimiquement stable dans les eaux naturelles. Le temps de demie vie du TBT sous irradiation UV est généralement long (plusieurs mois). Du fait de la faible pénétration des rayonnements UV pour les eaux de fond, ce processus de dégradation dans les écosystèmes marins semble mineur (Maguire et al. 1983).

En plus de cette dégradation du TBT qui peut générer comme produit de dégradation de l'étain inorganique, l'étain lui-même peut être méthylé et volatilisé (Craig 1986). La transformation en formes volatiles de l'étain (méthylétain, butylméthylétain, hydruure d'étain) par des microorganismes a été observée pour des systèmes aquatiques (Donard et Weber 1988, Amouroux et al. 2000). Cette formation d'espèces volatiles doit être considérée comme un phénomène significatif d'export de l'eau vers l'atmosphère. Des flux sédiment-eau et eau-atmosphère de butylméthylétain ont été mesurés pour des environnements estuariens. Des valeurs comparables ont été mesurées avec 80 à 790 nmol m<sup>-2</sup> an<sup>-1</sup> pour le transfert sédiment-eau et 20 à 510 nmol m<sup>-2</sup> an<sup>-1</sup> pour le transfert eau-atmosphère (Amouroux et al. 2000, Tessier et al. 2002).

#### A-III-2-2-2- Transferts eau/sédiment

La distribution du TBT à l'interface eau-sédiment est un processus environnemental primordial afin de définir le transport et le devenir de ce composé et de ses produits de dégradation dans les environnements côtiers. Du fait de sa faible solubilité et de son caractère hydrophobe, le TBT va être facilement adsorbé par les particules en suspension dans l'eau (Hoch 2001). Les concentrations dans l'eau vont donc être régulées par la sédimentation et le piégeage du TBT dans les sédiments. Les mécanismes d'adsorption sont régis par plusieurs paramètres physico-chimiques, comme la salinité, la composition chimique du sédiment et la teneur en matière organique (Fent 1996, Unger et al. 1988). Mais à l'heure actuelle, la question importante est de savoir si ce composé est persistant et surtout si les sédiments peuvent constituer une source de TBT pour la colonne d'eau. Plusieurs études montrent que le TBT est un composé persistant dans les sédiments sur de longues périodes évaluées à plusieurs années (De Mora et al. 1995, Sarradin 1993). La re-mobilisation du TBT vers la colonne est aussi possible par la remise en suspension des particules qui peut être due aux activités de draguage ou de pêche et par l'activité des organismes benthiques (bioturbation) (Point et al. Annexe n°3). Enfin la connaissance de l'adsorption sur les sédiments et de la partition entre les eaux interstitielles et la phase solide est essentielle pour définir la biodisponibilité du TBT pour les organismes aquatiques (Fent, 1996).

#### A-III-2-2-3- Bioaccumulation

Le caractère lipophile du TBT et de ses produits de dégradation (DBT et MBT) est forcément un paramètre important concernant la bioaccumulation par les organismes aquatiques. En raison de leur mode d'alimentation et de leur fort potentiel à accumuler les contaminants comme le TBT, les bivalves sont généralement utilisés comme organismes « sentinelles » pour suivre la contamination des écosystèmes aquatiques. Les mollusques comme les huîtres et les moules servent de bio-indicateurs pour la pollution en TBT

dans les écosystèmes marins car ces organismes présentent un intérêt commercial et sont largement consommés par l'homme ([RNO](#), [Ifremer](#)).

Des études ont démontré que les bivalves étaient capables d'accumuler des quantités importantes de TBT allant jusqu'à  $5 \mu\text{g g}^{-1}$  ([Laughlin 1996](#)). En revanche les crustacés et les poissons accumulent des quantités beaucoup plus faibles car ils possèdent des mécanismes de dégradation du TBT ([Laughlin 1996](#)).

Bien que la distribution du TBT dans les organismes aquatiques soit largement documentée, les mécanismes régulant la bioaccumulation et surtout la bioamplification le long de la chaîne trophique sont quant à eux relativement peu connus.

#### **A-IV- Spéciation chimique et isotopes stables**

Les analyses de spéciation, c'est-à-dire la détermination des formes chimiques d'un même élément, sont à l'heure actuelle largement utilisées et deviennent même des analyses de routine pour certains composés. Néanmoins, leurs résultats restent limités à la connaissance de la répartition des différentes espèces chimiques dans les différentes matrices environnementales et ne donnent pas accès à la réactivité et la mobilité de ces espèces chimiques dans les écosystèmes naturels. La toxicité et le transfert des composés organométalliques dépendent largement des formes chimiques et des réactions qui les régissent. Le développement d'outils analytiques et expérimentaux est donc nécessaire afin de déterminer la spéciation biogéochimique, qui comprend en plus de la spéciation chimique, la réactivité et la mobilité de chaque espèce, et de ce fait d'appréhender le réel impact de ces polluants sur l'environnement et sur la santé.

Le développement de nouveaux instruments de spectrométrie de masse performants tels que la spectrométrie de masse à plasma à couplage induit (ICPMS) permet à l'heure actuelle une analyse atomique multi-isotopique. Cette nouvelle performance analytique a conduit au développement de nouvelles techniques analytiques et expérimentales se basant sur l'utilisation des isotopes stables. Concernant l'étude de la spéciation du mercure et des butylétains dans l'environnement, l'utilisation des isotopes stables permet deux types de développement. Tout d'abord, à propos de la détermination des concentrations de chaque espèce dans les différentes matrices environnementales, l'utilisation d'espèces enrichies isotopiquement a conduit au développement de nouvelles techniques analytiques justes et précises par dilution isotopique. D'autre part les espèces enrichies isotopiquement offrent un potentiel important en tant que traceurs des processus environnementaux (transferts et transformations) de ces contaminants dans les écosystèmes.

##### **A-IV-1- Les défis analytiques pour la spéciation du mercure et des organo-étains**

D'un point de vue analytique, l'analyse de spéciation des composés organométalliques dans les échantillons environnementaux représente un réel défi analytique en raison, d'une part des très faibles concentrations présentes, et d'autre part de la complexité de matrices étudiées. De plus, cette analyse doit permettre la quantification efficace de chaque espèce chimique sans dégrader ou transformer les entités organométalliques. Toutes les étapes du protocole analytique représentent donc des phases délicates comme le prélèvement, le stockage, la préparation ou encore l'analyse de l'échantillon, et doivent faire l'objet de précautions particulières.

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#### A-IV-1-1- Les techniques analytiques classiques

Dans le cas de l'analyse de spéciation du mercure et des butylétains, le tableau 3 résume les différentes procédures qui sont utilisées pour la préparation ainsi que pour l'analyse des échantillons environnementaux. Généralement ces techniques analytiques combinent une méthode de séparation avec une détection spécifique. Les techniques chromatographiques telles que la chromatographie en phase gazeuse (GC) et la chromatographie en phase liquide (HPLC) sont les plus utilisées. Concernant la détection, différents détecteurs peuvent être utilisés tels que la spectrométrie d'absorption atomique (AAS) ou de fluorescence atomique (AFS), l'émission atomique à plasma induit par micro-ondes (MIPAES) ou à plasma à couplage induit (ICPAES) ou la spectrométrie de masse à plasma à couplage induit (ICPMS). Du fait de son excellente sensibilité, de sa spécificité et de sa possibilité d'analyse multi-élémentaire et multi-isotopique, l'ICPMS est le détecteur de choix pour les analyses de spéciation.

Concernant la préparation de l'échantillon pour les composés du mercure et des butyl-étains, elle comprend généralement plusieurs étapes. Pour des matrices solides, il est tout d'abord nécessaire d'extraire les analytes et de les mettre en solution. En fonction du type d'analyse utilisée, l'échantillon doit subir des étapes de pré-concentration, de purification ou de dérivation.

Un des grands problèmes des analyses de spéciation réside dans l'insuffisante assurance sur les résultats en terme de justesse et de traçabilité des protocoles analytiques. Une méthode analytique peut être contrôlée en utilisant des matériaux de référence certifiés possédant des caractéristiques similaires aux échantillons à analyser. Cependant peu de matériaux de référence sont à l'heure actuelle disponibles et ils sont souvent peu représentatifs des concentrations environnementales.

L'utilisation de la quantification par dilution isotopique s'avère donc une autre alternative pour valider et contrôler les méthodes analytiques. La dilution isotopique a été appliquée aux analyses de spéciation dans le but de contrôler les transformations des différentes espèces pendant l'analyse afin d'éviter des erreurs sur le résultat final. Cependant cette nouvelle approche est limitée à certains types d'analyses puisque que les standards de composés isotopiquement enrichis ne sont pas directement disponibles.

#### A-IV-1-2- La dilution isotopique

##### A-IV-1-2-1- Principe

La dilution isotopique est une technique de quantification qui, contrairement aux méthodes conventionnelles de quantification (étalonnage externe, ajouts dosés), permet de compenser des erreurs dues à des préparations

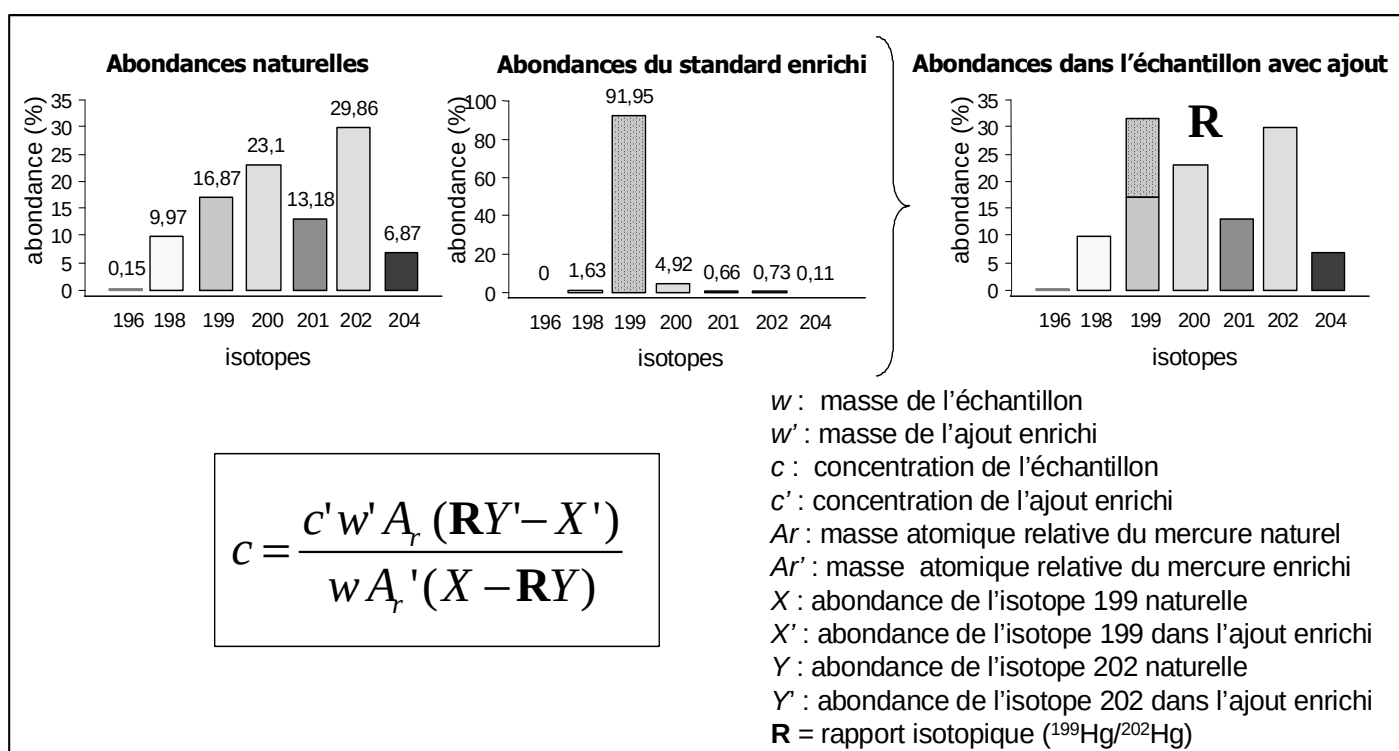
d'échantillon non quantitatives ou à des pertes pendant le traitement de l'échantillon (Kingston et al. 1998, Ruiz et al. 2003). Cette technique est basée sur l'ajout de l'espèce marquée isotopiquement (voir Figure 10).

| Composés                         | Technique       | Procédure                                                                                         | Limite de détection              | Référence               |
|----------------------------------|-----------------|---------------------------------------------------------------------------------------------------|----------------------------------|-------------------------|
| <b>Tissus biologiques</b>        |                 |                                                                                                   |                                  |                         |
| IHg, MMHg                        | GC-MIP-AED      | Extraction : NaCl+HCl (agitation)<br>Derivatization : Grignard (BuMgCl)                           | 0,4 pg (Hg)                      | Emteborg et al. 1994    |
| MMHg                             | GC-CV-AFS       | Extraction : KOH+méthanol (chauffage)<br>Derivatization : éthylation (NaBEt <sub>4</sub> )        | 0,08 ng/g                        | Liang et al. 1996       |
| IHg,MMHg                         | HG-CT-GC-QFAAS  | Extraction : TMAH (micro-ondes)<br>Derivatization : générateur d'hydrures (NaBH <sub>4</sub> )    | 50 pg (Hg)                       | Tseng et al.1997        |
| IHg,MMHg                         | GC-MIP-AED      | Extraction : TMAH (micro-ondes)<br>Derivatization : éthylation (NaBEt <sub>4</sub> )              | 3 pg/g                           | Gebersmann et al. 1997  |
| IHg,MMHg                         | GC-ICP-MS       | Extraction : TMAH (micro-ondes)<br>Derivatization : éthylation (NaBEt <sub>4</sub> )              | 0,15 pg (Hg)                     | Wasik et al. 1998       |
| IHg, MMHg                        | GC-AED          | Extraction : TMAH (micro-ondes)<br>Derivatization : éthylation (NaBEt <sub>4</sub> )              | 0,5 pg                           | Pereiro et al. 1998     |
| IHg,MMHg                         | GC-MIP-AED      | Extraction : TMAH (micro-ondes)<br>Derivatization : éthylation (NaBEt <sub>4</sub> )              | 0,1 ng/g                         | Pereiro et al. 1998     |
| MBT,DBT,TBT, TPhT                | GC-AED          | Extraction : acide acétique (micro-ondes)<br>Derivatization : éthylation (NaBEt <sub>4</sub> )    | 5-10 ng/g                        | Pereiro et al. 1996     |
| MBT, DBT, TBT, TPhT              | GC-MIP-AED      | Extraction : TMAH (micro-ondes)<br>Derivatization : éthylation (NaBEt <sub>4</sub> )              | 2-5 ng/g                         | Schmitt et al. 1997     |
| MBT,DBT,TBT, MPHT,DPhT,TPh       | GC-ICP-MS       | Extraction : TMAH (micro-ondes)<br>Derivatization : éthylation (NaBEt <sub>4</sub> )              | 0,05 pg                          | Pereiro et al. 1999     |
| <b>Sédiments</b>                 |                 |                                                                                                   |                                  |                         |
| IHg, MMHg                        | CT-GC-QFAAS     | Extraction : HCl 6N (micro-ondes)<br>Derivatization : éthylation (NaBEt <sub>4</sub> )            | 50 pg (Hg)                       | Tseng et al. 1997       |
| IHg, MMHg                        | CT-GC-QFAAS     | Extraction : HCl 6N (micro-ondes)<br>Derivatization : générateur d'hydrures (NaBEt <sub>4</sub> ) | 0,5 ng/g                         | Tseng et al. 1998       |
| MMT, MBT, DBT, TBT               | CT-GC-AAS       | Extraction : acide acétique (agitation)<br>Derivatization : éthylation (NaBEt <sub>4</sub> )      | 1 ng/g                           | Martin et al. 1994      |
| Methyl-, butyl-, phenyl-, octyl- | GC-FPD          | Extraction : acide acétique (micro-ondes)<br>Derivatization : éthylation (NaBEt <sub>4</sub> )    | 10 ng/g                          | Lalère et al. 1995      |
| MBT, DBT, TBT                    | GC-AED          | Extraction : acide acétique (micro-ondes)<br>Derivatization : éthylation (NaBEt <sub>4</sub> )    | 2 ng/g                           | Spuznar et al. 1996     |
| MMHg, IHg, MBT, DBT, TBT         | GC-ICPMS        | Extraction : acide acétique (Ultrasons)<br>Derivatization : propylation (NaBPr <sub>4</sub> )     | 52-170 fg (Sn)<br>210 fg (Hg)    | De Smaele et al. 1998   |
| <b>Eaux</b>                      |                 |                                                                                                   |                                  |                         |
| MMHg, IHg,                       | PT-GC-MIP-AES   | Extraction : purge<br>Derivatization : éthylation (NaBEt <sub>4</sub> )                           | 0,6 ng/l MMHg<br>2 ng/l IHg      | Ceulemans et Adams 1996 |
| MBT, DBT, TBT                    | GC-AED          | Extraction : solvant<br>Derivatization : propylation (NaBPr <sub>4</sub> )                        | 7-12 ng/l                        | Schubert et al. 2000    |
| MMHg, MBT, DBT, TBT              | SPME-GC-ICPMS   | Extraction : SPME<br>Derivatization : éthylation (NaBEt <sub>4</sub> )                            | 0,6 ng/l (Hg)<br>0,3-2 ng/l (Sn) | Moens et al. 1997       |
| MMHg, IHg, MBT, DBT, TBT         | SPME-GC-MIP-AES | Extraction : SPME<br>Derivatization : éthylation (NaBEt <sub>4</sub> )                            | 10 ng/l Hg<br>5 ng/l BuSn        | Carpinteiro et al. 2002 |

**Tableau 3 :** Techniques analytiques pour la spéciation du mercure et des organoétains dans différentes matrices environnementales.

Cette espèce chimique marquée isotopiquement a une composition isotopique anormale par rapport à la composition isotopique naturelle de l'élément. Généralement elle est enrichie artificiellement avec un des isotopes de façon à avoir une empreinte isotopique complètement différente de l'espèce naturelle.

Une quantité connue de la molécule marquée, de composition isotopique anormale par rapport à celle de l'espèce à mesurer, est ajoutée à l'échantillon, de préférence avant tout traitement chimique. Par une analyse de spectrométrie de masse, les abondances isotopiques de l'espèce marquée et de l'espèce à doser sont déterminées ainsi que le rapport isotopique résultant pour l'échantillon avec l'ajout d'espèce marquée. La concentration de l'espèce peut être alors directement déduite de ce rapport isotopique et de la quantité de traceur ajoutée par la formule donnée dans la figure 10.



**Figure 10** : Principe de la dilution isotopique (cas du mercure).

#### A-IV-1-2-2- Avantages

Le traceur isotopique est en fait un étalon interne idéal. Les isotopes considérés sont des isotopes du même élément et se comportent donc de la même façon dans la plupart des processus physico-chimiques. Toute perte d'échantillon après marquage va affecter de la même façon les isotopes et donc ne va pas modifier le rapport isotopique. Le traitement chimique après marquage n'a plus besoin d'être quantitatif. Pour les effets de matrice, ils affectent en général de la même façon les isotopes d'un même élément donc ne perturbent pas le dosage.

C'est pour ces raisons que la dilution isotopique est particulièrement performante pour les analyses de spéciation. En effet, pour une analyse de spéciation, la préparation de l'échantillon est souvent longue et complexe. Des pertes, des dilutions ou des réactions non quantitatives vont pouvoir être corrigées par la dilution isotopique. D'autre part, la conservation de la répartition des différentes espèces au cours de la préparation est souvent critique. Grâce au traceur isotopique, il est possible de suivre les transformations ou les dégradations des espèces analysées et ainsi de les prendre en compte dans la détermination de la concentration.

#### A-IV-1-2-3- Instrumentation

Pour l'analyse de spéciation des composés du mercure et des butylétains, le couplage GC-ICPMS est la technique la plus utilisée ([Bouyssié et al. 2002](#)). Elle offre de nombreux avantages tels qu'une excellente sensibilité et sélectivité permettant ainsi de détecter des concentrations très basses. Cette technique permet aussi de faire une détection multi élémentaire et donc de pouvoir analyser les composés du mercure et de l'étain simultanément. Enfin, elle permet de faire une analyse multi-isotopiques et donc de pouvoir utiliser les isotopes stables soit pour la quantification par dilution isotopique soit comme traceurs de processus.

#### A-IV-1-3- Applications aux analyses de spéciation

Depuis peu la technique de quantification par dilution isotopique a été adaptée aux analyses de spéciation. Ses applications sont répertoriées dans le tableau 3 en fonction des espèces et des matrices environnementales étudiées. Néanmoins certaines étapes de la procédure analytique pour la dilution isotopique, qui sont présentées dans la figure 11, peuvent être critiques et nécessitent une attention particulière ([Annexe n°1](#)).

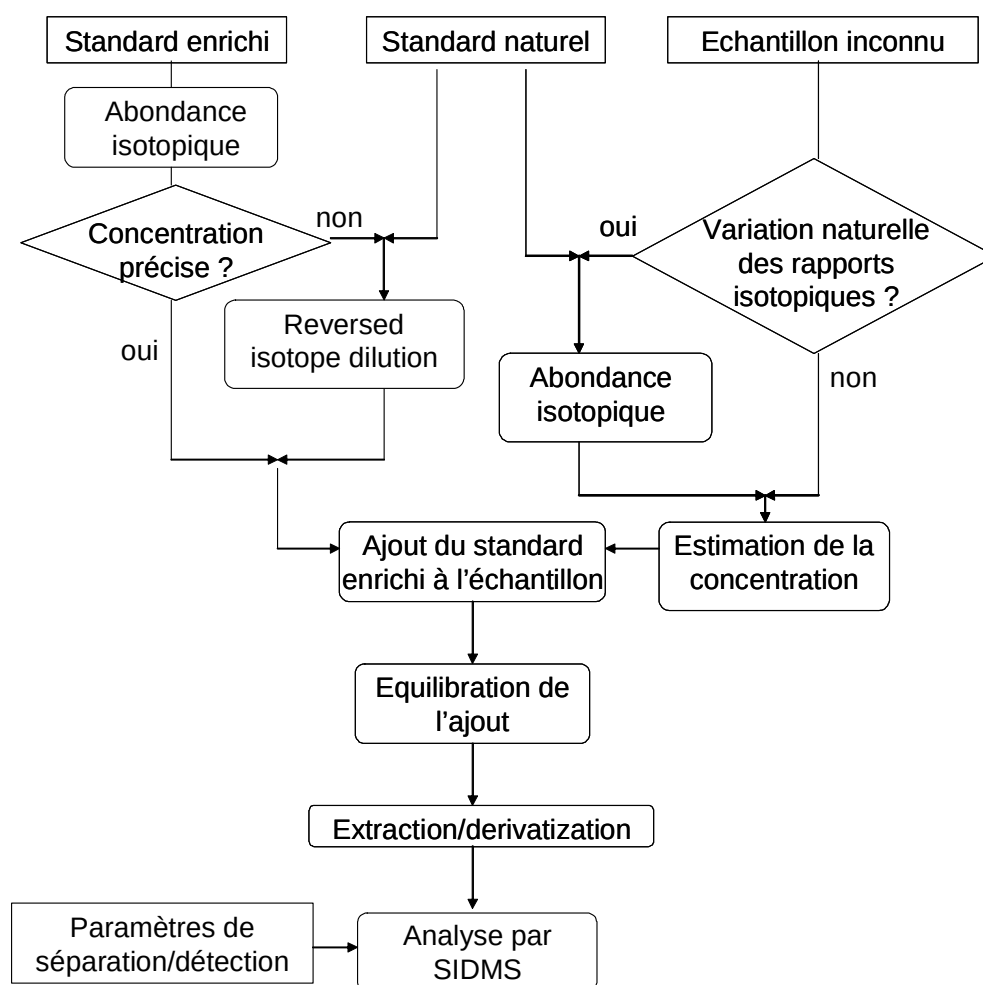
##### A-IV-1-3-1- Les étapes critiques de la dilution isotopique

*Espèces enrichies isotopiquement et caractérisation* - Le standard isotopiquement enrichi doit être exactement sous la même forme chimique que l'espèce à doser. L'enrichissement isotopique doit être choisi de façon à ce que l'isotope majeur soit non-interféré. A l'heure actuelle, aucune espèce organométallique enrichie isotopiquement n'est disponible commercialement. Il faut donc se procurer l'élément sur forme d'oxyde ou métallique puis transformer cet élément en l'espèce recherchée. Ces produits sont relativement chers. Il est donc nécessaire de développer des synthèses organiques à très petite échelle pour fabriquer des composés organométalliques le plus efficacement possible. [Rodriguez et al. \(2002\)](#) ont mis au point une méthode de synthèse du MMHg enrichi à partir de  $^{201}\text{HgO}$  avec un rendement d'environ 90%. [Ruiz Encinar et al. \(2000 et 2002\)](#) ont développé la synthèse du  $^{118}\text{DBT}$  et d'un mélange MBT, DBT et TBT enrichi avec l'isotope 119. Enfin



Sutton et al. (2000) ont développé la synthèse du TBT enrichi avec l'isotope 117 qui a été utilisé pour une campagne de certification d'un sédiment (Annexe n°3).

Afin de faire des analyses justes et précises, il est particulièrement important de connaître précisément la concentration du standard isotopiquement enrichi. Pour cela il faut doser le standard par la méthode de la dilution isotopique inverse (reversed isotope dilution). Le principe est le même que la dilution isotopique mais dans ce cas, le standard isotopiquement enrichi est considéré comme un échantillon auquel on ajoute une quantité connue de standard naturel (qui est alors lui considéré comme le traceur) (Ruiz Encinar et al. 2000, Rodriguez et al. 2001).



**Figure 11:** Etapes critiques de la dilution isotopique pour les analyses de spéciation

*Mise à l'équilibre de l'ajout avec l'échantillon* - De façon à pouvoir prendre en compte tout le protocole analytique, il est important que le standard soit introduit sous la même forme chimique que l'espèce à doser, que l'addition se fasse le plus tôt possible dans le protocole analytique car tout ce qui est perdu avant l'ajout entraîne des erreurs. Le rapport isotopique résultant entre les deux isotopes considérés doit être le proche possible de 1 pour une

meilleure précision sur la mesure du rapport isotopique (Rodriguez et al. 2002). De façon à ce que l'ajout se comporte similairement à l'espèce à analyser, l'équilibre entre les deux doit être optimum. Pour un échantillon sous forme liquide, une simple agitation après l'addition du traceur suffit généralement pour obtenir un mélange homogène et une bonne équilibration. Pour un échantillon solide, on peut utiliser une suspension ou « slurry » qui consiste à faire une suspension de l'échantillon dans un solvant. Le traceur isotopique est alors ajouté à ce mélange et le tout est agité puis séché et soumis à l'extraction (Annexe n°2)

Une des limitations importante de la dilution isotopique est qu'elle ne permet pas de corriger les problèmes de contamination pendant le protocole analytique. Des conditions d'analyse « propres » doivent donc être utilisées pour chaque étape de l'analyse et des « blancs » d'analyse doivent être analysés pour suivre les contaminations éventuelles. La concentration dans l'échantillon sera corrigée par la valeur du blanc.

*Optimisation des paramètres instrumentaux* - Pour l'application de la dilution isotopique aux analyses de spéciation, les paramètres instrumentaux doivent être contrôlés et optimisés soigneusement afin d'obtenir la meilleure précision sur les rapports isotopiques. La première étape de l'analyse consiste en une séparation chromatographique des différentes espèces. La séparation des différentes espèces chimiques se fait généralement soit par chromatographie gazeuse soit par chromatographie liquide (Tableau 4). Pour l'analyse par dilution isotopique, les conditions chromatographiques doivent être optimisées pour avoir la meilleure résolution pour chaque pic ainsi que des pics symétriques pour minimiser les erreurs sur l'intégration. La forme des pics est très importante car le rapport isotopique est calculé par la mesure des aires de pic.

La deuxième étape est l'analyse proprement dite réalisée par ICPMS. Différents paramètres nécessitent une optimisation : le temps mort du détecteur, le biais en masse et les paramètres d'acquisition (Rodriguez et al. 2002). Le temps mort du détecteur correspond au temps avant et après chaque pulse d'acquisition pendant lequel le détecteur n'enregistre rien (Held et Taylor 1999). Si des ions arrivent sur la surface du détecteur pendant ce temps « mort », ils ne seront pas détectés. Le temps mort du détecteur est surtout critique pour des concentrations trop importantes et peut affecter gravement la mesure d'un rapport isotopique. Le temps mort optimum d'un détecteur doit être fixé de manière à ce que les rapports isotopiques soient constants sur une gamme de concentration (Held et Taylor 1999).

Le biais en masse est caractérisé par la différence entre la mesure du rapport isotopique et le rapport théorique. Cette différence est due à une variation de transmission de l'instrument. Cette discrimination instrumentale est due au procédé d'ionisation ou à une différence de détection par le spectromètre de masse. Pour un ICPMS, ce biais est dépendant du temps donc le facteur correctif du biais de masse doit être déterminé périodiquement pendant l'analyse. Il existe deux méthodes pour le déterminer. La méthode du « bracketing » consiste à analyser un échantillon de composition isotopique connue ou certifiée entre chaque mesure de l'échantillon (Ruiz Encinar

et al. 2000). La méthode simultanée avec un standard interne consiste à injecter pendant l'analyse un étalon interne de composition isotopique certifiée (Rodriguez et al. 2002, Monperrus et al. 2003 Annexe n°2).

| Composés        | Méthode                | Isotope                            | Matrice                       | Référence                        |
|-----------------|------------------------|------------------------------------|-------------------------------|----------------------------------|
| DBT             | GC-ICPMS               | $^{118}\text{Sn}$                  | Sédiments                     | Ruiz Encinar et al. 2000         |
| MBT, DBT, TBT   |                        | $^{119}\text{Sn}$                  |                               | Ruiz Encinar et al. 2001         |
| MBT, DBT, TBT   | GC-ICPMS               | $^{118,119}\text{Sn}$              | Sédiments                     | Ruiz Encinar et al. 2002         |
| TBT             | GC-ICPMS               | $^{117}\text{Sn}$                  | Sédiments                     | Yang et al. 2002a                |
| TBT             | HPLC-ICPMS             | $^{117}\text{Sn}$                  | Sédiments                     | Yang et al. 2002b                |
| MBT, DBT, TBT   | GC-ICPMS               | $^{119}\text{Sn}$                  | Eau de mer                    | Rodriguez et al. 2002            |
| MBT, DBT, TBT   | GC-ICPMS               | $^{119}\text{Sn}$                  | Sédiments                     | Ruiz Encinar et al. 2002         |
| MBT, DBT, TBT   | GC-ICPMS               | $^{118}\text{Sn}$                  | Sédiments                     | Inagaki et al. 2003              |
| TBT             | GC-ICPMS               | $^{117}\text{Sn}$                  | Tissus biologiques, sédiments | Monperrus et al. 2003            |
| MMHg            | GC-ICPMS               | $^{201}\text{Hg}$                  | Tissus biologiques            | Rodriguez Martin et al. 2002     |
| MMHg, IHg       | GC-ICPMS<br>HPLC-ICPMS | $^{199,200,201}\text{Hg}$          | Sédiments                     | Hintemann et al. 1997            |
| MMHg            | HPLC-ICPMS             | $^{201}\text{Hg}$                  | Sédiments                     | Wilken et Falter 1998            |
| DMHg, MMHg, IHg | GC-ICPMS               | $^{200}\text{Hg}$                  | Condensas de gaz              | Snell et al. 2000                |
| MMHg, IHg       | GC-ICPMS               | $^{198,199,200,201}\text{Hg}$      | Sédiments                     | Lambersstsson et al. 2001        |
| MMHg            | GC-ICPMS               | $^{201}\text{Hg}$                  | Eau de mer                    | Demunth et Heumann 2001          |
| MMHg            | GC-ICPMS               | $^{198}\text{Hg}$                  | Tissus biologiques            | Yang et al. 2003                 |
| MMHg            | GC-ICPMS               | $^{200}\text{Hg}$                  | Sédiments                     | Hammerschmidt et Fitzgerald 2001 |
| MMHg            | GC-ICPMS               | $^{201}\text{Hg}$                  | Sédiments                     | Rodriguez Martin et al. 2004     |
| MMHg, TBT       | GC-ICPMS               | $^{201}\text{Hg}, ^{117}\text{Sn}$ | Tissus biologiques            | Monperrus et al. 2004            |
| TMPb, TETpB     | HPLC-ICPMS             |                                    | Pluie                         | Brown et al. 1994                |
| Cr(III), Cr(VI) | HPLC-ICPMS             | $^{50,53}\text{Cr}$                | Eaux                          | Kingston et al. 1998             |
| DMTI            | HPLC-ICPMS             |                                    | Eau de mer                    | Schedlbauer et Heumann 1999      |

**Tableau 4** : Applications de la dilution isotopique aux analyses de spéciation.

## A-IV-1-3-2- Les applications de la dilution isotopique

Plusieurs applications de la dilution isotopique pour les analyses de spéciation ont été publiées soit par GC-ICPMS soit HPLC-ICPMS et sont présentées dans le tableau 4. Ces études portent essentiellement sur le développement de la dilution isotopique pour l'analyse de spéciation du mercure et de l'étain ; on trouve aussi des applications pour la spéciation du plomb, du chrome et du thallium. Toutes ces études montrent le potentiel important de cette technique en terme de justesse et de précision.

## A-IV-1-3-3- Le suivi des transformations pendant l'analyse

Ces techniques de quantification par dilution isotopique sont utiles non seulement pour faire des analyses de routine justes et précises mais elles permettent aussi de tester et diagnostiquer les nouvelles méthodes analytiques. Grâce au marquage des différentes espèces, il devient ainsi possible de suivre les réactions de réarrangement entre les différentes espèces qui peuvent se produire pendant tout le protocole analytique. Chaque étape de la préparation de l'échantillon est critique et peut modifier la répartition des espèces.

[Rodriguez et al. \(2002\)](#) ont utilisé le MMHg isotopiquement enrichi avec l'isotope 201 pour suivre la dégradation du MMHg pendant la dérivation d'extraits de tissus biologiques. Ils ont pu mettre en évidence la formation de mercure élémentaire qui provient de la dégradation à la fois du MMHg et du IHg pendant l'éthylation. [Demunth et al. \(2001\)](#) ont aussi utilisé du MMHg enrichi isotopiquement pour valider l'étape de dérivation pour l'analyse d'échantillons d'eau. La transformation du MMHg en Hg<sup>0</sup> a été mise en évidence pendant l'éthylation et a pu être attribuée à la présence d'ions (Cl<sup>-</sup>, Br<sup>-</sup>) particulièrement importants dans les échantillons d'eau de mer.

Plusieurs études ont aussi mis en évidence la formation accidentelle de MMHg à partir du IHg pour l'analyse de sédiments ([Hintelmann 1995](#), [Falter et Wilken 1998](#), [Hammerschmidt et Fitzgerald 2001](#), [Rodriguez et al. 2003](#)). Dans les sédiments, la concentration en MMHg ne dépasse pas quelques pourcents du mercure total, donc même une méthylation du IHg très faible génère une erreur importante sur la concentration en MMHg. L'utilisation d'isotopes stables a permis de déterminer les facteurs et les étapes de la préparation responsables de cette transformation. [Hintelmann et al. \(1997a\)](#) ont montré que cette formation accidentelle se produisait pendant l'étape de distillation. [Wilken et Falter \(1998\)](#) ont montré que le MM<sup>200</sup>Hg généré par l'ajout de <sup>200</sup>IHg pendant la préparation de l'échantillon permettait de corriger l'artéfact. En comparant le MM<sup>200</sup>Hg formé pour différentes techniques de préparation de l'échantillon, [Hammerschmidt et Fitzgerald \(2001\)](#) ont montré que la formation de MMHg se produisait aussi bien en utilisant l'extraction acide que la distillation. Enfin une série d'expériences systématiques a été réalisée par [Rodriguez et al. \(2003\)](#) afin de localiser les sources de formation de MMHg. Différentes méthodes de dérivation et d'ajout de traceur isotopique ont été utilisées et ont montré que

la formation de MMHg ne se produit pas pendant l'extraction mais pendant la dérivation et dépend des concentrations en carbone organique et en soufre.

Concernant le TBT, plusieurs études ont examiné sa possible dégradation en DBT et MBT pendant la préparation de l'échantillon (Hill et al. 2000, Ruiz Encinar et al. 2002a, Ruiz Encinar et al. 2002b). Hill et al. (2000) ont étudié la dégradation du TBT pour l'analyse de sédiments et ont montré la formation de composés mixtes d'étain avec des groupements butyl et phényl. Différentes techniques d'extraction du TBT dans les sédiments telles que l'extraction par agitation, sous champ micro-ondes ou par ultrasons, ont été testées par Ruiz Encinar et al. (2002a) en utilisant un ajout mixte contenant du  $^{119}\text{MBT}$ ,  $^{118}\text{DBT}$ ,  $^{119}\text{TBT}$ . En mesurant les rapports isotopiques  $^{120}\text{Sn}/^{118}\text{Sn}$  et  $^{120}\text{Sn}/^{119}\text{Sn}$  pour chaque espèce et en développant un modèle mathématique, ils ont pu déterminer les facteurs de dégradation de chaque espèce. Les mêmes auteurs (Ruiz Encinar et al. 2002b) ont aussi contrôlé l'analyse des butylétains dans les sédiments par l'utilisation de la technique d'extraction accélérée par solvant (ASE).

#### A-IV-2- Utilisation des isotopes stables comme traceurs des processus environnementaux

Les traceurs isotopiques stables permettent aussi de suivre les processus environnementaux dynamiques tels que les échanges et les transformations des composés du mercure et des butylétains dans les environnements aquatiques. En effet, afin de bien comprendre le cycle biogéochimique de ces polluants, il est indispensable de connaître les conditions environnementales nécessaires pour leur transformation ainsi que le taux et la cinétique de ces transformations.

Les premières méthodes expérimentales utilisaient les espèces marquées radioactives mais du fait de leur faible activité, les concentrations à ajouter étaient souvent très grandes (Ramlal et al. 1986, Gilmour et al. 1998). Ces expériences ne reproduisaient pas réellement les processus naturels qui pouvaient être biaisés par une différence de partition ou de toxicité des espèces ajoutés par rapport aux espèces naturellement présentes dans l'environnement. De plus, la plupart des processus environnementaux sont des processus couplés et opposés comme par exemple l'adsorption et la désorption, l'oxydation et la réduction, l'assimilation et la dépuration, la méthylation et la déméthylation. Afin de connaître la contribution de chacun des mécanismes sur le résultat net, il est nécessaire de discriminer les deux processus antagonistes. En utilisant plusieurs espèces enrichies avec différents isotopes, il est alors possible de connaître le taux brut de chacun des processus.

Enfin, en utilisant ce type de méthode, il est aussi possible de contrôler simultanément le devenir des espèces ambiantes qui sont naturellement présentes dans l'environnement.

#### A-IV-2-1- Principe et mise en œuvre

La transformation d'espèces marquées isotopiquement est facilement détectable en mesurant les rapports isotopiques de chaque espèce chimique. La précision sur les rapports isotopiques étant généralement excellente, il est possible de travailler avec des concentrations de traceur très faibles afin de ne pas perturber le système tout en détectant les transformations même très minimes. [Hintelmann et Evans \(1997b\)](#) proposent des équations mathématiques pour calculer les taux de méthylation et de déméthylation du mercure basées sur la conservation des masses. Ils ont aussi déterminé les limites de détection pour ces taux de transformation. Les limites de détection pour la méthylation et la déméthylation du mercure sont dépendantes de la concentration naturellement présente du MMHg ou du IHg respectivement ainsi que de la précision sur le rapport isotopique. Le principe ainsi que les calculs sont résumés dans la figure 12 dans le cas de la méthylation/déméthylation du mercure. Les espèces marquées isotopiquement utilisées sont le  $^{201}\text{MMHg}$  pour suivre la déméthylation et le  $^{199}\text{IHg}$  pour suivre la méthylation. A la fin de la période d'incubation, des conversions d'espèces peuvent être observées modifiant les rapports isotopiques de chacune d'elles. Les équations sont données pour le calcul des concentrations des espèces formées. Les taux de méthylation et de déméthylation sont calculés directement en divisant la quantité d'espèce formée par la quantité d'espèce ajoutée.

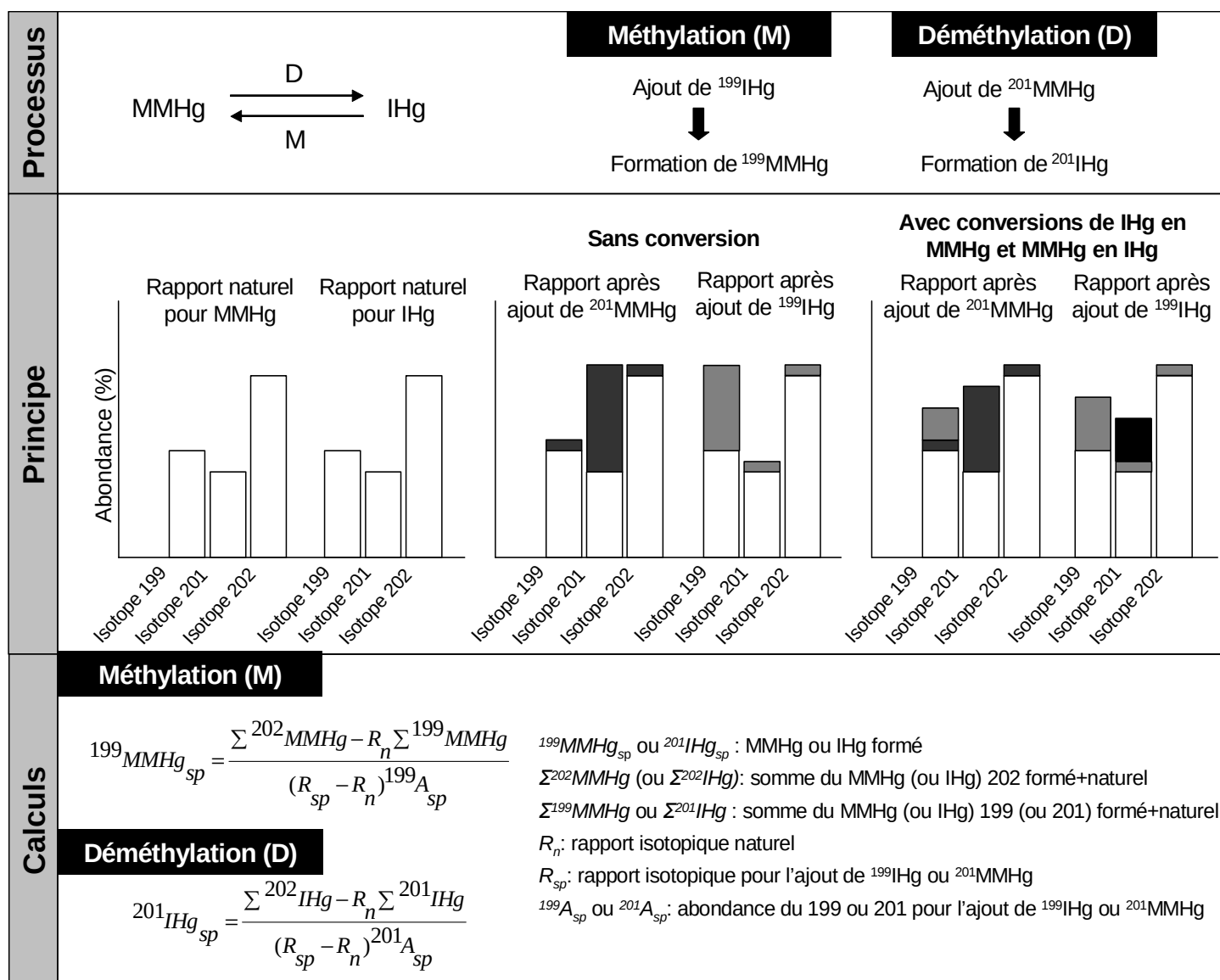
Ces méthodes expérimentales utilisant les traceurs isotopiques peuvent être mises en œuvre pour les différentes techniques d'incubation généralement utilisées telles que des incubations en laboratoire (aquarium, mésocosmes,...) ou in situ (carottes de sédiment, enceintes...).

#### A-IV-2-2- Applications environnementales

A l'heure actuelle, toutes les méthodes expérimentales utilisant les isotopes stables concernent l'étude des processus environnementaux des espèces du mercure. Ces applications sont récapitulées dans le tableau 5. La plupart de ces investigations ont porté sur l'étude des processus de méthylation et déméthylation dans les sédiments et les méthodes expérimentales utilisées sont de deux types.

Tout d'abord, des incubations de suspension de sédiments (ou « slurry ») permettent de déterminer les taux de méthylation et déméthylation dans un système où les paramètres tels que la température, l'oxygène, la lumière, l'activité biologique peuvent être parfaitement contrôlés ([Hintelmann et al. 2000](#), [Rodriguez et al. 2004](#)). De plus ce type d'incubation permet une étude cinétique des transformations en déterminant les potentiels à différents intervalles de temps. [Hintelmann et al. \(2000\)](#) ont réalisé des incubations de suspension de sédiment de lac en ajoutant du  $^{202}\text{MMHg}$  et du  $^{199}\text{IHg}$  à des concentrations proches des concentrations ambiantes. Ils ont pu déterminer les taux de transformations spécifiques des deux processus et les comparer avec les taux calculés pour les espèces naturellement présentes. Le traceur  $^{199}\text{IHg}$  semble être méthylé plus vite que le IHg ambiant ce

qui indique la plus grande disponibilité de l'ajout isotopique par rapport à l'espèce déjà présente. Pour le MMHg, la dégradation semble être du même ordre de grandeur pour le MMHg ajouté et le MMHg naturel. Enfin la disponibilité du IHg pour la méthylation a été examinée en ajoutant le IHg complexé à différents ligands susceptibles d'être présents dans l'environnement tels que les nitrates, les acides fulviques et les sulfures. Les ligands organiques tels que les acides fulviques semblent limiter la disponibilité du IHg pour la méthylation alors que les sulfures semblent l'augmenter.



**Figure 12** : Principe de l'utilisation des traceurs isotopiques stables pour la méthylation/déméthylation du mercure.

Rodriguez et al. (2004) ont aussi développé des expérimentations en utilisant des espèces de mercure enrichies isotopiquement ( $^{201}\text{MMHg}$ ,  $^{199}\text{IHg}$ ) sur une suspension de sédiment estuarien. Afin de comprendre les paramètres environnementaux régulant la méthylation et la déméthylation du mercure, des incubations en condition

d'aérobiose et d'anaérobiose ont été effectuées ainsi que des incubations en condition stériles et non stériles. La formation et la stabilité du MMHg pour ce type de sédiment semblent être optimum en condition d'anaérobiose et biotique ce qui corrobore avec une méthylation induite microbiologiquement par les BSR. En revanche, des conditions d'aérobiose stériles et non stériles semblent être propices à la dégradation du MMHg. Les produits de dégradation du MMHg ont été identifiés comme étant le IHg ainsi que des espèces de mercure volatiles.

| Compartiments           | Processus                                      | Mise en oeuvre                                                                                                           | Référence                        |
|-------------------------|------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------|----------------------------------|
| Sédiments               | Méthylation<br>déméthylation                   | Suspension de sédiment<br>Traceur du IHg complexé à différents ligands (nitrates, sulfures, acides fulviques)            | Hintelmann et al. 2000           |
| Sédiments               | Méthylation                                    | Carotte de sédiment<br>Partition eau interstitielle/phase solide                                                         | Hammerschmidt et Fitzgerald 2003 |
| Sédiments               | Méthylation<br>déméthylation<br>volatilization | Suspension de sédiment<br>Conditions oxygène/anoxygène<br>biotique/abiotique                                             | Rodriguez et al. 2004            |
| Algues, eau, périphyton | Méthylation<br>déméthylation                   | Incubation de différentes algues avec les bactéries associées                                                            | Mauro et al. 2002                |
| Forêt boréale           | Transferts végétation/sol/atmosphère           | Aspergiment de la végétation<br>échantillonnage air, végétation, sol et eau de ruissellement.                            | Hintelmann et al. 2004           |
| Eau                     | Formation et évaporation de DGM                | Enceinte dans un lac enfermant le sédiment et la colonne d'eau                                                           | Amyot et al. 2004                |
| Eau                     | Formation et évaporation de DGM                | Enceinte dans un lac enfermant le sédiment et la colonne d'eau<br>Etude des populations bactériennes et du phytoplancton | Poulain et al. 2004              |

**Tableau 5 :** Etudes sur les transformations des composés du mercure par l'utilisation de traceurs isotopiques.

Le deuxième type d'expérimentations environnementales est l'incubation de sédiment *in situ* (Hammerschmidt et Fitzgerald 2004). Pour cela le sédiment est échantillonné en carotte avec des tubes préalablement percés afin de pouvoir injecter les espèces marquées de mercure aux différentes profondeurs. Après le temps d'incubation, les potentiels de méthylation/déméthylation sont déterminés pour chaque tranche de sédiment. Les principaux



avantages de cette technique d'incubation par rapport à la méthode des « slurry », sont que les conditions d'incubation sont très proches des conditions environnementales et qu'elle permet d'avoir une connaissance de la répartition des processus sur la profondeur. Ainsi, [Hammerschmidt et Fitzgerald \(2004\)](#) ont montré que la méthylation du mercure était maximale pour les sédiments de surface et variait en fonction des saisons.

D'autres applications des isotopes stables pour tracer les processus environnementaux ont été effectuées dans le cadre du projet METAALICUS (Mercury Experiment To Assess Atmospheric Loading in Canada and the United States) ([Hintelmann et al. 2003](#), [Amyot et al. 2004](#), [Poulain et al. 2004](#)). Le but de ce projet était de déterminer la relation entre le taux de mercure dans l'atmosphère et le taux de mercure dans les poissons des lacs canadiens. Plusieurs expérimentations *in situ* à l'échelle d'un écosystème entier (forêt boréale) ont été effectuées en utilisant des ajouts de  $^{202}\text{IHg}$ ,  $^{201}\text{IHg}$  et  $^{198}\text{IHg}$  sur les trois composantes de l'écosystème (le lac, sa zone humide et son bassin versant). Les premiers résultats portaient sur la dynamique du mercure fraîchement déposé par aspergissement sur la forêt boréale par rapport au mercure anciennement déposé dans l'écosystème ([Hintelmann et al. 2003](#)). Des expérimentations supplémentaires ont été effectuées grâce à des mésocosmes disposés dans un lac enfermant le sédiment et la colonne d'eau afin de comprendre la formation d'espèces dissoutes gazeuses du mercure ([Amyot et al. 2004](#), [Poulain et al. 2004](#)).

## **A-V- Présentation du travail**

L'étude de la spéciation et du devenir du MMHg et du TBT dans les environnements côtiers a connu de nombreuses avancées durant les dernières décennies. Cependant, la connaissance du cycle biogéochimique du mercure et des butylétains dans ces environnements demeure incomplète non seulement du fait de sa complexité mais aussi d'un manque d'outils analytiques performants pour l'analyse de spéciation et le suivi des processus environnementaux.

Les objectifs de cette thèse peuvent être séparés en deux parties distinctes mais qui sont complémentaires. La première porte sur le développement de techniques analytiques justes et sensibles pour l'analyse de spéciation du mercure et des butylétains dans les différentes matrices de l'environnement par la technique de dilution isotopique. Le but de ce travail est d'appliquer ces nouvelles méthodes d'analyse de spéciation du mercure et des butylétains à des échantillons environnementaux réels pour lesquels les concentrations sont en général très basses. Ce travail tente de surmonter les problèmes analytiques généralement rencontrés pour l'analyse de spéciation (stockage, préparation de l'échantillon, détection) et de porter une attention particulière à l'analyse simultanée des espèces de mercure et de butylétains afin de simplifier et de réduire le temps d'analyse.

La deuxième partie de ce travail porte sur l'étude des processus environnementaux de transferts et transformations des composés de mercure et des butyl-étains dans les environnements côtiers et plus particulièrement sur l'étude des bio-transformations du mercure. L'objectif de cette étude est de développer des méthodes expérimentales d'échantillonnage et d'incubations de différentes composantes des environnements côtiers que sont les compartiments (sédiments, colonne d'eau) et les organismes (organismes benthiques, populations bactériennes et phytoplanctoniques). Par le couplage de méthodes expérimentales performantes grâce à l'utilisation de traceurs isotopiques stables avec l'étude des paramètres biologiques et microbiologiques, ce travail cherche à mettre en évidence l'importance et l'influence des activités biologiques sur le cycle biogéochimique du mercure.

### **A-V-1- Développement de techniques analytiques pour la spéciation du mercure et des butylétains**

L'analyse de spéciation du mercure et des butylétains comporte plusieurs étapes de préparation d'échantillon, de séparation des espèces et de détection. Toutes ces étapes représentent des manipulations critiques en terme de rendement, de contamination et de conservation des espèces. L'objectif de ce travail est d'appliquer la technique de quantification par dilution isotopique aux analyses de spéciation afin de corriger les pertes ou les transformations éventuelles lors de la procédure analytique.

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Toutes les étapes de préparation d'échantillon, comme l'extraction ou la dérivation, peuvent engendrer des rendements non quantitatifs ou des pertes ce qui génèrent des résultats faux. L'application de la dilution isotopique aux analyses de spéciation permet de contrôler et de corriger ces erreurs. Une revue des différentes applications des isotopes stables pour les analyses de spéciation est réalisée démontrant le potentiel de cette technique permettant d'améliorer les performances des techniques analytiques de spéciation et de tracer les processus environnementaux. (**Annexe, article E-I**).

Une partie importante dans cette étude est d'utiliser les atouts de la dilution isotopique pour développer des analyses de spéciation multi-élémentaires qui généralement nécessitent une méthode analytique spécifique à chaque élément afin que l'analyse soit quantitative. Dans le cas du mercure et des butyl-étains, ce travail consiste à optimiser une méthode simultanée à la fois pour la préparation d'échantillon et pour l'analyse des différentes espèces en utilisant la dilution isotopique pour corriger les rendements non quantitatifs possibles. Ces développements pour l'analyse de spéciation simultanée des composés du mercure et des butylétains sont réalisés pour des tissus biologiques (**Chapitre B, article B-I**) et pour des échantillons d'eaux naturelles (**Chapitre B, article B-II**). Des méthodes analytiques utilisant la technique de dilution isotopique pour l'analyse du TBT dans les différentes matrices environnementales (tissus biologiques et sédiments) sont développées (**Annexe, article E-II**). Différentes méthodes de mise à l'équilibre de l'ajout enrichi isotopiquement sont aussi étudiées afin d'optimiser cette étape critique. Ces techniques ont aussi été utilisées dans le cadre d'un programme, géré par le Comité Consultatif pour la Quantité de Matière, pour la certification de la concentration en TBT d'un sédiment marin par dilution isotopique (**Annexe, article E-III**).

D'autre part, dans le cas du mercure et des butylétains, des réactions d'alkylation/dé-alkylation peuvent avoir lieu pendant la préparation d'échantillon et ainsi modifier les concentrations initiales de chaque espèce chimique. L'intérêt de la dilution isotopique est de pouvoir suivre ces transformations et surtout de pouvoir les corriger. Dans le cas du mercure, la formation artificielle de MMHg à partir du IHg est critique surtout pour l'analyse de sédiments pour lesquels la proportion de MMHg par rapport à IHg est généralement très faible. Dans ce travail, l'utilisation d'espèces enrichies isotopiquement a été appliquée à la correction de cet artéfact ainsi qu'à la détermination de ses causes. (**Chapitre B, articles B-III**).

#### **A-V-2- Transferts et transformations du mercure et des butylétains dans les environnements côtiers**

Dans ce travail, les techniques analytiques par dilution isotopique développées précédemment sont ensuite utilisées pour l'analyse d'échantillons environnementaux réels afin de mieux comprendre les cycles biogéochimiques du mercure et des butylétains dans les environnements côtiers. Ces techniques analytiques justes et sensibles permettent le suivi des concentrations environnementales et surtout l'étude des processus de

transferts et de transformations qui régulent les concentrations de chaque espèce et donc les risques écologiques pour l'écosystème voire les risques pour la santé publique.

Tout d'abord, une des applications est de déterminer le transfert de ces polluants vers les organismes aquatiques en terme de bioaccumulation et de bioamplification. Dans les environnements côtiers, les organismes benthiques jouent un rôle important au niveau de la chaîne alimentaire et sont directement exposés aux composés du mercure et des butylétains stockés dans les sédiments. Une approche multi-disciplinaire entre chimistes et biologistes permet d'obtenir des informations non seulement sur les niveaux de contamination des populations benthiques et la possible bioamplification par ces premiers maillons de la chaîne alimentaire mais aussi sur l'impact écologique en terme de biodiversité des organismes benthiques (**Chapitre C, article C-I**).

Enfin la dernière partie de ce travail est plus focalisée sur les composés du mercure et leur transformations dans les différents compartiments des environnements côtiers avec comme but premier de déterminer et de prédire le devenir et les concentrations en méthylmercure. Un point important de cette étude réside dans l'utilisation d'espèces enrichies isotopiquement et le développement d'expérimentations en laboratoire ou *in situ* afin de quantifier les processus de méthylation/déméthylation et de mieux connaître les facteurs environnementaux régulant ces transformations.

L'élaboration de ces expérimentations doit prendre en compte la spécificité de chaque compartiment de l'environnement. Les différents compartiments que sont les sédiments et la colonne d'eau, sont des endroits avec des dynamiques et des paramètres différents tant sur le plan physico-chimique que sur le plan biologique. Par exemple, les sédiments constituent un compartiment important pour le devenir de ces composés en raison de leur rôle de réacteur pour l'activité bactérienne et pour les réactions chimiques. Ils jouent aussi le rôle de piège ou de source pour la colonne d'eau et les processus géochimiques associés sont ordonnés et stratifiés selon la profondeur. La colonne d'eau constitue aussi un compartiment privilégié pour les réactions chimiques notamment photochimiques ainsi que les transformations biotiques grâce à la présence d'organismes spécifiques comme le phytoplancton. L'influence de la lumière ainsi que l'intensité des activités biologiques sont alors des facteurs à prendre en compte pour l'étude des transformations dans ce compartiment.

Un des objectifs de cette thèse est donc de développer des procédures d'incubations, en utilisant les espèces enrichies isotopiquement, qui sont spécifiques à chacune de ces matrices afin de mettre en évidence les liens entre les processus de transformations du mercure et les activités biologiques de certains organismes tels que les bactéries et le phytoplancton présents dans l'environnement. Ce travail a tout d'abord consisté à étudier les réactions de méthylation et de déméthylation dans les sédiments et l'eau de surface d'un écosystème côtier, l'étang de Thau (**Chapitre C, article C-II**). Ces taux de transformations dans les sédiments et la colonne d'eau sont aussi mis en relation avec les échanges à l'interface eau-sédiment mesurés grâce à l'utilisation de chambres benthiques (**Annexe, article E-IV**).

Les techniques expérimentales utilisant les traceurs isotopiques stables sont aussi appliquées à l'étude des transformations des espèces de mercure (méthylation, déméthylation et réduction) dans les eaux côtières et marines de la Méditerranée. Différents modes d'incubations permettent de discriminer les facteurs contrôlant ces divers mécanismes tels que les réactions photochimiques et biotiques (**Chapitre C, article C-III**).

Enfin, contrairement à beaucoup d'études sur la méthylation du mercure par les bactéries qui utilisent des souches bactériennes commerciales, un des buts de ce travail est de déterminer, parmi les bactéries naturellement présentes dans un sédiment ayant un potentiel de méthylation, quelles sont celles qui méthylent le mercure et quel est l'impact direct sur l'écosystème étudié (**Chapitre C, article C-IV**).

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## **Chapitre B – I**

**Analyse simultanée du MMHg et du TBT par dilution isotopique  
pour un échantillon certifié d'huître**

## **Analyse simultanée du MMHg et du TBT par dilution isotopique pour un échantillon certifié d'huître**

Le méthylmercure (MMHg) et le tributylétain (TBT) sont reconnus comme des polluants majeurs des environnements aquatiques du fait de leur toxicité et de leur impact sur les écosystèmes. La prise de conscience de ces risques a stimulé le développement des analyses de spéciation mais celles-ci représentent un réel défi analytique en raison d'une part des faibles concentrations présentes, et d'autre part de la complexité des matrices étudiées. De plus, l'analyse de ces espèces chimiques doit permettre la quantification efficace sans dégrader ou transformer les entités organo-métalliques.

Malgré l'important développement des analyses de spéciation par des techniques analytiques de plus en plus performantes, certaines limitations sur la qualité des résultats sont néanmoins observées. En effet, en utilisant des techniques de quantification classiques telles que les ajouts dosés, la qualité des résultats peut être affectée par des pertes ou des transformations des espèces chimiques pendant la préparation de l'échantillon. La quantification par dilution isotopique représente une nouvelle alternative pour éliminer les problèmes habituellement rencontrés lors des analyses de spéciation. En effet, la dilution isotopique permet généralement de corriger des préparations d'échantillon non quantitatives, des pertes et des variations de sensibilité de l'instrumentation. Elle permet aussi d'améliorer les résultats en terme de justesse et de précision.

L'objectif de ce travail est de mettre à profit les avantages de la quantification par dilution isotopique afin de déterminer simultanément les concentrations en MMHg et TBT dans des tissus biologiques. Une méthode analytique a été développée proposant des conditions d'extraction optimum pour extraire simultanément le MMHg et le TBT d'un tissu d'huître ainsi que des conditions analytiques pour le couplage GC-ICPMS. La procédure de mise à l'équilibre du traceur isotopique avec l'échantillon a aussi été améliorée.

Le principal résultat de cette étude est de proposer une méthode rapide, juste et précise pour l'analyse simultanée du MMHg et du TBT dans les tissus biologiques. En effet, la préparation de l'échantillon est réalisée en moins de cinq minutes et les performances analytiques (justesse, précision) sont améliorées par rapport à la quantification par étalonnage externe.

*Anal. Chem.* 2003, 75, 4095–4102

## Simultaneous Sample Preparation and Species-Specific Isotope Dilution Mass Spectrometry Analysis of Monomethylmercury and Tributyltin in a Certified Oyster Tissue

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A rapid, accurate, sensitive, and simple method for simultaneous speciation analysis of mercury and tin in biological samples has been developed. Integrated simultaneous sample preparation for tin and mercury species includes open focused microwave extraction and derivatization via ethylation. Capillary gas chromatography–inductively plasma mass spectrometry (CGC–ICPMS) conditions and parameters affecting the analytical performance were carefully optimized both for species-specific isotope dilution analysis of MMHg and TBT and for conventional analysis of MBT and DBT. <sup>201</sup>Hg-enriched monomethylmercury and <sup>117</sup>Sn-enriched tributyltin were used for species-specific isotope dilution mass spectrometry (SIDMS) analysis. As important, accurate isotope dilution analysis requires equilibration between the spike and the analyte to achieve successful analytical procedures. Since the spike stabilization and solubilization are the most critical and time-consuming steps in isotope dilution analysis, different spiking procedures were tested. Simultaneous microwave-assisted spike stabilization and solubilization can be achieved within less than 5 min. This study originally introduces a method for the simultaneous speciation and isotope dilution of mercury and tin in biological tissues. The sample throughput of the procedure was drastically reduced by fastening sample preparation and GC separation steps. The accuracy of the method was tested by both external calibration analysis and species-specific isotope dilution analysis using the first biological reference material certified for multielemental speciation (oyster tissue, CRM 710, IRMM). The results obtained demonstrate that isotope dilution analysis is a powerful method allowing the simultaneous speciation of TBT and MMHg with high precision and excellent accuracy. Analytical problems related to low recovery during sample preparation are thus minimized by SIDMS. In addition, a rapid procedure allows us to establish a performant routine method using CGC–ICPMS technique.

The widespread use of organometallic compounds and their subsequent release into the environment has created great

environmental concern about the toxicity and effects of these pollutants. Tin and mercury compounds play an important role in environmental pollution, as they can be both anthropogenically introduced as well as naturally formed in the environment by biomethylation processes.<sup>1</sup> Tributyltin (TBT) has been extensively used as a biocide in antifouling paints ever since the early 1970s and has found its way into the marine environment causing extensive damage to nontarget organisms. Shell deformations in oysters,<sup>2,3</sup> sex changes (imposex) in whelks,<sup>4,5</sup> and harmful effects in marine life at very low concentrations have been reported.<sup>6</sup> As a result, several countries have introduced regulations over the last twenty years to limit the use of TBT in antifouling paints on vessels smaller than 25 m.<sup>7</sup> TBT breaks down in aqueous media under the action of light (photolysis) and microorganisms (biodegradation) into less toxic dibutyltin (DBT) and monobutyltin (MBT). They degrade slowly in water but are quite stable in sediments where they are concentrated. In the case of mercury, fish consumption is the major contribution to mercury risk for human and wild life. Fish tend to concentrate monomethylmercury by a factor of 10<sup>5</sup>–10<sup>7</sup>, which can lead to dangerous levels in their tissues even in areas with tolerable Hg concentrations.<sup>8</sup> This toxicity has led regulatory agencies to focus on fish as the target organisms to protect the health of humans and other sensitive organisms. For example, the U.S. Food and Drug Administration set an advisory standard of 1 ppm wet weight in fish flesh. Finding fish in a body of water that exceed established advisories led

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*Analytical Chemistry*, Vol. 75, No. 16, August 15, 2003 4095

health agencies to issue health warnings regarding fish consumption.

The recognition of these hazards has stimulated the interest of regulatory agencies and quality control laboratories in speciation analysis. The analysis of these metal(loid) species at low concentration levels requires extremely sensitive and selective separation and detection techniques. Inadequate sample preparation and analytical procedures may produce inaccurate data and inadequate interpretation about their biogeochemical cycle and ecological impact. Hyphenated techniques including species separation and detection offer a most promising approach in environmental analysis nowadays.<sup>9–12</sup> In combination with gas chromatography, inductively coupled plasma mass spectrometry provides excellent sensibility, selectivity, and multielemental<sup>9–13</sup> and multiisotope detection capabilities.<sup>14–17</sup>

Indeed despite the good performances of analytical techniques, several limitations concerning quality in species preservation and efficiency of recovery remain at the sample preparation level. Sample preparation for tin and mercury speciation requires extraction and derivatization steps often under different conditions for each element. Open microwave-assisted digestion has gained wide acceptance as a rapid and efficient method for sample extraction for speciation analysis.<sup>18–20</sup> The extraction of organotins from biological samples is generally performed with acetic acid.<sup>18–20</sup> Alkaline hydrolysis with tetramethylammonium hydroxide (TMAH) has been also successfully applied to biological tissues.<sup>21</sup> It was observed that, under microwave field, a short extraction time (3 min) allows the solubilization of biological tissues and the complete extraction of butyltin compounds. Mercury compounds are often extracted with nitric acid for sediment samples<sup>22</sup> and KOH/ methanol<sup>23</sup> or TMAH for biological samples.<sup>17,20,23</sup> Once the extraction step is performed, the organometallic species are derivatized in order to obtain volatile species. This step can be performed using Grignard reagent or hydride generation, but aqueous ethylation remains an accurate method for both mercury and tin compounds.<sup>24–26</sup> With the

introduction of microwave extraction and sodium tetraethylborate ( $\text{NaBEt}_4$ ) as derivatizing reagent, simultaneous sample preparation for tin and mercury compounds appears achievable.<sup>27–29</sup>

Isotope dilution analysis represents a good alternative method to avoid traditional problems related to nonquantitative recoveries and losses during the sample preparation procedure. Isotope dilution mass spectrometry (IDMS) analysis has been employed in a wide variety of samples for element trace analysis. If the equilibration of the spike and the analyte is fully achieved, IDMS is theoretically capable of compensating for nonquantitative sample preparation procedures, negating losses of analytes and instrument drift. However, the application of isotope dilution for speciation analysis is still limited by the commercial availability of isotopically enriched species. Another inconvenience remains the need for longer analytical time in comparison to conventional analysis, especially due to the spike equilibration step. Few applications of isotope dilution for speciation analysis have been published recently.<sup>14–17,30–35</sup> Species-specific isotope dilution mass spectrometry (SIDMS) analysis has been applied for the determination of MMHg in biological samples.<sup>17,31</sup> Gelaude et al.<sup>31</sup> have performed solid sampling electrothermal vaporization inductively plasma mass spectrometry (ICPMS) analysis with an isotopically enriched gaseous Hg spike. Rodriguez Martin Doimeadios et al.<sup>17</sup> have used an isotopically enriched MMHg standard synthesized from mercury oxide.<sup>36</sup> SIDMS was also applied for analysis of TBT in sediments<sup>15,16,32</sup> and biological tissues<sup>32</sup> with the first commercial enriched TBT standard and has shown an important enhancement of precision and accuracy. Encinar et al.<sup>16</sup> have developed a method for the determination of three butyltin compounds in sediments using homemade isotopically labeled spikes.

The objective of this work was to take advantage of the high accuracy and precision offered by ID in combination with the multielemental detection power of the ICPMS for the simultaneous speciation of mercury and tin in biological samples. To exploit this opportunity for speciation analysis, simultaneous extraction, derivatization, and detection of the different species need to be achieved. This approach is promising for saving analytical time and reducing analysis cost. External calibration and isotope dilution analysis were used throughout in order to get a better picture of the advantages and the limitations of this method. Since the most critical and time-consuming step is the equilibration of

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**Table 1. CGC–ICPMS Parameters for the Simultaneous Separation of Tin and Mercury Species**

| GC Parameters              |                                                                                        |
|----------------------------|----------------------------------------------------------------------------------------|
| column                     | M XT Silcosteel 30 m,<br>i.d. 0.53 mm, df 1 $\mu$ m                                    |
| injection port             | splitless                                                                              |
| injection port temperature | 200 °C                                                                                 |
| injection volume           | 2 $\mu$ L                                                                              |
| carrier gas flow           | He 25 mL/ min                                                                          |
| makeup gas flow            | Ar 300 mL/ min                                                                         |
| oven program               |                                                                                        |
| initial temperature        | 60 °C                                                                                  |
| initial time               | 0 min                                                                                  |
| ramp rate                  | 60 °C/ min                                                                             |
| final temperature          | 250 °C                                                                                 |
| Transfer Line              |                                                                                        |
| length                     | 1 m                                                                                    |
| inner                      | Silcosteel, i.d. 0.28 mm,<br>o.d. 0.53 mm                                              |
| outer                      | Silcosteel, i.d. 1.0 mm,<br>o.d. 1/16 in.                                              |
| ICPMS Parameters           |                                                                                        |
| rf power                   | 1250 W                                                                                 |
| gas flow                   |                                                                                        |
| plasma                     | 15 L/ min                                                                              |
| auxiliary                  | 0.9 L/ min                                                                             |
| nebulizer                  | 0.6 L/ min                                                                             |
| isotopes/ dwell times      | Hg: 202, 201; 30 ms<br>Sn: 117, 120; 30 ms<br>Tl: 203, 205; 5 ms<br>Sb: 121, 123; 5 ms |

the spike, two different spiking procedures have been tested and compared. The method was applied to the determination of mercury and tin species in a new multispecies certified biological reference materials (CRM 710) to demonstrate the high accuracy and enhanced precision offered by ID-CGC–ICPMS. To our knowledge, this is the first report of use of isotope dilution for simultaneous mercury and tin speciation in biological samples.

## EXPERIMENTAL SECTION

**Instrumentation.** A gas chromatograph (HP 6850) equipped with a capillary column and an automatic injector was coupled to an Elan 6000 inductively coupled plasma mass spectrometer (Perkin-Elmer) via a Silcosteel (Restek) transfer capillary. The instrumental configuration permits work under mixed wet and dry plasma conditions. A detailed description was previously published.<sup>17</sup> Briefly, the Silcosteel capillary was inserted into the torch injector through a particular design cyclonic spray chamber allowing the introduction of the transfer line, and the Meinhard nebulizer enabled continuous aspiration of standard (Tl + Sb) solution (10  $\mu$ g/ L). This configuration allowed optimization of the instrument performance and simultaneous measurement of <sup>203</sup>Tl/ <sup>205</sup>Tl and <sup>121</sup>Sb/ <sup>123</sup>Sb for mass bias correction during the chromatographic run. Operating conditions and instrumentation are listed in Table 1. GC separation parameters (temperature program and gas flow) were optimized in order to obtain symmetrical peaks in a way to minimize peak integration errors. The heating of the transfer line also improves the peak shapes, especially for butyltin compounds. The raw data of the transient isotope signals for the different species were further processed using Origin 6 software (Microcal Software) to obtain the peaks areas and the corresponding isotope ratios. An analytical balance,

Sartorius model BP211D (Goettingen, Germany), with a precision of 10<sup>−5</sup> g, was used for all the weighings. An open focused vessel microwave oven Prolabo A301 (Fontenay-sous-Bois, France) was used for the extractions of solid samples.

**Reagents.** Natural tributyltin chloride (TBTCl) and enriched [<sup>117</sup>Sn]TBTCl (90.5  $\mu$ g mL<sup>−1</sup>) were obtained from LGC Limited (Teddington, U.K.). The purification and synthesis of these standards is described in Sutton and al.<sup>37</sup> A stock solution of 1000  $\mu$ g of Sn g<sup>−1</sup> of TBTCl was prepared by dissolving TBTCl in methanol and kept in a fridge at dark. A 10 ng Sn mL<sup>−1</sup> dilution was daily prepared and used for the calibration. A dilution of the [<sup>117</sup>Sn]TBTCl spiking solution was prepared by weighing 0.1 g of a 90.5 mg g<sup>−1</sup> enriched solution and diluting into 10 g of water. Stock solutions of MBT and DBT (1000 mg/ L) of natural isotopic composition were prepared by dissolving BuSnCl<sub>3</sub> and Bu<sub>2</sub>SnCl<sub>2</sub> (Aldrich) in methanol. Stock solutions of IHg and MMHg (1000 mg/ L) of natural isotopic composition were prepared by dissolving mercury(II) chloride (Strem Chemicals) in 1% HNO<sub>3</sub> and methylmercury chloride (Strem Chemicals) in methanol, respectively. Working standard solutions were prepared daily by appropriate dilution of the stock standard solutions in 1% HNO<sub>3</sub> and stored in the refrigerator. Methylcobalamine (Sigma) used for enriched monomethylmercury synthesis was prepared by dissolution in an acetic acid/ acetate buffer solution (0.1 M, pH 5). <sup>201</sup>HgO was obtained from Oak Ridge National Laboratory (Oak Ridge, TN). The methylmercury synthesis is described in previous work.<sup>36</sup> The concentrations of enriched TBT and MMHg solutions obtained were calculated by reverse isotope dilution mass spectrometry at the same time as the sample spiking procedure. Three independent isotope dilution experiments were carried out, and each solution was injected five times. A 0.260 ± 0.003  $\mu$ g TBT g<sup>−1</sup> (<sup>117</sup>Sn abundance of 96.2%) solution and a 0.119 ± 0.002  $\mu$ g MMHg g<sup>−1</sup> (<sup>201</sup>Hg abundance of 97.1%) solution were obtained.

**WARNING:** MMHg and TBT are highly toxic compounds and must be handled with appropriate personal protection.

Inorganic antimony, tin, and thallium were obtained from Spex Certiprep (Metuchen, NJ). Inorganic antimony and thallium were used for the mass bias correction for tin and mercury, respectively. Inorganic tin was used for the detector dead time correction.

Sodium tetraethylborate (purity higher than 99%) was obtained from Strem Chemicals (Newburyport, CT). NaBEt<sub>4</sub> solutions (1% w/ w) were daily prepared and kept in the dark. Tetramethylammonium hydroxide (TMAH; 25%w/ w) was obtained from Fluka (Buchs, Switzerland) and methanol from Merck (Darmstadt, Germany).

All other reagents were of analytical reagent grade. Ultrapure water was obtained from a Milli-Q system (Quantum EX, Millipore).

**Reference Material.** The reference material BCR 710 (oyster tissue) was obtained from the Institute for Reference Materials and Measurements (IRMM; Geel, Belgium). This biological tissue is the first reference material certified for multielemental speciation analysis.<sup>38</sup> Indeed, interlaboratory studies have been organized

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**Table 2. Calibration Curves and Detection Limits in Simultaneous Speciation of Tin and Mercury by CGC-ICPMS**

| species                                   | <sup>202</sup> MMHg | <sup>202</sup> Hg <sup>2+</sup> | <sup>120</sup> MBT | <sup>120</sup> DBT | <sup>120</sup> TBT |
|-------------------------------------------|---------------------|---------------------------------|--------------------|--------------------|--------------------|
| linearity R <sup>2</sup><br>(5–100 pg)    | 0.997               | 0.995                           | 0.997              | 0.999              | 0.998              |
| slopes<br>(counts/ pg)                    | 169.2               | 245.2                           | 345.1              | 312.6              | 316.5              |
| detection limit<br>(μg kg <sup>-1</sup> ) | 0.11                | 0.24                            | 0.30               | 0.17               | 0.15               |

**Table 3. Concentrations of Mercury and Tin Compounds (in μg kg<sup>-1</sup> Species), Determined in Oyster Tissue CRM 710 Reference Material by Speciated Isotope Dilution (SIDMS) and External Calibration (EC) Analysis after Successive and Simultaneous Spike Microwave Extraction Protocols (Mean Value ± SD)<sup>a</sup>**

| compd | certified value                    | successive spike-microwave extraction (method I) |                      | simultaneous spike-microwave extraction (method II) |                      |
|-------|------------------------------------|--------------------------------------------------|----------------------|-----------------------------------------------------|----------------------|
|       |                                    | SIDMS                                            | EC                   | SIDMS                                               | EC                   |
| MMHg  | 115.0 ± 9.0<br>(7.8)               | 113.0 ± 1.9<br>(1.7)                             | 75.1 ± 4.1<br>(5.5)  | 114.0 ± 3.2<br>(2.8)                                | 53.3 ± 2.9<br>(5.5)  |
| TBT   | 133.0 ± 19.0<br>(14.3)             | 126.7 ± 1.6<br>(1.3)                             | 112.2 ± 7.2<br>(6.4) | 148.4 ± 3.7<br>(2.5)                                | 113.9 ± 6.8<br>(6.0) |
| DBT   | 82.0 ± 15.0<br>(18.3)              |                                                  | 89.7 ± 3.60<br>(4.0) |                                                     | 93.8 ± 2.3<br>(2.4)  |
| MBT   | 50.0 ± 14.0 <sup>b</sup><br>(28.0) |                                                  | 40.4 ± 0.9<br>(2.3)  |                                                     | 50.3 ± 2.6<br>(5.2)  |

<sup>a</sup> The values in parentheses are RSDs (%). <sup>b</sup> Indicative value.

in the past 10 years to systematically evaluate the performance of methods used in speciation analysis and to produce Certified Reference Materials (CRMs). The range of CRMs available for the quality control of speciation analysis is constantly increasing. However, these materials are generally certified for chemical species of a single element, which makes it difficult for quality to be controlled in multispecies determination. For this aim, a group of 18 European laboratories was formed, under the coordination the Community Bureau of Reference (BCR) of the Commission of the European Communities, to certify an oyster tissue that is of interest to the human food sector and to environmental monitoring. The batch of oysters (*Crassostrea gigas*) was collected in Arcachon Bay (France) near Arcachon Harbor. Feasibility was first checked, including homogeneity and stability tests and intercomparison. Each laboratory received two bottles and was requested to report the results of six independent replicate determinations. After two rounds of intercomparison in this feasibility study, the material was finally certified for MMHg, TBT, DBT, and AsBet. The certified values are presented in Table 3.

**Procedures. Microwave Extraction Procedure.** The biotissue extraction procedure using microwave heating has been described elsewhere.<sup>19–23</sup> Briefly, ~0.25 g of sample is accurately weighed in the extraction vessel. Then 5 mL of TMAH is added, and a condenser is placed on the top of the extraction vessel so that sample losses are limited. The mixture is gently stirred, placed in the open microwave cavity, and extracted for 2 min at 20% of irradiation power (40 W). After the extraction period, the sample is allowed to cool at room temperature. The extract is quantitatively transferred to a glass tube with a Teflon cap and centrifuged

for 5 min at 2500 rpm. The supernatant is then transferred to a clean tube with a Teflon cap and submitted to ethylation.

**Derivatization of the Extract.** The organometallic species have to be derivatized in order to obtain volatile species. Concentrated ammonium hydroxide and 5 mL of acetic acid/ sodium acetate buffer (0.1 M) are added to 2 mL of the extract to set the pH at 5. Two milliliters of isooctane and 5 mL of 0.5% (w/w) sodium tetraethylborate are added. The tube is immediately capped and hand shaken for 5 min. The organic phase is finally transferred to an injection vial and stored at –18 °C until measurement.

**Spiking Procedures for Isotope Dilution.** Isotope dilution is based on the addition of a precise amount of an isotopically labeled form of the analyte to the sample. When the natural abundances of the analyte in the sample and in the spike are known, the concentration of the analyte in the sample can be calculated if a known amount of the spike is added and equilibrated with the analyte in the sample. The spiking procedure is a critical stage to ensure full equilibrium and the same behavior for both the analyte and the analogue during the analytical procedure. This is the most critical and time-consuming step in isotope dilution analysis. Therefore, two different spiking procedures were tested to know the influence of the equilibrium step between the spike and the analyte. A flowchart of the simplified isotope dilution spike and extraction procedures is shown in Figure 1.

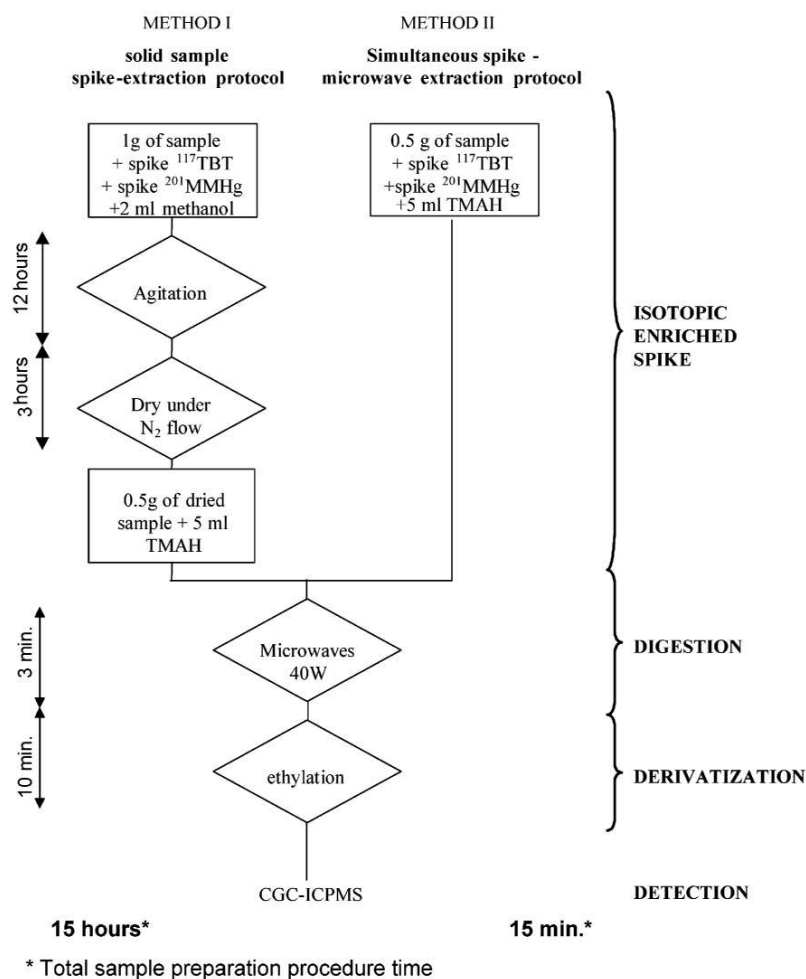
**Successive Solid Sample Spike-Extraction Protocol (Method I).** One gram of dry sample is accurately weighed in a glass tube and spiked with known amounts of the enriched [<sup>117</sup>Sn]TBTCl solution and the enriched [<sup>201</sup>Hg]MMHg solution. Two milliliters of methanol is then added; the tube is capped and mechanically shaken overnight in the dark. After shaking, the methanol is evaporated using a gentle stream of nitrogen (2 mL/min, 3 h). The dried subsample of the sediment is then stirred for homogenization before microwave extraction with TMAH. For this procedure, the spike and the extraction steps are successive. A long equilibration time and a recombination of the initial matrix is used to ensure full equilibration.

**Simultaneous Microwave Extraction-Spike Protocol (Method II).** About 0.5 g of dried sample is accurately weighed in a microwave extraction vessel and spiked with known amounts of the enriched [<sup>117</sup>Sn]TBTCl solution and the enriched [<sup>201</sup>Hg]MMHg solution. Five milliliters of TMAH is added, and a condenser is placed on the top of the extraction vessel. The mixture is then submitted to microwave extraction. Spiking and extraction procedures are simultaneous without equilibration time for the spike.

For all spiking procedures, to minimize errors on the isotope ratio in the final determination, the amount of enriched standard added to the sample is adjusted in order to obtain a spike-to-analyte isotopic ratio close to unity. Each experiment is performed three times with five GC injections. Blanks for each spiking method are also determined to check for any contamination.

## RESULTS AND DISCUSSION

**CGC-ICPMS Optimization for Simultaneous Mercury and Tin Species Detection.** For species-specific isotope dilution analysis, peak shape and acquisition times are critical for the determination of the isotope ratios based on the peak area measurements. The optimal chromatographic separation and ICPMS detection conditions for all the studied compounds are given in Table 1.



**Figure 1.** Flowchart of the sample preparation procedures.

**CGC–ICPMS Conditions.** The mixture of organometallic compounds investigated included the following: ethylated methylmercury and IHg species ( $\text{MeEtHg}$ ,  $\text{Et}_2\text{Hg}$ ) and ethylated butyltin compounds ( $\text{BuEt}_3\text{Sn}$ ,  $\text{Bu}_2\text{Et}_2\text{Sn}$ ,  $\text{Bu}_3\text{EtSn}$ ). The objective of GC–ICPMS optimization was to conduct simultaneous detection in a reasonable time with optimized peak profile for accurate isotope ratio measurements. Three principal chromatographic parameters were taken into account during the optimization of the separation conditions for the above organometallic compounds: baseline resolution between the adjacent peaks, effect of the baseline perturbation on the signal of lighter species, due to the elution of the solvent in the plasma, and peak shape.

GC separation parameters were first optimized with a standard solution containing MMHg, IHg, MBT, DBT, and TBT to obtain symmetrical peaks. GC conditions were chosen in a way that the elution of the species is sufficiently away from the zone disturbed by the solvent elution (isooctane). The temperature program and gas flow were optimized to get high chromatographic resolution for all the species. Peak shape was first improved by heating the transfer line especially for the heavy species such as TBT. The makeup gas flow through the transfer line was optimized to obtain both best symmetric peak shape and high sensitivity.

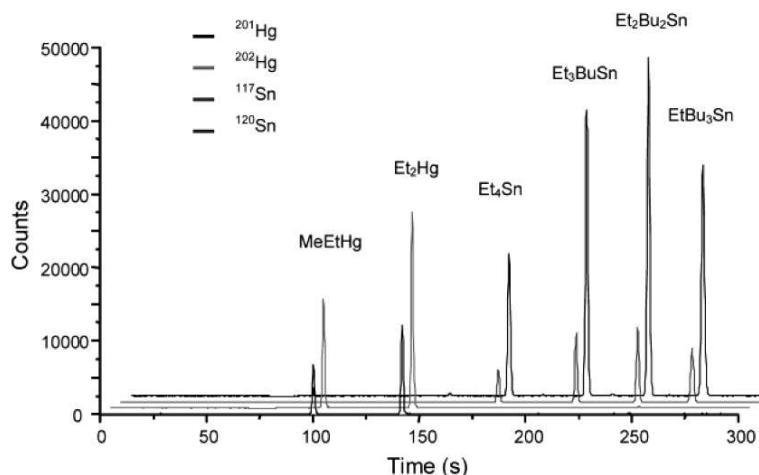
Parameters affecting the precision and accuracy of the isotopic ratio measurements such as detector dead time and mass bias

were carefully evaluated. Detector dead time was calculated by applying a method proposed by Held and Taylor.<sup>39</sup> This method gave a detector dead time of 45 ns, which was chosen for the rest of measurements. In previous works, mass bias for mercury has been corrected by using the  $^{205}\text{Tl}/^{203}\text{Tl}$  isotope ratio<sup>17</sup> and for tin the  $^{123}\text{Sn}/^{121}\text{Sn}$  isotope ratio.<sup>31</sup> According to these methods, simultaneous isotope dilution analysis of MMHg and TBT was performed under wet plasma conditions with a standard solution containing thallium and antimony ( $10\ \mu\text{g/L}$ ) introduced in the cyclonic chamber during all CGC measurements.

For isotope dilution analysis, data acquisition parameters are important to well define chromatographic peaks. Dwell times of 30 ms for mercury and tin isotopes (201, 202, 117, 120) were chosen, and 5 ms for thallium and antimony isotopes (203, 205, 121, 123). The total integration time was 140 ms. Considering a normal peak width around 3 s, the peaks were defined by ~22 point measurements. Each sample was injected five times. Isotopes ratios were always measured as peak area ratios and corrected by a mass bias factor.

A chromatogram obtained under optimal CGC–ICPMS conditions for a standard solution containing  $10\ \mu\text{g/L}$  MMHg, IHg, MBT, DBT, and TBT is presented in Figure 2. This chromatogram

(39) Held, A.; Taylor, P. D. *P. J. Anal. At. Spectrom.* 1999, 14, 1075–1079.



**Figure 2.** Chromatogram obtained for mercury and tin species (natural isotope abundance) obtained with the reported CGC–ICPMS method.

demonstrates that an optimized separation of mercury and tin compounds is achieved.

**Derivatization Conditions.** NaBEt<sub>4</sub> was chosen as the derivatizing reagent for Hg and Sn compounds. Derivatization conditions described in the literature using NaBEt<sub>4</sub> for mercury and tin are rather similar.<sup>19,25</sup> The most important differences between the ethylation procedures remains in the derivatization reagent concentration and in the pH. Since an excess of derivatization reagent should be kept, the most concentrated NaBEt<sub>4</sub> (0.5%) was selected for subsequent analysis. The effect of pH on the efficiency of derivatization has been studied and evaluated by others for organotins<sup>19,23</sup> and mercury compounds.<sup>17,21,22,25</sup> the optimum condition for ethylation of mercury compounds was found with a pH between 4 and 5, and for organotins the optimum pH is 5. For pH adjustment of the simultaneous speciation, different ethylations of a standard solution with all the compounds in MQ water were performed at pH 4 and 5. No significant difference of the peak area was observed for the inorganic mercury between the two experiments. For methylmercury, the peak area was slightly reduced for pH 5. The degradation of MMHg during the derivatization at pH higher than 4 was previously described.<sup>17,25</sup> For tin, the derivatization efficiency was drastically improved at pH 5 compared to pH 4. pH 5 was thus used throughout our experimental work. No transalkylation problem was detected, neither artifact MMHg formation nor the appearance of other intermediate species such as MeBuSn.

**Analytical Performances of Ethylation-CGC–ICPMS.** Reproducibility was evaluated by injecting 10 times a 10 µg/L standard solution with all species of interest. The reproducibility of <sup>202</sup>Hg/<sup>201</sup>Hg isotope ratio measurements was 0.60% for MMHg. A relative standard deviation of 0.70% was obtained for TBT with the <sup>120</sup>Sn/<sup>117</sup>Sn isotope ratio.

Calibration curves established at four different concentration levels (5, 10, 20, and 100 pg) showed excellent linearity between peak areas and concentrations within the tested concentration range for both mercury and tin compounds (Table 2). The slopes of the calibration curves found for the butyltin compounds are not significantly different with 345.1, 312.6, and 316.5 counts/pg as Sn for BuEt<sub>3</sub>Sn, Bu<sub>2</sub>Et<sub>2</sub>Sn, and Bu<sub>3</sub>EtSn, respectively. Calibration curves exhibit lower slopes for mercury compounds with 169.2

and 245.2 counts/pg as Hg for MeHgEt and HgEt<sub>2</sub> respectively. The different slopes are a result of different ICPMS responses and of nonquantitative recovery for mercury species.

The detection limit for the CGC–ICPMS was estimated by the equation  $DL = 3\sigma/p$ , where DL is the detection limit,  $\sigma$  the standard deviation of six blank measurements, and p is the slope of the calibration curve calculated from spiked standards with the different species of Hg and Sn. Based on this equation, the lowest detectable quantities for the different species are presented in Table 2.

For isotope dilution analysis, the real detection limit depends on the precision on the isotope ratio. Yu and al.<sup>40</sup> have developed a complete formulation for the determination of IDMS detection limits that is a function of the enrichment of the isotopic spike (eq 1), where  $L_{Da}$  and  $L_{Db}$  are the linear calibration detection limits

$$LD = \frac{\sqrt{L_{Da}^2 + R_p^2 L_{Db}^2}}{|A_x - R_p B_x|} \quad (1)$$

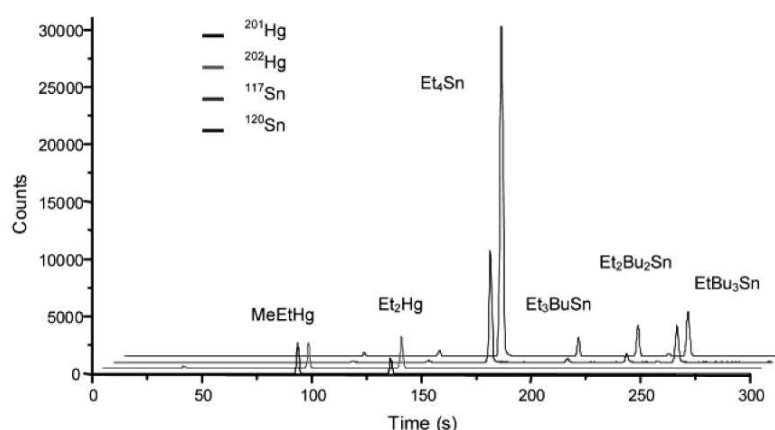
for isotopes A and B, respectively,  $R_p$  is the ratio of isotopes A and B for the spike, and  $A_x$  and  $B_x$  are the atom fraction of isotopes A and B in the sample. IDMS detection limits, calculated according to this equation, were 0.35 µg kg<sup>-1</sup> for MMHg and 0.45 µg kg<sup>-1</sup> for TBT.

High precision on isotope ratio and low detection limits using the CGC–ICPMS allows the best conditions for speciated isotope dilution analysis of MMHg and TBT and for conventional analysis of MBT and DBT.

**Microwave Extraction of Mercury and Tin Species from Biological Samples.** Since simultaneous mercury and tin species derivatization and detection are possible, simultaneous extraction from the solid sample was attempted.

The extraction of mercury compounds in biological samples by open microwave systems has been previously examined. For this purpose, TMAH was found to be an excellent extractant.<sup>19,20,23</sup> Tseng et al.<sup>23</sup> reported a complete optimization of the different extraction parameters (TMAH concentration, heating time, mi-

(40) Yu, L. L.; Fassett, J. D.; Guthrie, W. F. *Anal. Chem.* 2002, 74, 3887–3891.



**Figure 3.** Chromatogram obtained for the standard mussel tissue CRM 710 spiked with  $^{201}\text{MMHg}$  and  $^{117}\text{TBT}$ .

crowave power). A 25% TMAH solution was found efficient to ensure complete solubilization after heating for 2–4 min at a power of 40–60 W. For organotins, acid acetic is generally used as extractant.<sup>18,20</sup> However, it is not appropriate for mercury compounds, causing methylmercury degradation.<sup>23</sup> Szpunar et al.<sup>21</sup> reported an efficient procedure to extract butyltin compounds using TMAH. In this work, taking into account the previous optimizations and the fact that TMAH allows complete alkaline hydrolysis of biomaterials (i.e., proteins, lipids, and sugars),<sup>23</sup> this extractant was chosen. The results obtained for the CRM 710 exhibit excellent agreement with the certified values for MBT, DBT, and TBT quantified by external calibration. Recoveries are between 82 and 114% and agree within the limits of the certified values given by the Institute for Reference Materials and Measurements. Extraction using TMAH appears to be as efficient for organotin compounds in biological tissues as for mercury species.

Simultaneous Species-Specific Mercury and Tin Isotope Dilution. Once simultaneous mercury and tin species microwave extraction, derivatization, and detection was possible, the next step was to attempt simultaneous species-specific isotope dilution.

Comparison of the Spiking Methods. Using isotope dilution analysis, full equilibrium between the enriched spike and the sample must be achieved at an early step of the sample treatment to ensure the same chemical behavior. For solid samples, the spike was directly added to a slurry of the solid sample with a solvent. This was then shaken a long time to ensure the best equilibration between the spike and the analyte. This first spiking step, necessary for species-specific isotope dilution analysis, is very time-consuming. Therefore, two different spiking procedures were tested: a successive spiking and extraction procedure (method I) and a simultaneous and integrated spiking and extraction procedure (method II) to minimize drastically sample preparation protocol and time (Figure 1). Three independent spiking experiments were carried out for each tested spiking procedure. The species-specific isotope dilution analysis of MMHg and TBT and conventional determination by external calibration of MBT and DBT were applied to the certified oyster tissue reference material CRM 710. Each sample was injected five times into the CGC–ICPMS. Figure 3 shows a chromatogram obtained for the CRM 710 spiked with  $^{201}\text{Hg}$ MMHg and  $^{117}\text{Sn}$ TBT. The results are given in Table 3.

From the results obtained by external calibration, the MBT and DBT recoveries are always slightly lower with the two-step spiking extraction procedure (method I). Losses or degradations could occur during the first spiking step in a volatile solvent (methanol). MBT and DBT are lighter compounds compared to TBT and could undergo volatilization loss during the agitation or the drying of the sample under  $\text{N}_2$  flow. With TBT, the results are equivalent for the two sample preparation procedures. For methylmercury, the results quantified by external calibration are clearly lower than the certified value. 65.8% of the MMHg were recovered for the successive procedure and 46.7% for the simultaneous procedure. These low recoveries could be due to a nonquantitative extraction or a nonquantitative ethylation. TMAH has been already found to be a good extractant for MMHg in biological materials.<sup>22</sup> A nonquantitative ethylation at pH 4.9 seems thus to be responsible for these low recoveries, as previously demonstrated.<sup>17</sup> For a standard solution in MQ water, a significant difference was found with an ethylation between pH 4 and 5 since degradation of MMHg is occurring at pH 5. In addition, it has been shown that some interferences in biological matrixes may also decrease the ethylation yield.<sup>17</sup>

Validation of Simultaneous SIDMS Determination. The concentrations of TBT and MMHg in CRM 710 were calculated by species-specific isotope dilution. Results, summarized in Table 3, show that there is insignificant difference between the two spiking and extraction procedures and that the measured concentrations of the two species are in excellent agreement with the certified values and their confidence intervals. Taking into account the previous recovery problems detected by external calibration, the advantages of isotope dilution to correct for losses or low recoveries during the sample preparation steps then become obvious.

For methylmercury, speciated isotope dilution analysis allows us to avoid low ethylation recoveries due to matrix interference at pH 5. The enriched  $^{201}\text{Hg}$ MMHg spike presents the same behavior despite the spiking procedure used. This result shows that equilibrium has been achieved between the spike and the analyte species within the mixture during the sample preparation procedure even for the one-step spiking extraction procedure. No additional equilibration time is required that allows drastic time

reduction (from 15 h to 15 min) and one step for the sample preparation.

For TBT, the results are in good agreement with the certified value for the two different spiking procedures. For the simultaneous spike and extraction procedure, 148.4 mg/kg TBT is found whereas for the successive procedure the value is lower with 126.7 mg/kg. Using a successive method, the equilibration between spike and analyte is improved. In these conditions, the spiked species behave similarly to the analyte during sample preparation. Using a short procedure, the spiked species are most probably free in solution and can be affected by chemical degradation or volatilization loss within the microwave system. Thus, a minor loss of spiked species during extraction overestimates the true concentration. However, the two results obtained are within the confidence intervals of the certified values. This agreement, without any spike equilibration, is certainly due to a quantitative extraction with TMAH and a quantitative ethylation for TBT.

Regarding the uncertainty in the results, speciated isotope dilution analysis is also much more precise than traditional external calibration analysis. The precision for MMHg and TBT is always better with speciated isotope dilution analysis with relative standard deviations (RSD) between 1.3 and 2.8% against RSD between 5.5 and 6.4% with external calibration. This result clearly demonstrates the higher capability of the ID technique for improvement of the analytical precision in speciation analysis.

Moreover, the accuracy of the two spiking procedures is similar, but the precision is always lower with the simultaneous spike extraction method. For this method, weighing of both sample and spike occurs in the microwave extraction vessel, which is too heavy to get high precision on the spike mass.<sup>32</sup> The higher relative standard deviation could be explained by a systematic error in the weight of the sample and the spike. Nevertheless, using the isotope dilution technique for quantification improves the precision of the results and allows us to set an accurate method recommended for good traceability and quality on experiments.

## CONCLUSIONS

A fast, accurate, and precise simultaneous determination method for mercury and tin species in biological tissues by CGC-ICPMS has been developed. A significant improvement in the precision of MMHg and TBT determination using ID, as opposed to external calibration, was obtained, clearly demonstrating its superior capability in overcoming poor precision problems. The paper has demonstrated the possibility of radically accelerating each of the critical steps of isotope dilution sample preparation for speciation analysis (spike equilibration, sample decomposition, extraction) by carrying them out in a low-power focused microwave field. The 3-min single-step sample preparation developed is equivalent in terms of recovery and accuracy to hours-long multisteps procedures commonly reported in the literature for isotope dilution analysis. This study is the first to present a method for simultaneous mercury and tin species-specific isotope dilution in biological tissues. Taking into account that ID is considered to be an absolute analytical method and the achievement in reducing analysis time, the proposed procedure could be used as a reference method in control laboratories.

## ACKNOWLEDGMENT

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## **Chapitre B – II**

**Analyse simultanée des espèces de mercure et butylétains par dilution isotopique pour des échantillons naturels d'eau et de neige**



## **Analyse simultanée des espèces de mercure et butylétains par dilution isotopique pour des échantillons naturels d'eau et de neige**

Le méthylmercure (MMHg) et le tributylétain (TBT) sont reconnus comme des polluants majeurs des environnements aquatiques et sont présents dans les différents compartiments environnementaux. Même à des concentrations très faibles, ils représentent un risque pour les organismes aquatiques du fait de leur capacité à se bioaccumuler. En effet, des concentrations importantes ont été révélées pour des organismes vivants dans des écosystèmes peu impactés. Les océans et les dépositions humides représentent des modes de transport importants pour ces polluants d'où la nécessité de contrôler les concentrations dans ces matrices.

D'un point de vue analytique, la détermination de ces formes chimiques dans des eaux présente des difficultés importantes du fait des très faibles concentrations généralement rencontrées dans ces matrices et de la nécessité de conserver la répartition des espèces.

L'objectif de ce travail est de développer une méthode analytique pour l'analyse simultanée des espèces de mercure et des butylétains. Les différentes étapes de préparation de l'échantillon sont soigneusement étudiées, notamment les conditions de l'étape de dérivation. La quantification par dilution isotopique est appliquée afin d'améliorer les performances analytiques et de détecter les possibles transformations des différentes espèces pendant l'analyse.

Les résultats montrent que la méthode de propylation est la technique de dérivation la plus appropriée à l'analyse d'échantillons environnementaux car son efficacité est peu affectée par les effets de matrice. La méthode est appliquée à des échantillons réels d'eaux de mer et de neiges montrant son important potentiel pour l'analyse de ces composés à des concentrations environnementales très faibles. L'utilisation de la dilution isotopique permet l'amélioration de la précision. Sa capacité à pouvoir valider les techniques analytiques est aussi démontrée ce qui fait d'elle une méthode de référence.

## Simultaneous speciation of mercury and butyltin compounds in natural waters and snow by Propylation and Species Specific Isotope Dilution Mass Spectrometry Analysis

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### ABSTRACT

A robust method for the simultaneous determination of mercury and butyltin compounds in aqueous samples has been developed. This method is capable of providing accurate results for analyte concentrations in the  $\text{pg.l}^{-1}$  to  $\text{ng.l}^{-1}$  range. The simultaneous determination of the mercury and tin compounds is proposed by species specific isotope dilution, derivatization and gas chromatography-ICPMS (GC-ICPMS). Derivatization by ethylation-propylation, reaction parameters such as pH and the chloride effect were carefully studied. Ethylation was found to be more sensitive to matrix effects, especially for mercury compounds. Propylation was thus the preferred derivatization method for the simultaneous determination of organo-mercury and -tin compounds in environmental samples. The analytical performance of this method provides a high level of accuracy and precision, with RSD values of 1 and 3% for analyte concentrations in the  $\text{pg.l}^{-1}$  and  $\text{ng.l}^{-1}$  range. Using cleaned protocols and SIDMS blank measurements, detection limits in the range of 10-60  $\text{pg.l}^{-1}$  were achieved, which are suitable for the determination of background levels of these contaminants in environmental samples. This has been demonstrated by the analysis of real snow and sea water samples using this method. This work illustrates the great advantage of species specific isotope dilution technique for the validation of speciation analytical method offering the possibility to overcome species transformations and non quantitative recoveries. Analysis time is saving by the simultaneous method allowing a single sample preparation and analysis.

**KEYWORDS:** speciation, mercury, butyltin, isotopic dilution, propylation, natural waters.



## 1 INTRODUCTION

Butyltin and mercury compounds are the organometallic species most studied in the environment due to their high toxicity and their widespread distribution. They are found mainly in aquatic environments as a result of their anthropogenic uses, such as tin compounds in the shipping industry, and as a consequence of biotransformation, like methylation of inorganic mercury [1]. The important bioaccumulation ability of these compounds in the food chain is also of concern even for clean compartments of the biosphere. These contaminants can be transported via oceanic waters or atmospheric deposition and even at low concentration levels; these species can pose a real risk to marine organisms. High concentrations of butyltin compounds were observed in marine mammals and birds [2], even for non polluted ecosystems, methylmercury concentrations could be 1 million times the concentration found in the water [3]. The natural concentrations of these compounds in aquatic systems are typically in the ng.l<sup>-1</sup> range or less [2,3]. For these reasons, sensitive analytical methods are required to determine these low concentration levels.

Gas chromatography (GC) is a widely used separation technique for organo-mercury and -tin compounds [4,5]. GC hyphenation with an inductively coupled plasma mass spectrometer (ICPMS) is often used as a sensitive and multi-isotopic technique [5-7]. To date it is well established that ICPMS allows high sensitivity and multi-isotope capability for tin and mercury speciation analysis. In addition this technique allows the application of isotope dilution calibration [8,9]. For GC-ICPMS, the analyte compounds have to be transformed into volatile species prior to the analysis. Rapsomanikis et al. [10] have reviewed derivatization using NaBEt<sub>4</sub> showing its significant capabilities for the analysis of organo-mercury, -tin and -lead. Recently derivatization using NaBPr<sub>4</sub> has been reported [11-14] to exhibit promising results for organo-mercury and organo-tin species. De Smaele et al. [11] were the first to report the similar behaviour of NaBPr<sub>4</sub> than NaBEt<sub>4</sub> as derivatization reagent as well as the study of Schubert et al. [12] for the analysis of organo-tin and organo-lead. Demuth et al. <sup>13</sup> have demonstrated the advantages of NaBPr<sub>4</sub> for the determination of MMHg in aqueous samples avoiding MMHg artefactual transformations when using NaBEt<sub>4</sub>.

Despite significant improvements in instrumentation, the quality of results is mainly associated with sample pre-treatment stages. There are traditional problems related to non-quantitative recoveries, and more recently, questions have arisen about the artefact formation and the transformations of species during sample preparation [13,15,16]. In the case of mercury in environmental samples, the detection of inorganic mercury and methylmercury needs to take into account the possible transformations by methylation, demethylation or reduction. For butyltin compounds, debutylation is possible. The use of isotope dilution techniques addresses these issues since quantitative recoveries are not necessary and rearrangement reactions can be easily detected

[13,15,16]. Several studies have shown the high potential of this technique for the speciation analysis of mercury [17-22], tin [21, 23-26], and chromium [27].

The objective of this work was to develop a robust method for the simultaneous detection of mercury and butyltin compounds in aqueous samples, and to provide accurate results even for very low concentration levels. Derivatization conditions were carefully studied for all the organo-metallic compounds taking into account environmental conditions (matrix interferences, concentration levels).

## 2 EXPERIMENTAL SECTION

### 2.1 Instrumentation

A new interface developed in collaboration with Thermo Elemental has been used coupling a Thermo Electron GC (Focus) with a Thermo Electron ICP-MS (X7 series). The instrumental configuration permits a dual sample introduction that enables the system to operate under mixed wet and dry plasma conditions. The silcosteel capillary transfer line was inserted into a particular design torch injector allowing the introduction of the transfer line and the use of the impact ball nebulizer enabled continuous aspiration of standards (Tl + Sb) solution (10  $\mu\text{g/L}$ ). This configuration allowed optimization of the instrument performance and simultaneous measurement of  $^{203}\text{Tl}/^{205}\text{Tl}$  and  $^{121}\text{Sb}/^{123}\text{Sb}$  for mass bias correction during the chromatographic run. Operating conditions and instrumentation are listed in Table 1. An analytical balance Sartorius model BP211D with a precision of  $10^{-5}$  g (Goettingen, Germany) was used for all the weighings.

### 2.2 Reagents

Ultrapure water was obtained from a Milli-Q system (Quantum EX, Millipore, USA). Analytical reagent grade isooctane, glacial acetic acid and sodium acetate were obtained from Sigma Aldrich (Belgium).

Buffer solution of pH 5 was prepared by dissolving sodium acetate in MQ water and adjusting to pH 5 with glacial acetic acid. Sodium tetraethylborate ( $\text{NaBEt}_4$ ) (purity higher than 99%) and sodium tetrapropylborate ( $\text{NaBPr}_4$ ) were purchased from GALAB (Germany). 0.5% (w/v)  $\text{NaBEt}_4$  and  $\text{NaBPr}_4$  solutions were prepared every 6 hours and kept in the dark.

### 2.3 Standards and samples

*Butylin standards* - Natural tributyltin chloride (TBTCI) and enriched  $[^{117}\text{Sn}]\text{TBTCI}$  (90.5  $\mu\text{g ml}^{-1}$ ) were obtained from LGC Limited (United Kingdom). The purification and synthesis of these standards is described by Sutton et al. [28] A stock solution of 1000 mg (Sn)  $\text{l}^{-1}$  of TBTCI was prepared by dissolving TBTCI in methanol and kept in a fridge at dark. A 10  $\text{ng.ml}^{-1}$  as Sn dilution was prepared daily and used for the calibration. A dilution of the  $[^{117}\text{Sn}]\text{TBTCI}$  spiking solution was prepared by weighing 0.1 g of a 90.5  $\text{mg g}^{-1}$  enriched solution and diluting into 10 g of water. Stock solutions of MBT and DBT (1000 mg (Sn)  $\text{l}^{-1}$ ) of natural isotopic composition were prepared by dissolving  $\text{BuSnCl}_3$  and  $\text{Bu}_2\text{SnCl}_2$  (Sigma-Aldrich, Belgium) in methanol.

*Mercury standards* - Stock solutions of  $\text{IHg}$  and  $\text{MMHg}$  (1000 mg (Hg)  $\text{l}^{-1}$ ) of natural isotopic composition were prepared by dissolving mercury (II) chloride (Strem Chemicals, USA) in 1%  $\text{HNO}_3$  and methylmercury chloride

(Strem Chemicals, USA) in methanol, respectively. Working standard solutions were prepared daily by appropriate dilution of the stock standard solutions in 1% HNO<sub>3</sub> and stored in the fridge until use.

| GC parameters              |                                       |       |  |
|----------------------------|---------------------------------------|-------|--|
| Column                     | MXT Silcosteel 30m. id 0.53mm. df 1µm |       |  |
| Injection port             | Splitless                             |       |  |
| Injection port temperature | 250°C                                 |       |  |
| Injection volume           | 3 µl                                  |       |  |
| Carrier gas flow           | He 25 ml/min.                         |       |  |
| Make up gas flow           | Ar 300 ml/min.                        |       |  |
| Oven program               |                                       |       |  |
| Initial temperature        | 60°C                                  |       |  |
| Initial time               | 0 min                                 |       |  |
| Ramp rate                  | 60°C/min.                             |       |  |
| Final temperature          | 250°C                                 |       |  |
| Transfer line              |                                       |       |  |
| Temperature                | 280°C                                 |       |  |
| Length                     | 0.5 m                                 |       |  |
| Inner                      | Silcosteel. i.d. 0.28mm. o.d. 0.53mm  |       |  |
| Outer                      | Silcosteel. i.d. 1.0 mm. o.d. 1/16"   |       |  |
| ICP-MS parameters          |                                       |       |  |
| Rf Power                   | 1250 W                                |       |  |
| Gas flow                   |                                       |       |  |
| Plasma                     | 15 L/min.                             |       |  |
| Auxiliary                  | 0.9 L/min.                            |       |  |
| Nebulizer                  | 0.6 L/min.                            |       |  |
| Isotopes/dwell times       | Hg : 202. 201.199                     | 30 ms |  |
|                            | Sn : 117. 120                         | 30 ms |  |
|                            | Tl : 203. 205                         | 5 ms  |  |
|                            | Sb : 121. 123                         | 5 ms  |  |

**Table 1:** Operating conditions for the GC/ICPMS coupling system.

Methylcobalamine (Sigma-Aldrich, Belgium) used for enriched monomethylmercury synthesis was prepared by dissolution in an acetic acid-acetate buffer solution (0.1 M, pH 5). <sup>201</sup>HgO was obtained from Oak Ridge National

Laboratory (USA). The description of the methylmercury synthesis is described in a previous work [29].  $^{199}\text{HgCl}_2$  was prepared by dissolving  $^{199}\text{HgO}$  (Oak Ridge National Laboratory, USA) in HCl.

The concentrations of the enriched TBT and MMHg solutions obtained were calculated by reverse isotope dilution mass spectrometry (RIDMS) at the same time as the sample spiking procedure. Three independent isotope dilution RIDMS analysis were carried out and each solution was injected five times.

WARNING : MMHg and TBT are highly toxic compounds and must be handled with appropriate personal protection.

Inorganic Antimony, tin and thallium were obtained from Spex Certiprep (USA). Inorganic antimony and thallium were used for the mass bias correction for tin and mercury, respectively. Inorganic tin was used for the detector dead time correction.

*Cleaning procedure* - Working under clean conditions is also an important consideration for the successful analysis at very low concentration levels. Special attention has been paid to the cleaning procedure of all vessels used for the sampling and the sample preparation. Briefly, all the containers were first washed using a detergent and rinse with MQ water. They were then cleaned in successive acid baths (nitric acid and hydrochloric acid). After rinsing with MQ water, they were dried under a laminar flow hood and stored in double sealed polyethylene bags until use. Blank determinations were also performed by SIDMS to check for any contamination during the sample preparation.

*Samples* - To date, no aqueous certified reference material exists for the speciation of mercury and tin compounds due to the instability of these compounds. To test the accuracy of the analytical procedure, the method was applied to synthetic and real aquatic samples (natural sea water and snow samples).

Synthetic sea water samples were obtained by dissolving NaCl in MQ water at 13 and 35 g.l<sup>-1</sup> concentration levels. Samples were then spiked with natural species (MMHg, IHg, MBT, DBT and TBT) and submitted to derivatization.

Fresh natural snow deposits samples were collected in the Pyrenees Mountain (South West, France) in February 2003 using cleaned HDPE bottles. Snow 1 was collected at an altitude of 788m and snow 2 at 800m. They were melted under a laminar flow hood, transferred to cleaned Teflon bottles, acidified with HCl 1% and stored at -20°C prior to the analysis.

Natural sea water samples were collected in the Thau Lagoon (Mediterranean Sea, France) in May 2003 using pre-cleaned Teflon bottles at two different locations. Water 1 was collected in the middle of the lagoon, water 2 near shellfish farming area. The samples was filtered through 0.45µm PVDF filter, acidified with HCl 1% and then stored at 4°C until measurements.

## 2.4 Procedures

In the proposed procedure, 100 ml of sample is accurately weighed in a flask and spiked with known amounts of the enriched  $[^{117}\text{Sn}]\text{TBTCl}$ ,  $[^{201}\text{Hg}]\text{MMHg}$  and  $[^{199}\text{Hg}]\text{IHg}$  solutions. The mixture is then directly submitted to derivatization. Each experiment is performed three times with three GC injections.

*Spiking procedure for isotope dilution* - Isotope dilution is based on the addition of a precise amount of an isotopically labeled form of the analyte to the sample. The concentration of the analyte in the sample can be calculated from the observed isotope ratios when the natural and enriched isotope ratios, the mass of sample and spike are respectively known. The spiking procedure is a critical stage that requires full equilibrium and the same behaviour for both the analyte and the analogue during the analytical procedure. For aqueous samples, equilibration step is less critical than for solid matrix for which the solubilization should be quantitative to be sure of the full equilibrium. To minimize errors on the isotope ratio in the final determination, the amount of enriched standard added to the sample is adjusted in order to obtain a spike to analyte isotopic ratio close to unity.

*Derivatization of the extract* - 5 ml of acetic acid / sodium acetate buffer (0.1M) was added to 100 ml of the water sample. The pH was adjusted at 5 using concentrated ammonium hydroxide. 0.2 ml of iso octane and 1 ml of 0.5% (w/v) sodium tetraethylborate (or sodium tetrapropylborate) were added, and the flask was immediately capped and vigorously hand shaken by hand for 5 min. The organic phase was then transferred to an injection vial and stored at  $-18^{\circ}\text{C}$  until measurement.

*SIDMS-GC-ICPMS analysis* - Optimized operating conditions used for the GC separation and the ICPMS detection for the simultaneous speciation of organomercury and organotin compounds has been described elsewhere [21]. Briefly, GC conditions were chosen in a way that the elution of the species are sufficiently away from the zone disturbed by the solvent elution (iso-octane). Peak shape was improved by heating the transfer line especially for the heavy species like TBT. The make up gas flow through the transfer line was optimized to obtain the best symmetric peak shape and the highest sensitivity. Parameters affecting the precision and accuracy of the isotopic ratio measurements such as detector dead time and mass bias were carefully evaluated. In previous works, mass bias for mercury has been corrected by using the  $^{205}\text{Tl}/^{203}\text{Tl}$  isotope ratio [17] and for tin by  $^{123}\text{Sb}/^{121}\text{Sb}$  [26]. According to these methods, simultaneous isotope dilution analysis was performed under wet plasma conditions with a standard solution containing thallium and antimony ( $10\text{ }\mu\text{g l}^{-1}$ ) introduced in the cyclonic chamber during all GC measurements. All GC/ICPMS parameters are presented in Table 1.

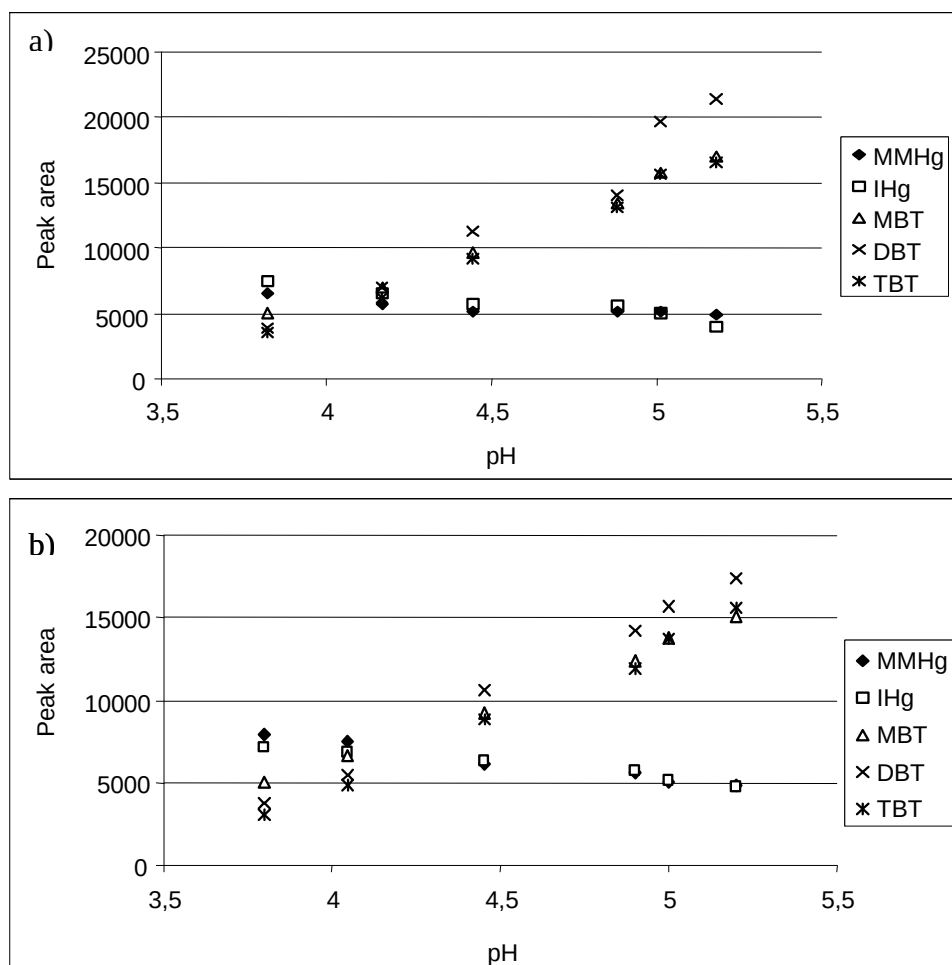
MMHg, IHg and TBT were analyzed by SIDMS whereas MBT and DBT were analyzed by external calibration. Each sample was injected three times and the chromatographic peak integration was performed using homemade software. Isotopes ratios were always measured as peak area ratios and corrected by mass bias factor.

*Blanks determination* - Blanks were also determined to check for any contamination which could be an important error source at low concentration levels. Each step of the procedure was carefully evaluated in terms of contamination. First, sample conditioning was tested by storing MQ water in the same condition as the real samples. For snow samples, blanks were carried through the storage and melting, and for water samples, blanks were carried through the storage and filtration. Steps of the entire sample preparation procedure were also checked carefully. Blanks were carried through the entire derivatization procedure with the analysis of samples. All these different blanks were quantified by species specific isotope dilution assuring accuracy and precision for these low values.

### 3 RESULTS AND DISCUSSION

#### 3.1 Simultaneous determination of mercury and tin species in water samples

*Derivatization pH* - The derivatization of organometallic compounds with tetraalkylborates is known to be strongly pH dependent and needs to be optimized for each compound. Figure 1 shows the influence of the pH value on the relative derivatization efficiency of butyltin and mercury compounds by ethylation (1a) and propylation (1b). In the series of experiments, the pH was set between 3.8 to 5.2 for spiked solutions with 10 ng.l<sup>-1</sup> of each compound. For ethylation of mercury compounds, the intensity decreases slightly as the pH increases. For butyltin, the derivatization efficiency increases significantly as the pH increases. According to this pH optimization study, a pH value of 5 allows simultaneous derivatization of all investigated organotin and organomercury compounds with satisfactory derivatization yields. A similar series of experiments were carried out for derivatization using sodium tetrapropylborate and similar results were obtained. For this reason, derivatization for both ethylation and propylation were carried out under identical conditions.



**Figure 1:** Influence of pH on the peak area of organomercury and organotin compounds using NaBEt<sub>4</sub> (a) and NaBPr<sub>4</sub> (b) as derivatizing reagents.



*Chloride effect* – De Diego et al. [30] mentioned that possible transformations of mercury species can be promoted by the presence of sodium chloride using ethylation. This attribution has been highlighted with the study of Demuth et al. [13] with the help of isotopically enriched species. Results have shown that methylmercury transformation into elemental mercury takes place exclusively with the presence of halide ions in the water even at low concentrations. Mercury and tin compounds responses were followed by varying the sodium chloride concentration. Table 2 gives recoveries for mercury and butyltin compounds in sodium chloride solutions (13 and 35 g.l<sup>-1</sup> of NaCl) containing 1 ng.l<sup>-1</sup> of each compound. Results are given for quantification both by external calibration, and specific isotope dilution, and show the strong dependence of the derivatization efficiency for mercury compounds on the presence of sodium chloride. Methylmercury is the most affected compound with only 30% recovery whereas inorganic mercury recoveries are greater than 80%. These results agree with the study of Carpinteiro et al. [31]. In this work, a calibration in sea water exhibits a slope 3 times lower than for the calibration in MQ water. De Diego et al. [30] attributed this chloride interference to the formation of highly stable chlorocomplexes of MMHg. A reduction of these complexes into elemental mercury has been demonstrated by Demuth et al. [13] during ethylation in a salt water matrix.

| calibration | NaCl 13 g.l <sup>-1</sup> |             |                    |             | NaCl 35 g.l <sup>-1</sup> |             |            |             |
|-------------|---------------------------|-------------|--------------------|-------------|---------------------------|-------------|------------|-------------|
|             | EC <sup>1</sup>           |             | SIDMS <sup>2</sup> |             | EC                        |             | SIDMS      |             |
|             | Ethylation                | Propylation | Ethylation         | Propylation | Ethylation                | Propylation | Ethylation | Propylation |
| MMHg        | 30                        | 96          | 101                | 100         | 30                        | 94          | 101        | 100         |
| IHg         | 83                        | 98          | 100                | 99          | 81                        | 95          | 100        | 99          |
| MBT         | 101                       | 102         | ND*                | ND          | 102                       | 102         | ND         | ND          |
| DBT         | 104                       | 101         | ND                 | ND          | 108                       | 105         | ND         | ND          |
| TBT         | 111                       | 105         | 100                | 102         | 117                       | 105         | 100        | 101         |

(All the RSD are below 5%), <sup>1</sup>External Calibration. <sup>2</sup>Species Specific isotope dilution, ND\* : not determined

**Table 2:** Chloride ions interferences on derivatization efficiency. Recoveries ± standard deviation (n=3).

No interference by sodium chloride can be observed for butyltin compounds but on the contrary with mercury compounds, efficiency seems to be improved, especially for heavier species. TBT recoveries increase with the salinity and reach of 117% for a sodium chloride concentration of 35 g.l<sup>-1</sup>. By adding sodium chloride, the solubility of butyltin compounds decrease. For pH 5, Inaba et al. [32] have determined solubility for TBTCl of 70 mg.l<sup>-1</sup> in distilled water and 2 mg.l<sup>-1</sup> in seawater. This allows a more efficient extraction of butyltin compounds in the organic phase for sodium chloride solutions.

Recovery values are also given for the analysis by specific isotope dilution for MMHg, IHg and TBT. The results show that isotope dilution allows correcting differences between derivatization efficiencies in MQ water and more complex matrix. Using species specific isotope dilution allows avoiding the calibration by standard addition which is also time consuming. Accuracy as well as the precision (RSD ranging from 0.9 to 2.1%) are simply and remarkably improved.

*Derivatization reagent-* An alternative for derivatization of such compounds in water containing salts has been proposed by several authors. Demuth et al. [13] have shown that propylation do not lead to artifact transformation of methylmercury in sea water. Schubert et al. [12] have compared derivatization using NaBEt<sub>4</sub> and NaBPr<sub>4</sub> for the analysis of organotin and organolead compounds and similar performances were observed. Lastly, propylation was applied by De Smaele et al. [11] for the simultaneous determination of organo-mercury, -lead and -tin compounds with equivalent efficiency than ethylation.

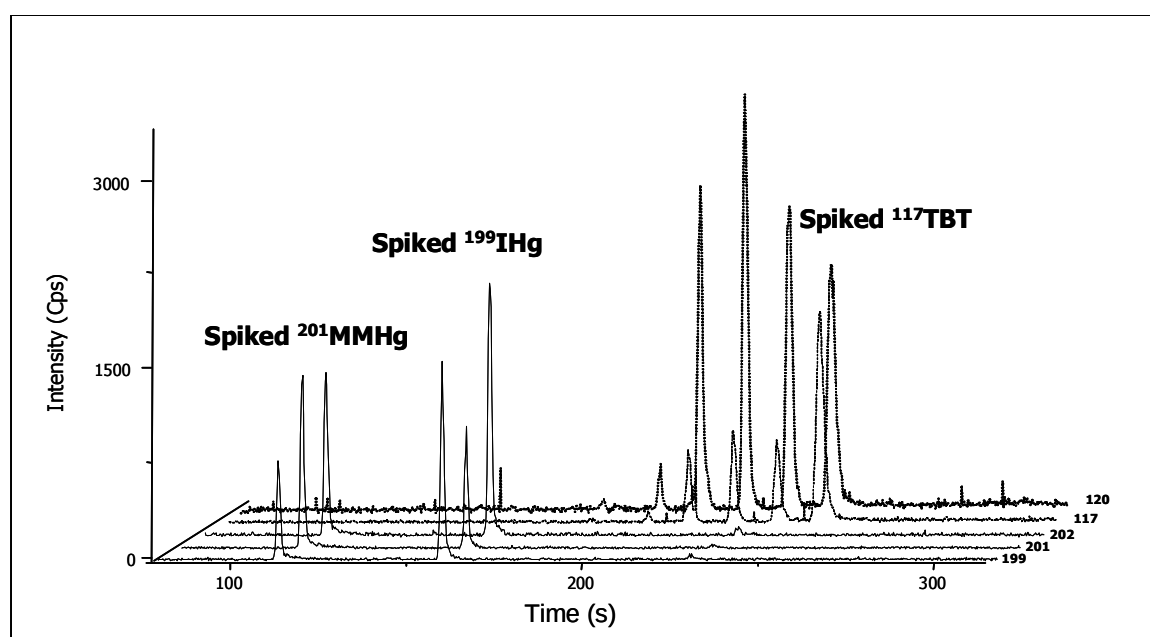
In this study, the identical series of experiments done by ethylation on salt effects has been repeated using propylation. Results are presented in Table 2. The same sensitivity for mercury compounds was achieved regardless of the sodium chloride concentration. As previously found by Demuth et al. [13], no methylmercury transformation takes place using sodium tetrapropylborate as derivatizing reagent. For butyl tin, the sensitivity is equivalent with those found by ethylation. This alternative for derivatization is thus preferential for the analysis of environmental samples which can exhibit high salinity and very low concentrations in the range of the pg.l<sup>-1</sup>.

*Species specific isotope dilution analysis-* In addition to providing the highest level of accuracy and precision, SIDMS also allows for the tracking and correction of possible species transformations during the analytical procedure. Figure 2 presents a chromatogram of a standard solution (2 ng.l<sup>-1</sup>) spiked with enriched [<sup>117</sup>Sn]TBT, [<sup>201</sup>Hg]MMHg and [<sup>199</sup>Hg]IHg solutions, derivatized by propylation. As reported by Demuth et al. [13], methylmercury can be reduced into Hg<sup>0</sup>. Alkyl addition or removal is also possible such as methylation of inorganic mercury and debutylation of butyltin compounds. Neither methylation of inorganic mercury, nor demethylation of methylmercury has been observed in standards and natural samples. Similarly, no debutylation of tributyltin has been detected. Species specific isotope dilution allows the correction of mercury species losses for matrix containing chloride ions.

### 3.2 Performances of the analytical technique

The performance of this method was investigated using spiked MQ water and synthetic sea water (35g.l<sup>-1</sup> NaCl) samples both for ethylation and propylation as derivatizing reactions. The LODs were determined by calculating 3 times the standard deviation for 5 blank solutions divided by the slope of the calibration curve (0.1-5 ng.l<sup>-1</sup>).

Relative standard deviations were calculated from the standard deviation of 3 independent measurements of solutions containing  $1 \text{ ng.l}^{-1}$  of each species. As Shown in Table 3, the characteristics for both derivatization methods in MQ water are comparable in terms of detection limits and precision. For sea water, propylation is the more sensitive derivatization technique, particularly for mercury species where detection limits were 3 times lower for MMHg. This is especially important for the analysis of natural samples from non polluted environments where MMHg concentration levels are not expected to exceed few tenth  $\text{pg.l}^{-1}$ . Analysis of real sea water samples will show that, in some cases, measurements of MMHg are feasible by propylation whereas no signal is detectable using ethylation.



**Figure 2:** Chromatogram obtained by GC-ICPMS for a standard solution containing mercury and butyltin compounds ( $2 \text{ ng.l}^{-1}$ ) spiked with enriched  $^{201}\text{MMHg}$ ,  $^{199}\text{IHg}$  and  $^{117}\text{TBT}$  and derivatized with  $\text{NaBPr}_4$ .

| <b>Ethylation</b>  |                             | MMHg  | IHg   | MBT   | DBT   | TBT   |
|--------------------|-----------------------------|-------|-------|-------|-------|-------|
| MQ water           | LOD* ( $\text{ng l}^{-1}$ ) | 0.016 | 0.053 | 0.030 | 0.023 | 0.020 |
|                    | RSD%**                      | 1.6   | 1.9   | 1.8   | 4.2   | 1.2   |
| Sea water          | LOD ( $\text{ng l}^{-1}$ )  | 0.045 | 0.075 | 0.028 | 0.020 | 0.017 |
|                    | RSD%                        | 3.1   | 2.6   | 2.0   | 4.2   | 1.8   |
| <b>Propylation</b> |                             | MMHg  | IHg   | MBT   | DBT   | TBT   |
| MQ water           | LOD ( $\text{ng l}^{-1}$ )  | 0.010 | 0.060 | 0.033 | 0.027 | 0.022 |
|                    | RSD%                        | 1.7   | 1.4   | 1.8   | 2.5   | 1.3   |
| Sea water          | LOD ( $\text{ng l}^{-1}$ )  | 0.012 | 0.063 | 0.032 | 0.024 | 0.019 |
|                    | RSD%                        | 1.1   | 2.3   | 1.6   | 2.1   | 1.8   |

\*LOD=3\*SD/calibration slope, \*\*RSD% relative standard deviation (n=3.  $1 \text{ ng l}^{-1}$ )

**Table 3:** Analytical performances for ethylation and propylation of spiked water (MQ water and sea water ( $\text{NaCl } 35\text{g l}^{-1}$ ))

### 3.3 Application to real aquatic samples

#### A- Snow samples

The described method was applied to the determination of mercury and tin compounds in natural environmental samples. Table 5 presents results for two snow samples collected in the Pyrenees (South West, France). Levels of MMHg, IHg and TBT in samples were obtained by species specific isotope dilution analysis. Values found by external calibration are also added for comparison. In addition, table 4 presents the assessment of derivatization by ethylation and propylation for mercury compounds and TBT.

*Blank controls* – As previously indicated, blank contamination could be the limiting factor for obtaining accurate results for low concentration levels. In the case of snow samples, blanks of the whole sample preparation (storage, melting, derivatization) were carefully investigated (see Table 4). For mercury compounds and TBT, quantification of blanks by SIDMS allows high reproducibility on blank values even for very low concentrations assuring very low detection limits (0.015, 0.108 and 0.138 ng l<sup>-1</sup> respectively). For MBT and DBT, blanks quantifications have shown an important and non-reproducible contamination certainly due to the storage in HDPE bottles. Results for MBT and DBT are not valid and are not presented here.

| Procedural blanks |                             | MMHg* | IHg*  | MBT** | DBT** | TBT* |
|-------------------|-----------------------------|-------|-------|-------|-------|------|
| Snow sample (n=6) | Value (ng l <sup>-1</sup> ) | 0.028 | 0.774 | 0.250 | 1.45  | 1.02 |
|                   | SD (ng l <sup>-1</sup> )    | 0.005 | 0.036 | 0.046 | 0.47  | 0.29 |
|                   | LOD (ng l <sup>-1</sup> )   | 0.015 | 0.108 | 0.138 | 1.41  | 0.87 |
| Sea water (n=8)   | Value (ng l <sup>-1</sup> ) | 0.056 | 0.775 | 0.218 | 0.22  | 0.19 |
|                   | SD (ng l <sup>-1</sup> )    | 0.007 | 0.042 | 0.014 | 0.03  | 0.04 |
|                   | LOD (ng l <sup>-1</sup> )   | 0.021 | 0.126 | 0.042 | 0.10  | 0.13 |

\* determined by IDA, \*\* determined by external calibration

**Table 4:** Blanks values of the entire sample preparation procedure for sea water and snow samples with associated standard deviations (SD) and detection limits (LOD).

*Natural background concentrations* - The results by SIDMS for MMHg obtained by ethylation and propylation are in good agreement, with low relative standard deviations (2.6-6.3%) even at this low concentration levels. The results found by ethylation show that application of this derivatization method using an external calibration leads to inaccurate results (78 and 76% recoveries for snow 1 and 2 respectively). These results confirm that ethylation is more sensitive to such matrix effect than propylation, even when the matrix doesn't contain high concentrations of chloride ions. Further systematic investigations are necessary to identify other components that could promote transformation mechanisms such as humic acids or others halide ions.

| MMHg           |                       |                       |                        |                       |
|----------------|-----------------------|-----------------------|------------------------|-----------------------|
| Derivatization | Propylation           |                       | Ethylation             |                       |
| Calibration    | EC <sup>1</sup>       | SIDMS <sup>2</sup>    | EC                     | SIDMS                 |
| Snow 1         | 0.416±0.021<br>(5.0%) | 0.386±0.018<br>(2.6%) | 0.305±0.027<br>(8.8%)  | 0.390±0.016<br>(4.1%) |
| Snow 2         | 0.251±0.025<br>(9.9%) | 0.238±0.017<br>(7.1%) | 0.181±0.020<br>(11.0%) | 0.236±0.015<br>(6.3%) |

| IHg            |                       |                       |                        |                       |
|----------------|-----------------------|-----------------------|------------------------|-----------------------|
| Derivatization | Propylation           |                       | Ethylation             |                       |
| Calibration    | EC                    | SIDMS                 | EC                     | SIDMS                 |
| Snow 1         | 0.446±0.025<br>(5.6%) | 0.423±0.014<br>(3.3%) | 0.482±0.053<br>(10.9%) | 0.412±0.014<br>(3.3%) |
| Snow 2         | 1.868±0.122<br>(6.5%) | 2.011±0.050<br>(2.4%) | 1.837±0.154<br>(8.4%)  | 1.986±0.065<br>(3.3%) |

| TBT            |                       |                       |                       |                       |
|----------------|-----------------------|-----------------------|-----------------------|-----------------------|
| Derivatization | Propylation           |                       | Ethylation            |                       |
| Calibration    | EC                    | SIDMS                 | EC                    | SIDMS                 |
| Snow 1         | 0.400±0.023<br>(5.7%) | 0.399±0.005<br>(1.2%) | 0.412±0.019<br>(4.6%) | 0.409±0.006<br>(1.5%) |
| Snow 2         | 0.294±0.015<br>(5.1%) | 0.311±0.007<br>(2.2%) | 0.322±0.022<br>(6.8%) | 0.304±0.007<br>(2.3%) |

<sup>1</sup>External Calibration. <sup>2</sup>Species Specific isotope dilution

**Table 5:** Determination of mercury species in snow samples using NaBEt<sub>4</sub> and NaBPr<sub>4</sub> as derivatization reagent. Mean values (ng.l<sup>-1</sup>) ± standard deviation (n=3) and relative standard deviation.

Results for IHg, values found by external calibration and SIDMS are in a good agreement both for ethylation and propylation. Precision on the results is improved with RSD ranging from 2.5 to 3.3% for SIDMS determination whereas with external calibration RSD were between 5.6 to 10.9%. As the same way, TBT concentrations found by ethylation and propylation well agree. Using SIDMS allow improving precision on the results with RSD ranging from 1.2 to 2.3% whereas by external calibration RSD are ranging from 4.6 to 6.8%.

## B- Sea water samples

*Blank controls* – Blank contamination of the whole procedure for sea water analysis including filtration, storage and derivatization were carefully controlled (see Table 4). Coupling a meticulous cleaning procedure with blank quantification by SIDMS have led to very low and reproducible contamination permitting very low detection limits with 0.021, 0.126 and 0.042 ng l<sup>-1</sup> for MMHg, IHg and TBT, respectively.

*Natural background concentrations* - Table 6 presents concentrations of mercury and butyltin compounds found in the sea water samples both by ethylation and propylation. MMHg, IHg and TBT concentrations were determined by species specific isotope dilution, and MBT and DBT by external calibration.

In the case of mercury, some notable disparities can be observed between the two derivatization procedures. For IHg, low values were found by external calibration for the ethylation procedure with about 86% of recovery in comparison with SIDMS quantification. As mentioned previously, ethylation is more sensitive to matrix interferences but can be overcome by using isotope dilution. Propylation exhibits an excellent agreement between values found by external calibration and SIDMS but precision is greatly improved using SIDMS with RSD divided by a factor 2.

Using ethylation, methylmercury is below the detection limit whereas using propylation, it can be determined at very low concentration levels with a good precision. Figure 3 illustrates these results showing chromatograms obtained for the sea water sample (water 1) using ethylation and propylation. Ethylation suffers from matrix interferences for MMHg and no peak of MMHg is detected. Using propylation for the same sample, a significant peak of MMHg is detected allowing the quantification of MMHg in this sample. Regarding precision on the results, the use of propylation in connection with SIDMS quantification allow to drastically improve RSD with 24 and 32% for water 1 and 2 respectively instead of 62 and 76% found by external calibration.

Regarding results for butyltin compounds, values are in a good agreement between ethylation and propylation both by external calibration and SIDMS for TBT. Again, species specific isotope dilution allows a very good precision at very low concentrations with RSD ranging between 11 and 13% for TBT whereas using external calibration RSD are ranging between 27 and 36%. For MBT and DBT, the use of SIDMS analysis should improve the poor precision found using external calibration with RSD ranging between 10 and 27%.

| MMHg           |                      |                      |            |       |
|----------------|----------------------|----------------------|------------|-------|
| Derivatization | Propylation          |                      | Ethylation |       |
| Calibration    | EC <sup>1</sup>      | SIDMS <sup>2</sup>   | EC         | SIDMS |
| Water 1        | 0.053±0.033<br>(62%) | 0.042±0.010<br>(24%) | <LOD       | <LOD  |
| Water 2        | 0.033±0.025<br>(76%) | 0.037±0.012<br>(32%) | <LOD       | <LOD  |

| IHg            |                   |                     |                      |                     |
|----------------|-------------------|---------------------|----------------------|---------------------|
| Derivatization | Propylation       |                     | Ethylation           |                     |
| Calibration    | EC                | SIDMS               | EC                   | SIDMS               |
| Water 1        | 2.14±0.15<br>(7%) | 2.31±0.06<br>(2.6%) | 1.89±0.22<br>(11.6%) | 2.19±0.11<br>(5.0%) |
| Water 2        | 1.09±0.10<br>(9%) | 1.11±0.05<br>(4.5%) | 0.88±0.11<br>(12.5%) | 1.03±0.07<br>(6.8%) |

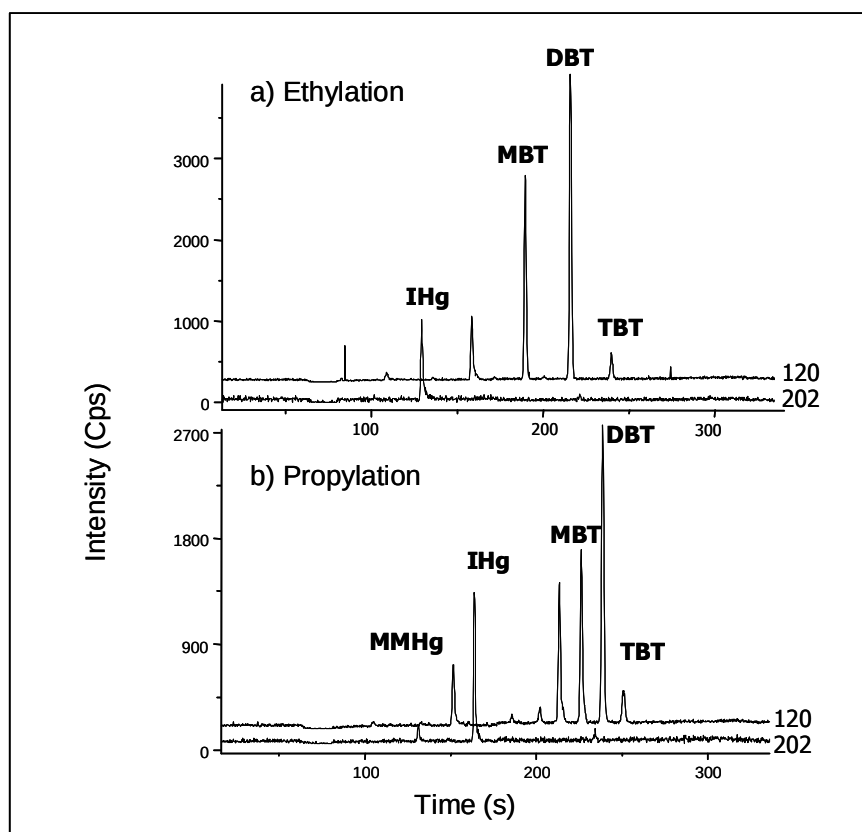
| TBT            |                    |                    |                    |                    |
|----------------|--------------------|--------------------|--------------------|--------------------|
| Derivatization | Propylation        |                    | Ethylation         |                    |
| Calibration    | EC                 | SIDMS              | EC                 | SIDMS              |
| Water 1        | 0.45±0.12<br>(27%) | 0.47±0.05<br>(11%) | 0.46±0.15<br>(33%) | 0.46±0.06<br>(13%) |
| Water 2        | 0.25±0.09<br>(36%) | 0.27±0.03<br>(11%) | 0.23±0.08<br>(35%) | 0.25±0.03<br>(12%) |

| DBT            |                    | MBT                |                    |                    |
|----------------|--------------------|--------------------|--------------------|--------------------|
| Derivatization | Propylation        | Ethylation         | Propylation        | Ethylation         |
| Calibration    | EC                 | EC                 | EC                 | EC                 |
| Water 1        | 1.42±0.36<br>(25%) | 1.57±0.33<br>(21%) | 0.92±0.18<br>(19%) | 1.19±0.25<br>(21%) |
| Water 2        | 1.12±0.25<br>(22%) | 1.09±0.30<br>(27%) | 0.72±0.09<br>(12%) | 0.69±0.07<br>(10%) |

<sup>1</sup>External Calibration. <sup>2</sup>Species Specific isotope dilution

**Table 6:** Determination of mercury species and butyltin compounds in sea water samples using NaBEt<sub>4</sub> and NaBPr<sub>4</sub> as derivatization reagent. Mean values (ng.l<sup>-1</sup>) ± standard deviation (n=3)



**Figure 3:** Chromatograms obtained by GC-ICPMS for a sea water sample using ethylation (a) and propylation (b) as derivatizing reagent.

#### 4. CONCLUSIONS

An accurate and precise method for the simultaneous determination of mercury and tin species in aqueous samples by propylation CGC-ICPMS has been developed. For environmental samples, propylation is preferred over ethylation for derivatization since it is less affected by matrix effects. Precision values using SIDMS were significantly better than those obtained by external calibration, clearly demonstrating the superior capabilities of this approach for low level determinations. Taking into account that no certified reference material is available for the speciation of mercury and butyltin compounds in aqueous samples, SIDMS is considered as an absolute analytical method controlling species transformations and low recoveries. The proposed procedure could be used as a reference method in control laboratories.

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## **Chapitre B – III**

### **Détermination des espèces de mercure et de la formation accidentelle de MMHg par dilution isotopique pour des sédiments certifiés**

## **Détermination des espèces de mercure et de la formation accidentelle de MMHg par dilution isotopique pour des sédiments certifiés**

Le MMHg présente un risque écologique fort pour les écosystèmes aquatiques du fait de sa toxicité et de sa capacité à se bioamplifier dans les organismes aquatiques. Le MMHg est produit dans les environnements aquatiques à partir du mercure inorganique mais les mécanismes gouvernant cette méthylation sont à l'heure actuelle peu connus. Néanmoins les sédiments sont reconnus comme un compartiment important jouant le rôle de réacteur pour les réactions chimiques et biologiques. Ils sont aussi un piège pour ces polluants représentant ainsi un risque pour les organismes benthiques qui sont les premiers maillons de la chaîne trophique. L'analyse du MMHg dans les sédiments s'avère donc nécessaire afin de mieux comprendre son devenir dans l'environnement.

A l'heure actuelle, l'analyse du MMHg dans les sédiments est problématique du fait d'un artéfact pendant la préparation de l'échantillon. En effet, le méthylmercure peut être formé accidentellement à partir du IHg pendant la procédure analytique ce qui génère un biais sur le résultat. De plus, la concentration en MMHg ne dépasse pas quelques pourcents du mercure total, donc même une méthylation très faible du IHg génère une erreur importante sur la concentration en MMHg. L'utilisation des isotopes stables permet de déterminer les transformations des espèces pendant l'analyse et donc de les corriger. Dans le cas du sédiment, plusieurs études ont mis en évidence l'artéfact mais n'ont pas permis sa correction.

L'objectif de ce travail est donc d'utiliser les traceurs isotopiques stables afin de mieux comprendre la formation de MMHg pendant les différentes étapes de préparation du sédiment. Une étude systématique de chaque étape de la procédure analytique est réalisée afin de localiser et de contrôler les causes de cet artéfact de méthylation. Différentes procédures de mise à l'équilibre du traceur isotopique, d'extraction et de dérivation sont testées pour des sédiments certifiés.

Le principal résultat de cette étude est la mise au point d'une technique analytique juste pour la détermination du MMHg dans les sédiments. Cette technique fait intervenir l'extraction sous champ micro-ondes, l'éthylation et l'analyse par couplage GC-ICPMS. Une extraction supplémentaire dans un solvant organique est aussi nécessaire afin de réduire la quantité de IHg avant l'éthylation. Grâce à l'utilisation de traceurs isotopiques, il est possible de suivre les transformations et il apparaît que l'artéfact de méthylation ne se produit pas pendant les étapes de mise à l'équilibre de l'ajout et d'extraction. Les réactions de transalkylation semblent plutôt se produire dans la phase organique finale.

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## Using Speciated Isotope Dilution with GC–Inductively Coupled Plasma MS To Determine and Unravel the Artificial Formation of Monomethylmercury in Certified Reference Sediments

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Speciated isotope-dilution mass spectrometry (SID-MS) is claimed to be an absolute method; however, it has been found to be affected by artifact monomethylmercury (MMHg) formation in sediments. The determination of MMHg in sediments was carried out by SID-MS after open-focused microwave extraction. The extracted mercury species were then ethylated and separated by capillary gas chromatography (CGC). Isotope ratios (peak area ratios at different masses) were measured by on-line ICP-MS detection of the CGC-separated compounds. Reproducibility of  $^{202}\text{Hg}/^{201}\text{Hg}$  isotope ratio measurements were 0.60% for MeEtHg and 0.69% for Et<sub>2</sub>Hg; for  $^{202}\text{Hg}/^{199}\text{Hg}$ , 0.43 and 0.46%, respectively, were determined. The absolute detection limits for CGC–ICPMS measurements were better than 26 fg for  $^{202}\text{Hg}$ , 20 fg for  $^{201}\text{Hg}$ , and 24 fg for  $^{199}\text{Hg}$ . For the direct determination of MMHg in sediment reference materials (CRM 580, IAEA 356, and IAEA 405), higher values than the certified were always found. Systematic experiments were carried out to localize the sources of the unintentional abiotic methylmercury formation during analysis. Different spiking and derivatization procedures (either ethylation, propylation, or derivatization by Grignard reagents) were tested. In addition, isotopically enriched inorganic mercury was spiked. The amount of inorganic mercury initially present in the sample was found to be the critical factor that should be known and carefully controlled. A simple solvent extraction technique involving no critical cleanup steps was applied in order to reduce high Hg<sup>2+</sup> amounts. The method was applied to the determination of MMHg in sediment reference material IAEA-405 with satisfactory results after organic solvent extraction. The limitations of applicability of the proposed method are evaluated as related to inorganic mercury, organic carbon, and sulfur contents. The results obtained confirmed that

available sediment reference materials are adequate to achieve traceable mercury speciation analysis and to detect potential sources of MMHg artifact formation.

The ecological and human health effects of mercury are generally related to the environmental transformations of inorganic mercury to the toxic and biomagnification-prone compound monomethylmercury (MMHg).<sup>1</sup> Indeed, more than 80% of the mercury accumulated in fish tissues is present as MMHg, whereas in sediments, it does not exceed 1.5% of the total Hg content.<sup>2,3</sup> Processes leading to MMHg production in natural aquatic systems still need to be investigated. If microorganisms are well recognized for their potential to methylate inorganic mercury, the contribution of abiotic mercury methylation is still unresolved.<sup>1</sup> The methylation and demethylation processes preferably seem to occur in sediments. This compartment would act as the source for mercury methylation, subsequently leading to bioaccumulation.<sup>4</sup> Therefore, accurate and precise determination of monomethylmercury (MMHg) in sediments is the basis to better understanding the biogeochemical cycling of this contaminant and to estimate the associated exposures of aquatic food webs.

To date, many analytical techniques have been developed for the speciation of mercury.<sup>5–12</sup> The analysis of solid samples such as sediments or biotissues requires a leaching/digestion step prior

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to analysis. Detection of methylmercury is mostly carried out by atomic spectrometry so that the different mercury species must first be separated. Separation is often performed by GC after conversion of MMHg into a peralkylated volatile compound. Ethylation by sodium tetraethylborate is the most common derivatization technique used. Among the methods available, the hyphenation of CGC to ICP-MS appears to be one of the most promising techniques to carry out this type of speciation work because of its extremely high sensitivity and multielemental and multispecies-isotopic capabilities.

Despite significant improvements in instrumentation, MMHg determination is hindered by traditional problems related to nonquantitative recoveries and to questions about the possibility of artifact formation and transformations of methylmercury during the sample preparation step.<sup>13–19</sup> The production of artificial MMHg during the analytical procedure is a problem reported specifically for sediments. These facts may result in significant bias of measurements. Natural sediments often contain very low amounts of MMHg, representing only 0.5–2% of total mercury.<sup>2,3</sup> Therefore, even if artificial mercury methylation occurs in the small proportion of 0.02–0.03% of inorganic mercury only, this can result in 30–80% overestimation of MMHg concentrations in a sediment.<sup>13</sup>

In the aftermath of these early investigations, critical comments concerning the certified MMHg values in reference materials were made. The controversy was serious enough for the European Commission to finance a Workshop, of which the conclusions were summarized in a special issue of *Chemosphere*, published in 1999. The causes and factors involved in methylmercury formation during analysis were systematically evaluated.<sup>19</sup> A series of different techniques commonly used to extract MMHg from various matrixes were screened and tested to evaluate their potential to accidentally generate MMHg from inorganic Hg<sup>2+</sup> during sample preparation. The results highlighted the assumption that mercury species transformations were occurring during the sample pretreatment step and, more specifically, with distillation-based methods. The quantity of MMHg produced during extraction was related to the amount of inorganic mercury in the sample and the extraction method.<sup>13</sup> These observations were made on the basis of methylation of spiked inorganic mercury and not of spiked methylmercury. Experts participating in the workshop agreed that “at present, existing CRMs fulfill the purpose of verifying the accuracy of current methods and achieving data comparability; the doubts expressed on analytical measurements should, however, be considered seriously and further research should be pursued to possibly confirm the experiments”.<sup>20</sup>

Further experiments have confirmed artificial MMHg as a function of the inorganic mercury content present in the sample. The artifact of MMHg has been reported to be preferably formed during derivatization,<sup>14</sup> separation,<sup>15</sup> and extraction procedures.<sup>13,16</sup>

Since the levels of total mercury in most sediment reference materials far exceed those found in natural sediments, the formation of artifact in such reference materials may well occur whereas this artificial production in less contaminated natural sediments will not take place at a detectable level. However, even for a reference material that resembles mercury concentrations found in natural estuarine regions, such as IAEA 405 (5.49 and 810 ng g<sup>-1</sup> for MMHg and total Hg, respectively), artificial MMHg formation has been recently reported using distillation extraction.<sup>21</sup>

Taking into account the present problem of mercury speciation data validation in sediments, isotope dilution analysis may prove a useful strategy.<sup>22</sup> Not only is this technique highly accurate and precise, but also the isotopically enriched isotopes can be used as tracers to check for species transformations. The use of species-specific enriched stable isotopes could greatly assist in the testing and diagnostics of analytical methods. The isotope dilution approach has the potential to provide several types of information not available with other techniques, not only by spiking inorganic mercury but also by mercury species labeled with enriched mercury isotopes.

Research effort to better understand the area of speciation analysis by using speciated isotope dilution mass spectrometry (SID-MS) and appropriate experimental design is a question of major interest, and it will be attempted. In this paper, a SID-MS procedure for MMHg determination in sediments has been developed using extraction by acid leaching, derivatization by ethylation with NaBEt<sub>4</sub> and CGC–ICP-MS analysis. Systematic experiments have been carried out with certified reference sediments (IAEA, IRMM) to localize the sources of unintentional abiotic methylmercury formation during analysis and to determine the limitations of the proposed method. Different spiking and derivatization procedures were tested. Finally, potential factors controlling MMHg formation have been studied, with special attention to the total organic carbon and sulfur content of the sediments.

## EXPERIMENTAL SECTION

**Instrumentation.** A gas chromatograph (HP5890) was equipped with a capillary column and coupled to an Agilent model HP-7500 inductively coupled plasma mass spectrometer via a Silcosteel (Restek) transfer capillary. The instrumental configuration allows working in wet plasma conditions. A detailed description has been previously published.<sup>12,23</sup> Briefly, the silcosteel capillary was inserted into the torch injector, and the connection to the torch was realized by means of glass T-piece. A Scott cooled (2 °C) spray chamber and a conventional Babington nebulizer were connected to this T-piece and enabled continuous aspiration of a standard solution (TI, 10 µg/L). This configuration allowed optimization of instrument performance and simultaneous measurement of <sup>203</sup>Tl and <sup>205</sup>Tl for mass bias correction during the chromatographic run. Operating conditions and instrumentation are listed in Table 1.

Extraction of mercury species from solid samples was undertaken by a Microdigest A301 (2450 MHz; maximum power, 200 W) microwave digester (Prolabo, Fontenay-sous-bois, France).

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**Table 1. CGC and ICP-MS Operating Conditions for the Separation of MeEtHg and Et<sub>2</sub>Hg**

| GC Parameters        |                                                                 |
|----------------------|-----------------------------------------------------------------|
| column               | MXT Silcosteel 30 m, i.d. 0.53 mm, df 1 $\mu$ m                 |
| injection            | splitless                                                       |
| injector temp        | 200 °C                                                          |
| injection volume     | 2 $\mu$ L                                                       |
| carrier gas flow     | He 25 mL/min                                                    |
| makeup gas flow      | Ar 300 mL/min                                                   |
| oven program         |                                                                 |
| init temp            | 60 °C                                                           |
| init time            | 0 min                                                           |
| ramp rate            | 50 °C/min                                                       |
| final temp           | 180 °C                                                          |
| Transfer Line        |                                                                 |
| length               | 1 m                                                             |
| inner                | Silcosteel, i.d. 0.28 mm, o.d. 0.53 mm                          |
| outer                | Silcosteel, i.d. 1.0 mm, o.d. 1/16 in.                          |
| ICP-MS Parameters    |                                                                 |
| rf power             | 1250 W                                                          |
| gas flow             |                                                                 |
| plasma               | 15 L/min                                                        |
| auxiliary            | 0.9 L/min                                                       |
| nebulizer            | 0.9 L/min                                                       |
| isotopes/dwell times | Hg: 202, 201, 199; 30 ms<br>Tl: 203, 205; 5 ms<br>Xe: 126; 5 ms |

**Reagents.** Stock solutions of Hg<sup>2+</sup> and MMHg (1000 mg/L) of natural isotopic composition were prepared by dissolving mercury (II) chloride (Strem Chemicals 99.9995% Hg) in 1% HNO<sub>3</sub> (Merck) and methylmercury chloride (Strem Chemicals) in methanol (Merck), respectively. Working standard solutions were prepared daily by appropriate dilution of the stock standard solutions in 1% HNO<sub>3</sub>; all solutions were stored at 4 °C in the dark. Methylcobalamine (Sigma) used for synthesis was prepared by dissolution in an acetic acid–acetate buffer solution (0.1 M, pH 5). <sup>201</sup>HgO and <sup>199</sup>HgO were obtained from Oak Ridge National Laboratory (USA). The sodium tetraethylborate (98%) was purchased from Strem Chemicals (Bischheim, France), and the sodium tetrapropylborate (98%), from Galab (Geesthacht, Germany). BuMgCl (2.0 M in THF, Sigma Aldrich) was used as Grignard reagent.

The organic solvents used (2-propanol, isooctane, methylene chloride) were of HPLC grade. All other reagents were of analytical reagent grade. Ultrapure water was obtained from a Milli-Q system (Quantum EX, Millipore, USA).

**Reference Materials.** A series of sediment reference materials presenting a large difference in total inorganic mercury and MMHg concentration was used. Sediment reference material CRM 580 (Estuarine Sediment) was obtained from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). The reference sediments IAEA-356 (Polluted Marine Sediment) and IAEA-405 (Trace Elements and Methylmercury in Estuarine Sediment) were purchased from the International Atomic Energy Agency (IAEA, Vienna, Austria).

Organic carbon and total sulfur contents in the sediment reference materials (IAEA 405, IAEA 356, and CRM 580) were measured using a LECO CS 125 induction furnace with infrared detection.<sup>24</sup> The organic carbon was measured after removal of

the carbonates with 2 M HCl. The procedure for the determination of the total sulfur was similar, except that no HCl was added to the sample.

**Real Samples.** A surface intertidal sediment was collected from the Adour River estuary (France) in October 2000. The sediment sample was immediately sieved at 2 mm, subsequently frozen at −18 °C, freeze-dried, and finally, ground to fine particle size. The dry sediment was then stored in the dark at +4 °C until analysis. Total mercury concentration was found to be 115 ng/g.

**Study Design.** Potential formation of artifact MMHg in CRM 580, IAEA 356, and IAEA 405 reference materials and sediment from the Adour River estuary (France) was investigated. Isotopically enriched monomethylmercury (MM<sup>201</sup>Hg) was spiked to perform SID-MS. Since derivatization, separation, and extraction steps have been previously reported for its potential implication in MMHg artifact formation, the experiments were designed to examine each analysis step. A schematic diagram of the systematic experiments carried out is shown in Figure 1. First, the spiking step was evaluated, both with respect to the homogenization effect (solvent used for homogenization) and the timing of spiking (before or after microwave extraction). Different derivatization conditions were then tested. Finally, the effect of leached inorganic mercury during the extraction in the subsequent steps of analysis was evaluated.

Replicate samples were prepared and analyzed for each treatment (*n* ranged from 2 to 5 per treatment). Standard errors of mean values, as shown in the tables, are based on the standard deviation of replicate samples.

**Procedures.** *Microwave Acid Extraction of Mercury Species from the Sediments.* The extraction of mercury species in sediments was performed by open microwave-assisted extraction techniques previously described in the literature.<sup>5</sup> Briefly, a sample of 1 g of dry sediment was suspended in 10 mL HNO<sub>3</sub> (6 N) and exposed to microwave irradiation at a power of 60 W for 3 min. The supernatant solution was separated after centrifugation at 2000 rpm for 5 min, poured into 22-ml Pyrex vials with Teflon caps (Supelco), and stored in a refrigerator until analysis.

*Isotopically Enriched Monomethylmercury Spike.* SID-MS is based on the addition of a known amount of the isotopically enriched species to the sample. <sup>201</sup>Hg-enriched MMHg was not commercially available and was synthesized in-house from <sup>201</sup>HgO using methylcobalamine. Initial conditions were selected from Filipelli and Baldi's work,<sup>25</sup> but were optimized in order to obtain maximum methylmercury yield while minimizing dimethylmercury formation. Optimization of the synthesis conditions has been described elsewhere.<sup>26</sup>

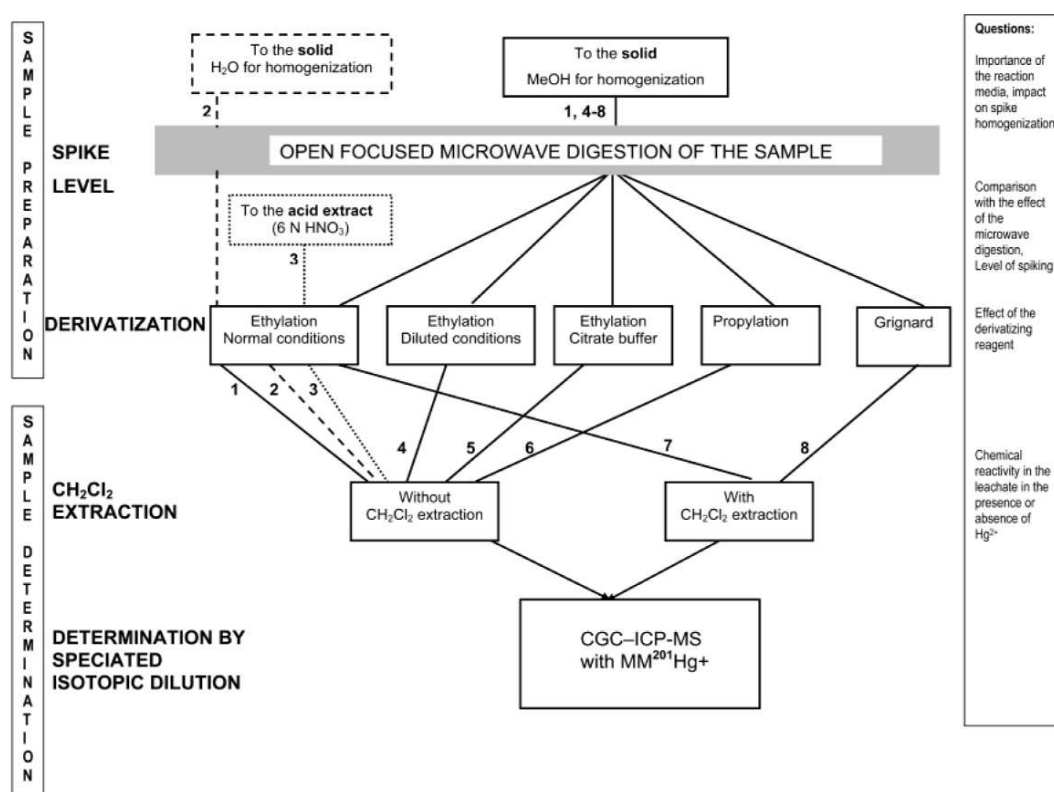
Equilibration of the spike isotope with the natural element/species in the sample alters the isotope ratio that is measured and used for calculation. SID-MS relies purely on isotope ratios and assumes that the equilibrium between analyte and spike has been reached. Therefore, the spiking procedure appears a critical stage to ensure full equilibrium and the similar behavior of the analyte and its analogue during the analytical procedure. To find

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**Figure 1.** Scheme summarizing the speciated isotope dilution procedures evaluated in this work for potential MMHg artifact formation evaluation.

possible artifact sources, different spiking procedures were tested (Figure 1):

- *Direct Spike to the Solid in a Methanol Slurry before Extraction.*

This spiking procedure was previously used for SID-MS analysis of biological reference materials, giving satisfactory results.<sup>12</sup> Approximately 1 g of sample was spiked with a known amount of <sup>201</sup>Hg-enriched methylmercury solution, the concentration of which had been determined beforehand by reversed speciated isotope dilution. Two milliliters of methanol was added, and the resultant slurry was shaken mechanically overnight at ambient temperature in the dark. The remaining solvent was evaporated under a stream of nitrogen. The dry sample was subsequently dissolved following the microwave-extraction procedure.

- *Direct Spike to the Solid in an Aqueous Slurry before Extraction.*

The solid sample was spiked with the isotopically enriched solution. A 30-mL portion of water was added, and the slurry was mechanically shaken overnight. The sample was frozen (−18 °C), and the water was eliminated by freeze-drying. The dry sample was then extracted following the microwave-extraction procedure.

- *Direct Spike to the Acid Extract after Microwave Heating.*

The spike was added to the acid leachate of CRM 580 after acid leaching and the removal of sediment particles.

**Derivatization Procedures.** A series of derivatization procedures were tested to evaluate their effect on artificial MMHg formation (Figure 1).

*Ethylation in Normal Conditions.* Mixed standard solutions of different mercury compounds were buffered to pH 3.9 with 5 mL of a 0.1 M acetic acid–sodium acetate buffer. For the sediment sample extracts (6 N HNO<sub>3</sub> or CH<sub>2</sub>Cl<sub>2</sub> extracts), the pH was adjusted to 3.9 by addition of NH<sub>3</sub> (concentrated) and 5 mL of 0.1

M acetic acid–sodium acetate buffer. A 5-mL portion of a 0.5% sodium tetraethylborate solution was added as derivatization reagent. When the HNO<sub>3</sub> extract was used, 2 mL of isooctane was also added to extract the alkyl compounds formed. When CH<sub>2</sub>Cl<sub>2</sub> extract is used, no isooctane addition is necessary, because the reaction is taking place directly in the organic solvent. After 5 min of manual shaking and 5 min of further centrifugation (2500 rpm), the organic layer was transferred to a glass vial and stored at −18 °C until measurement.

*Ethylation in Diluted Conditions.* A 2-mL portion of the extract was diluted in 40 mL of MQ water; 1 mL of 0.1 M acetate buffer (pH = 3.9), 1 mL of KOH, and 50  $\mu$ L of NaBEt<sub>4</sub> 0.5% were added with 2 mL of isooctane. This procedure (without adding isooctane) was previously used by Tseng et al. for the determination of MMHg in sediments by cryotrapping-gas chromatography–quartz furnace atomic absorption spectrometry (CT-GC–QFAAS) and gave good results with certified sediments.<sup>5</sup>

*Propylation.* A 2-mL portion of the extract was buffered with 5 mL of a 0.1 M acetic acid–sodium acetate buffer and adjusted to pH 3.9 by addition of NH<sub>3</sub> (concentrated). A 5-mL portion of 0.5% sodium tetrapropylborate and 2 mL of isooctane were added in order to derivatize the mercury species. After 5 min of manual shaking and 5 min of further centrifugation (2500 rpm), the organic layer was transferred to a glass vial and stored at −18 °C until measurement.

*Grignard Reaction.* A 2 mL portion of the extract was submitted to solvent extraction with CH<sub>2</sub>Cl<sub>2</sub> as described below. Na<sub>2</sub>SO<sub>4</sub> was added to the organic phase in order to remove water traces. To perform the Grignard reaction, the organic phase was transferred into a vial with 50  $\mu$ L of BuMgCl and gently shaken for 10 min.

The organic phase was then transferred into a GC vial and stored at  $-18^{\circ}\text{C}$  until measurement.

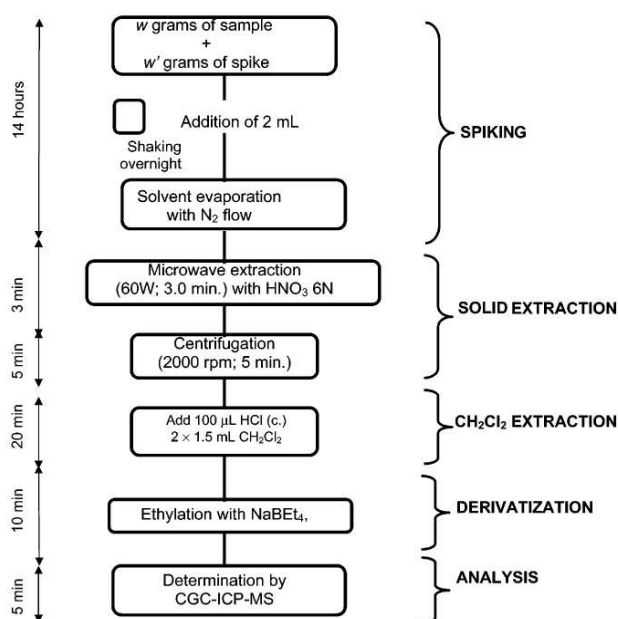
**Solvent Extraction with  $\text{CH}_2\text{Cl}_2$ .** Organic solvent extraction with methylene chloride was necessary to reduce the amount of inorganic mercury present during the derivatization step. Depending on the MMHg concentration, an appropriate aliquot of 6 M  $\text{HNO}_3$  sediment extract was weighed into a glass vial. A 1.5 mL portion of  $\text{CH}_2\text{Cl}_2$  and 100  $\mu\text{L}$  of  $\text{HCl}$  were added to the digest. The mixture was capped and shaken manually for 10 min. After the phases had separated, the lower solvent-phase  $\text{CH}_2\text{Cl}_2$  was collected. This operation was repeated one more time, and the combined extract (3 mL) was taken for derivatization.

**Capillary GC–ICP–MS Analysis.** Previously established CGC–ICP–MS conditions were used with minor modifications.<sup>12</sup> GC separation parameters were optimized in order to obtain symmetrical peaks, thus minimizing peak integration errors. A total integration time of 123 ms for all masses monitored was found to be optimum to accurately follow the chromatographic peak profile with a minimum peak skew. A 30-ms integration time for mercury isotopes (202, 201, 199) and 5 ms for thallium (203, 205) and xenon (126) isotopes were chosen. The peak width at half peak height was typically about 1.2 s; thus, 24 points define a full width peak of 3 s. Detector dead time was calculated according to Held and Taylor's method and applied for the subsequent isotope ratio measurements.<sup>27</sup> Mass bias was corrected by using the simultaneously measured thallium signal at  $^{203}\text{Tl}$  and  $^{205}\text{Tl}$ .<sup>12</sup> The raw data of the transient isotope signals for the different mercury species were further processed using the chromfile software (HP) to obtain the corresponding isotope ratios. Reproducibility of the  $^{202}\text{Hg}/^{201}\text{Hg}$  isotope ratio measurements was 0.60% for MeEtHg and 0.69% for Et<sub>2</sub>Hg. For the ratio  $^{202}\text{Hg}/^{199}\text{Hg}$ , 0.43 and 0.46%, respectively, were determined. The detection limits for the CGC–ICP–MS were estimated using three times the standard deviation of the background noise close to the chromatographic peak and were better than 26 fg for  $^{202}\text{Hg}$ , 20 fg for  $^{201}\text{Hg}$ , and 24 fg for  $^{199}\text{Hg}$ . The good precision on isotope ratio and the low detection limits using the CGC–ICP–MS allows good conditions for SID–MS.

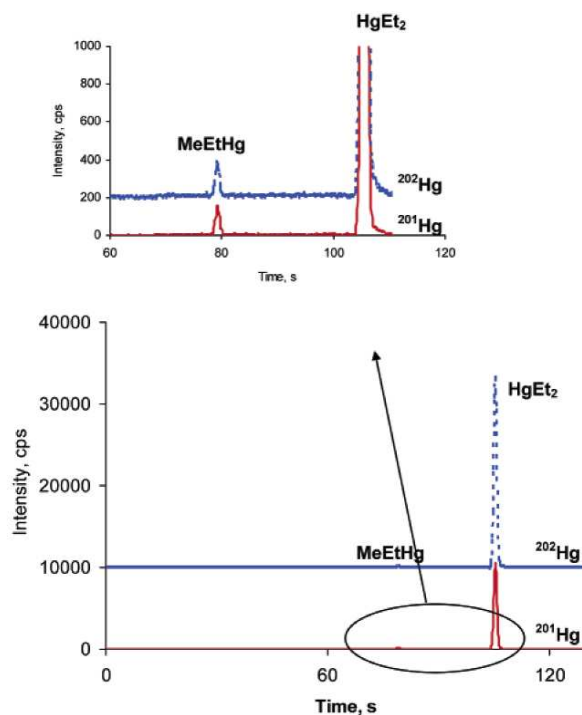
## RESULTS AND DISCUSSION

A flowchart of the simplified SID–MS analytical procedure (i.e., spike, MW extraction, derivatization, determination) used is presented in Figure 2.

**1. Isotope Dilution Analysis of MMHg in Sediments by Acid Leaching Extraction, Ethylation and CGC–ICP–MS.** SID–MS analysis was first applied to the quantification of MMHg from certified reference sediments CRM 580 (estuarine sediment), IAEA 356 (polluted marine sediment) and IAEA 405 (estuarine sediment), covering a wide range of total mercury (from 810 to 132 000 ng/g) and monomethylmercury concentrations (from 5.49 to 75.3 ng/g). The initial conditions were those named no.1 in Figure 1. A chromatogram obtained for IAEA 405 spiked with MM $^{201}\text{Hg}$  is shown in Figure 3 for isotopes  $^{201}\text{Hg}$  and  $^{202}\text{Hg}$ . As can be seen, good chromatographic separation and peak profile were obtained. The isotopic composition of methylmercury has drastically been altered; an isotope ratio of 1.172 for  $^{202}\text{Hg}/^{201}\text{Hg}$  was determined instead of the natural value of 2.26.



**Figure 2.** Schematic flow diagram of the isotope dilution protocol for mercury speciation analysis in sediments.



**Figure 3.** Chromatogram obtained for the IAEA 405 sediment reference material spiked with  $^{201}\text{Hg}$ -enriched monomethylmercury solution. The chromatogram at mass 202 was shifted for clarity.

The results obtained for five injections of each solution into the CGC–ICP–MS system are given in Table 2. Higher values than the certified ones are systematically found for all materials tested, MMHg recoveries ranged from 148 to 413% in excess of the certified value. In addition, the high standard deviation values obtained should be pointed out. The % RSD values are around 10% for all of the materials tested, which provide rather poor precision for isotope dilution analysis.

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**Table 2. MMHg Analysis in Certified Reference Sediments by SID-MS under Different Derivatization Conditions<sup>a</sup>**

| material | cert values                                  | ethylation          |                     |
|----------|----------------------------------------------|---------------------|---------------------|
|          |                                              | normal conditions   | diluted conditions  |
| CRM 580  | 75.5 ± 3.7 ng/g MMHg<br>132 ± 3 µg/g Hg      | 311.7 ± 33.6 (413%) | 193.8 ± 19.9 (257%) |
| IAEA 356 | 5.87 ± 0.89 ng/g MMHg<br>7.62 ± 0.65 µg/g Hg | 19.8 ± 1.7 (337%)   | 23.6 ± 3.0 (402%)   |
| IAEA 405 | 5.90 ± 0.57 ng/g MMHg<br>0.81 ± 0.04 µg/g Hg | 8.72 ± 1.16 (148%)  | 7.69 ± 0.82 (130%)  |

<sup>a</sup> Determined MMHg concentration (ng/g) ± SD (% recovery). Number of independent replicates = 3, each injected five times.

SID-MS has been claimed to provide high precision and to be mostly interference-free. SID-MS with labeled methylmercury can adequately correct for variations in the solid–liquid (from the solid to the aqueous phase) and liquid–liquid (aqueous to organic phase) extraction efficiency, the derivatization yield and instrumental sensitivity drift. On the other hand, reference materials have been certified on the basis of several extractions as well as detection techniques. A series of independent and well-experienced laboratories and producers of CRMs were agreed upon and contributed to establish the certified values. In the case of MMHg certifications in sediment, an excellent near-agreement was found among the expert laboratories for the IAEA 405, IAEA 356, and CRM 580 sediment reference materials. However, comparability of data on its own is not an absolute assurance of true values. Therefore, the high values found in the sediment reference materials by SID-MS pose questions on both certified values and SID-MS procedures.

**2. Influence of Analytical Steps on Artifact MMHg Formation.** From the literature, methylation artifacts may be produced during the extraction step,<sup>13,16,21</sup> by ethylating reagents<sup>14</sup> or during the gas chromatographic separation.<sup>15</sup> In our protocol, microwave acid extraction followed by capillary GC–ICP-MS detection is used. This acid microwave extraction procedure has already been tested for MMHg artifact formation by Tseng et al. without significant problems, as long as all analytical parameters are well-controlled.<sup>15</sup> In this case, the determination was performed by aqueous ethylation and CT-GC–QFAAS. Extraction of MMHg from CRM 580 sediment was performed with various concentrations of HNO<sub>3</sub> and HCl by microwave leaching. The concentrations were found to be in agreement with the certified values for extractions carried out with 2–8 M HNO<sub>3</sub> or HCl. In addition, the validation of MMHg measurements was achieved by using spiked recovery experiments prepared with a wet procedure before microwave extraction. Spike recovery of 96 ± 8% was obtained for CRM 580 after extraction with 6 M HNO<sub>3</sub>. Concerning the consensus values with respect to certified values, potential methylation artifact formation during the microwave-assisted acid extraction step was considered as nonexistent or negligible.

Thus, potential formation of artificial MMHg during spiking, derivatization, or separation steps in our procedure but not the previous microwave extraction step will be evaluated. The different tested conditions are schematically shown in Figure 1.

**Effect of Spiking Procedures on MMHg Formation.** The results shown in Table 2 were obtained on the basis of the <sup>201</sup>Hg-enriched monomethylmercury spiked to the solid in a methanol medium for homogenization (Figure 1, experiment no. 1). Any

sample pretreatment will bring not only methylmercury and inorganic mercury into solution, but also the matrix components (e.g. organic matter, organic, and reactive ligands, ...), including potential methylating agents, which may induce artifact formation. The methylating compounds could, thus, be introduced by the solvent (methanol) in a reactive form so that methylation reactions take place more easily.

Therefore, different MM<sup>201</sup>Hg spike procedures were evaluated for CRM 580 reference sediment material (Figure 1, experiments nos. 1–3). Methanol was substituted by water in the homogenization step (Figure 1, experiment no. 2), but similar artifact MMHg formation was obtained. Thus, methanol is apparently not responsible for such an analytical artifact.

To further clarify if the spike stabilization step is responsible for artifact formation, the CRM 580 reference material was acid microwave-extracted, with neither methanol nor water addition. The sediment particles were removed, and the MM<sup>201</sup>Hg spike was directly added to the extract (Figure 1, experiment no. 3). Artifact MMHg was also found in similar yield.

Consequently, from the above experiments, it seems that the artificial MMHg formation is related neither to the spike stabilization nor to the microwave extraction step, but most probably is due to further analytical steps in solution.

**Effect of Derivatization Conditions on MMHg Formation.** The derivatization step has previously been related to artificial MMHg formation. The presence of small impurities of methyl groups in the ethylation reagent used has been previously reported to provide erroneous methylmercury results when high concentrations of inorganic mercury are present in the sample.<sup>14</sup> In addition, acetic acid was found to be a very good methylation agent of inorganic mercury in methods using water vapor distillation.<sup>21</sup> Thus, potential formation of artifact MMHg during the derivatization steps was evaluated, and different ethylation conditions (reagent concentration and buffer) and derivatization procedures (ethylation and propylation) were tested together with methanol homogenization (Figure 1, experiments nos. 1 and 4–6).

Derivatization by ethylation was carried out using the same reagents but under more diluted conditions (experiment no. 4), and the final concentrations selected were the same as previously used by Tseng et al. for purge-and-trap GC–QFAAS detection (see derivatization procedures section).<sup>15</sup> The final volume was 40 mL of water instead of 12 mL, and the buffer and NaBEt<sub>4</sub> concentration were significantly reduced (50 µL of NaBEt<sub>4</sub> 0.5% instead of 5 mL, for instance). It was assumed that under these conditions, the probability of the methylation agent to react with mercury would be reduced. However, even under these more dilute conditions,



artificial mercury methylation was observed for all of the studied reference materials, as shown in Table 2. Recoveries were between 130 and 402% with respect to the certified values, and the precision was not as good as expected for an isotope dilution analysis procedure.

The effect of the buffer used during ethylation was also tested. Citrate instead of acetate buffer was used to control the pH (experiment no. 5). No improvement in the artifact formation was observed ( $215 \pm 15.3$  ng/g of MMHg for three independent determinations of CRM 580, i.e., recovery of 284%).

Since ethylation in different conditions gave similar results, a different derivatization reagent was tested. Recently, a propylation reagent (NaBPr<sub>4</sub>) has been commercialized. Similar to NaBEt<sub>4</sub>, the propylation reagent is used in aqueous media, and in the case of mercury, it allows the distinction between ethyl and inorganic mercury derivatives. No information about methylmercury artifacts has been previously reported. Thus, propylation instead of ethylation was attempted (experiment no. 6). Again, high MMHg artifact formation was found ( $134.9 \pm 22.5$  ng/g MMHg for CRM 580; three independent replicates, each injected three times).

According to these series of results, the methylation artifact formation must occur during the ethylation/organic extraction step, and it does not appear to be related to direct methylation by the derivatization reagents. This is supported by the fact that very different derivatization conditions lead to the same results. Another parameter is, thus, probably controlling this artifact formation during derivatization and organic solvent extraction.

**3. Importance of Inorganic Mercury Contents in Sediments. Relationship between MMHg Formation and Hg<sup>2+</sup> Content in CRMs.** Artifact methylmercury formation has previously been related by other authors to the amount of inorganic mercury in the sample.<sup>13–15,21</sup> In our case, artifact formation during MW extraction has been excluded because of the results of previous experiments, and mostly seems to occur in the following steps. It is, thus, relevant to investigate the importance of inorganic mercury content on artificial MMHg formation.

First evidence indicating the importance of inorganic mercury is the clear relationship between the monomethylmercury formed and the amount of inorganic mercury leached from reference sediment materials. The relation between the MMHg formed and the concentration of Hg<sup>2+</sup> gives a slope of  $13 \times 10^{-4}$  for the ethylation with normal conditions and  $7 \times 10^{-4}$  for the ethylation in diluted conditions with regression coefficients ( $r^2$ ) of 1.00 and 0.999, respectively. Thus, 0.13 and 0.07%, respectively, of the inorganic mercury in solution was methylated after acid leaching, both in normal and in diluted ethylation conditions. This level is similar to those described in the literature. Hintelmann et al. found a methylation yield of ~0.03% using distillation, aqueous phase ethylation followed by Tenax preconcentration, thermodesorption, GC separation, and analysis by ICP-MS.<sup>13</sup> Using a similar preparation procedure and cold-vapor atomic fluorescence spectrometric detection (CVAFS), Hammerschmidt and Fitzgerald found ~0.17% for a low concentration of inorganic mercury, and 0.026% was obtained when total mercury was higher than 1550 ng/g.<sup>21</sup> Horvat et al. found a yield ranging from 0.08 to 0.94%, depending on the ethylation reagent when alkaline digestion, aqueous phase ethylation, Carbotrap adsorption, thermal desorption onto GC column, pyrolysis, and CV-AFS detection were applied. Similar results were

**Table 3. Artifact Formation of MM<sup>199</sup>Hg in Adour River Sediment after Addition of Enriched <sup>199</sup>Hg to the Clean Acid Extract**

| <sup>199</sup> Hg <sup>2+</sup> added, ng | <sup>199</sup> MMHg found, ng | specific methylation rate, % | isotope ratio ( <sup>202</sup> Hg/ <sup>199</sup> Hg) |
|-------------------------------------------|-------------------------------|------------------------------|-------------------------------------------------------|
| 73.6                                      | 0.27                          | 0.3                          | 0.907                                                 |
| 935                                       | 0.53                          | 0.05                         | 0.070                                                 |
| 1912                                      | 1.68                          | 0.09                         | 0.044                                                 |

obtained when distillation instead of alkaline digestion was used.<sup>14</sup> Tseng et al. obtained yields ranging between 0.005 and 0.022% with MW acid extraction, aqueous phase ethylation, cryo-trapping, GC separation, quartz furnace pyrolysis, and atomic absorption spectrometry. In this case, artificial MMHg formation was attributed to the silanizing agent (dimethyldisilazane) used to prepare the chromatographic packed column.<sup>15</sup> Thus, similar methylation yields are observed using very different preparation procedures, even though artifact formation has been associated with different analysis stages.

*Artificial MMHg Formation from Spiked Inorganic Mercury in Natural Sediments.* To confirm the potential formation of the artifact MMHg during either derivatization or separation steps, isotopically enriched inorganic mercury (<sup>199</sup>Hg<sup>2+</sup>) was added to a natural nonpolluted estuarine sediment (natural total mercury concentration was 115 ng/g). The enriched <sup>199</sup>Hg<sup>2+</sup> was added at three concentration levels to the leachate of this sample after microwave extraction with 6 N HNO<sub>3</sub> and separation of the acid from the sediment particles by centrifugation. The results are shown in Table 3. When different spike levels are compared, an increase in MM<sup>199</sup>Hg is observed with rising <sup>199</sup>Hg spike. It is also interesting to point out the change in the MMHg isotope ratio switch: MM<sup>199</sup>Hg increases with respect to natural MM<sup>202</sup>Hg, and the measured <sup>202</sup>Hg/<sup>199</sup>Hg isotope ratio ranged between 0.907 and 0.044 for the different spiking levels, while the natural isotope ratio is 1.77. Table 3 presents also the methylation yield (%) calculated by dividing the mass of MM<sup>199</sup>Hg produced by the total mass of <sup>199</sup>Hg in solution (spiked + unspiked). The higher yield (in %) of artifacts formation corresponds to the lower amount of spiked <sup>199</sup>Hg<sup>2+</sup>. Since the enriched isotope was added to the extract after MW extraction and centrifugation, the only explanation for this observation is an artificial formation of MMHg during or after the derivatization step. This result also indicates that the artifact is not produced at a major extension during the MW extraction procedure.

Additionally, a potential factor controlling artifact formation is the amount of inorganic mercury present in the solution at this step of analysis. However, in both cases (reference materials and spiked natural sediments), higher inorganic mercury levels correspond to lower methylation yield. Similar results were reported by others in distillates of IAEA-405<sup>21</sup> and for spiked distillates of IAEA-356.<sup>13</sup> Consequently, methylation yield does not seem to be controlled either by the total amount of natural inorganic mercury or by the availability of spiked mercury. Most probably, it will be exhaustion or quenching of the methylating agent.

**4. Isotope Dilution Analysis of MMHg by Acid Leaching and CH<sub>2</sub>Cl<sub>2</sub> Extraction, Derivatization and CGC-ICP-MS.** Regardless of the reactivity of inorganic mercury, artifact methy-

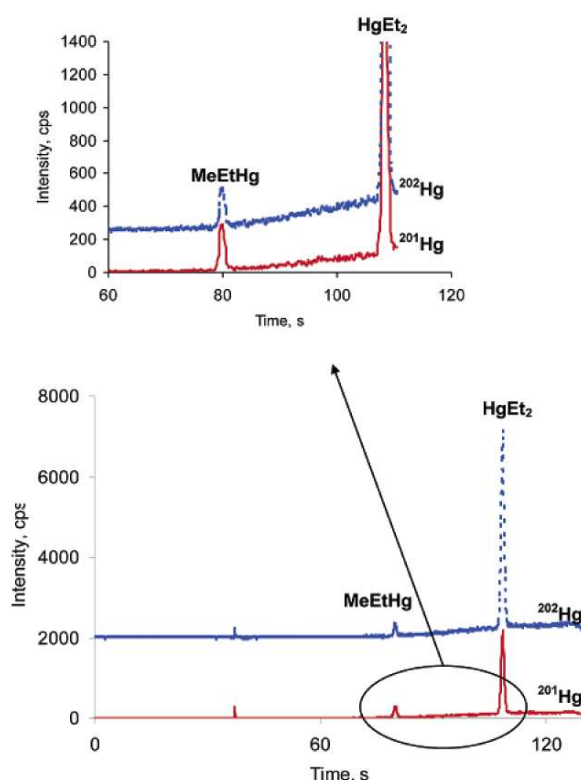


lation yield is not acceptable, since under natural conditions, the percentage of MMHg in sediments is usually below 1.5%. This interference represents a real limitation, especially when samples from mercury-contaminated areas are to be analyzed. Thus, since the amount of inorganic mercury extraction by the leaching procedure and its presence during derivatization seems to be a critical parameter, one approach to overcome the artifact MMHg could be to discriminate MMHg from inorganic mercury before derivatization.

**MMHg Selective Extraction by  $\text{CH}_2\text{Cl}_2$ .** There are several options to try to remove or reduce inorganic mercury from the extract before starting the derivatization, such as distillation, extraction, or ion exchange procedures. The optimum conditions should be those at which the recovery of MMHg is maximized, while  $\text{Hg}^{2+}$  transferred to the end product is minimized. Separation techniques, such as distillation, are not well-suited, since during distillation of samples rich in inorganic mercury, large amounts of  $\text{Hg}^{2+}$  are transferred into the distillate together with MMHg.<sup>14</sup> Another approach is the use of a simple organic solvent extraction with methylene chloride ( $\text{CH}_2\text{Cl}_2$ ) involving no further specific cleanup steps (Figure 1, experiment no. 7).<sup>28</sup> This procedure has been adapted to our working conditions using nitric acid for extraction instead of a mixture of KOH and methanol. Though in our case, neutralization is not required, the use of HCl was maintained, since it has been shown that in the presence of this acid, the amount of inorganic mercury transferred to the organic solvent decreases with increasing HCl amount.<sup>28</sup> With high  $\text{Cl}^-$  concentrations, the relatively stable  $\text{Hg}^{2+}$  and MMHg complexes ( $\text{HgCl}_4$ )<sup>2-</sup> and  $\text{MeHgCl}$  are formed. Thus, anionic inorganic Hg will be much less soluble in an organic solvent than the neutral (and polar) organic MMHg. Recoveries using this procedure were ~75% for MMHg and <1% for inorganic mercury, for both standards and real samples.

First, the screening test with the natural sediment spiked with  $^{199}\text{Hg}^{2+}$  was repeated but using the methylene chloride extraction before ethylation. The natural isotopic composition was not so drastically altered as previously observed ( $^{202}\text{Hg}/^{199}\text{Hg}$  measured isotope ratio of 1.51 instead of 1.77 theoretical), even if the spike level was much higher (5621 ng/g instead of 1914 ng/ $^{199}\text{Hg}^{2+}$ ). There was also no increase in  $\text{MM}^{199}\text{Hg}$  when the  $^{199}\text{Hg}^{2+}$  amount added was increased. This means that in these working conditions, artifact MMHg formation is not observed or is negligible as a result of the reduced amount of inorganic mercury during the ethylation procedure. This confirms that artifact formation is mostly related to the presence of both inorganic mercury and methylating agents during the derivatization/organic extraction step.

**Method Evaluation with CRMs.** Reference materials were analyzed using  $\text{CH}_2\text{Cl}_2$  extraction before ethylation (Figure 1, experiment no. 7). Significant inorganic mercury diminution with respect to the theoretical values was found. Figure 4 shows a chromatogram obtained for IAEA 405 sediment reference material spiked with enriched  $\text{MM}^{201}\text{Hg}$ . The measured  $^{202}\text{Hg}/^{201}\text{Hg}$  isotope ratio is 1.03 instead of the natural ratio of 2.26 ratio. The results for MMHg determinations are given in Table 4. MMHg concentrations for the reference sediment IAEA 405 (the less



**Figure 4.** Chromatogram obtained for the spiked IAEA 405 sediment reference material after  $\text{CH}_2\text{Cl}_2$  extraction. The chromatogram at mass 202 was shifted for clarity.

polluted sediment) were in good agreement with the certified value. The recovery found was 101%, with a relative standard deviation on three independent measurements of 5.8%. For the same material, Hammerschmidt and Fitzgerald found a recovery of 133% using both distillation and acid leaching techniques without SID-MS.<sup>21</sup> The highly precise determination (below 5% RSD in most cases) obtained by SID-MS for this multistep procedure should be pointed out here (see Figure 2).

However, MMHg artifact formation was still observed when highly polluted reference sediments (CRM 580 and IAEA 356) were analyzed using one single  $\text{CH}_2\text{Cl}_2$  extraction. Recoveries up to 216% were obtained for IAEA 356 (Table 4). Since the artifact seems to be related to the derivatization/organic extraction step, an alternative organic phase derivatization procedure using Grignard reagent ( $\text{BuMgCl}$ ) was tested after methylene chloride solvent extraction (Figure 1, experiment no. 8). Three independent spiking procedures were carried out to analyze each certified sediment, and each sample solution was injected four times into the CGC-ICP-MS. The results are also shown in Table 4. The derivatization with Grignard reagent gives the same result as the ethylation procedure. Recoveries are in good agreement with the certified value for the IAEA 405 but significantly higher for the CRM 580 and IAEA 356 reference materials.

In our procedure, artifact methylethylmercury (i.e., MMHg derivative), can be formed from either the mercuric ion ( $\text{Hg}^{2+}$ ) during derivatization by methylation pathways or diethylmercury (i.e., the  $\text{Hg}^{2+}$ ) derivative during separation with organic solvent by transalkylation pathways for CGC-ICP-MS injection. A similar process would occur with dibutyl-Hg after Grignard derivatization.

(28) Liang, L.; Horvat, M.; Cernichiar, E.; Gelein, B.; Balogh, S. *Talanta* **1996**, *43*, 1883–1888.

**Table 4. MMHg Determination in Certified Reference Sediments by SID-MS with CH<sub>2</sub>Cl<sub>2</sub> Extraction before Different Derivatization Procedures<sup>a</sup>**

| material | cert values                                  | CH <sub>2</sub> Cl <sub>2</sub> extraction/ethylation | CH <sub>2</sub> Cl <sub>2</sub> extraction/Grignard |
|----------|----------------------------------------------|-------------------------------------------------------|-----------------------------------------------------|
| CRM 580  | 75.5 ± 3.7 ng/g MMHg<br>132 ± 3 µg/g Hg      | 104.9 ± 1.3 (139%)                                    | 101.4 ± 5.2 (134%)                                  |
| IAEA 356 | 5.87 ± 0.89 ng/g MMHg<br>7.62 ± 0.65 µg/g Hg | 12.7 ± 1.8 (216%)                                     | 11.3 ± 0.8 (192%)                                   |
| IAEA 405 | 5.90 ± 0.57 ng/g MMHg<br>0.81 ± 0.04 µg/g Hg | 5.98 ± 0.35 (101%)                                    | 6.02 ± 0.14 (102%)                                  |

<sup>a</sup> Determined MMHg concentration (ng/g) ± SD (% recovery). Number of independent replicates = 3, each injected four times.

**Table 5. Percent C<sub>org</sub>, Percent S, Hg<sup>2+</sup> Content, and Percent Methylation**

|              |      |      | A. Without CH <sub>2</sub> Cl <sub>2</sub> Extraction <sup>a</sup> |               |                               |               |
|--------------|------|------|--------------------------------------------------------------------|---------------|-------------------------------|---------------|
|              |      |      | derivatization                                                     |               |                               |               |
|              |      |      | ethylation normal conditions                                       |               | ethylation diluted conditions |               |
| ref material | % C  | % S  | Hg <sup>2+</sup> <sup>b</sup>                                      | % methylation | Hg <sup>2+</sup> in soln      | % methylation |
| IAEA 405     | 1.75 | 0.25 | 820 ± 35                                                           | 0.343         | 860 ± 45                      | 0.208         |
| IAEA 356     | 6.40 | 0.88 | 12500 ± 850                                                        | 0.111         | 13400 ± 900                   | 0.132         |
| CRM 580      | 6.0  | 0.24 | 143400 ± 2100                                                      | 0.073         | 140000 ± 2000                 | 0.084         |

|              |      |      | B. With CH <sub>2</sub> Cl <sub>2</sub> Extraction <sup>a</sup> |               |                              |               |
|--------------|------|------|-----------------------------------------------------------------|---------------|------------------------------|---------------|
|              |      |      | derivatization                                                  |               |                              |               |
|              |      |      | ethylation normal conditions                                    |               | Grignard                     |               |
| ref material | % C  | % S  | Hg <sup>2+</sup> <sup>b</sup>                                   | % methylation | Hg <sup>2+</sup> in solution | % methylation |
| IAEA 405     | 1.75 | 0.25 | 90 ± 8                                                          | -             | 80 ± 6                       | -             |
| IAEA 356     | 6.40 | 0.88 | 520 ± 50                                                        | 1.296         | 630 ± 60                     | 0.855         |
| CRM 580      | 6.0  | 0.24 | 550 ± 50                                                        | 5.074         | 610 ± 50                     | 4.073         |

<sup>a</sup> No. independent replicates = 3; each injected five times. <sup>b</sup> ng/g.

Taking into account the very different ethylation procedures tested (ethylation in normal and diluted conditions and propylation without CH<sub>2</sub>Cl<sub>2</sub> extraction; ethylation in normal conditions and Grignard with CH<sub>2</sub>Cl<sub>2</sub> extraction), our hypothesis is that the artifact occurs from the ethylated derivatives in the last organic phase. One additional fact supporting this hypothesis is that the "ethylation in diluted conditions" are the same conditions used by Tseng et al.,<sup>15</sup> but they do not find any artifact related to this step. The difference is that Tseng et al. perform the determination of the ethylated species directly in the aqueous phase by a purge-and-trap system, whereas in our case, the organic extraction is required for the injection into the CGC-ICP-MS system. Therefore, it seems that MMHg is not really synthesized, but rather, methylmercury is produced and interpreted as MMHg during detection as a result of transalkylation reactions in the organic phase. Transalkylation of the formed dialkylmercury have been reported in the literature, and the transformation rate was found to be dependent on the amount of inorganic mercury.<sup>29</sup> The question is to determine the methylating agent(s). Unspecific compounds from sediment matrix could be responsible for it, but it is still difficult to confirm this point. One additional experiment would be to spike the final organic extract after derivatization with isotopically enriched Et<sub>2</sub>Hg. However, this standard is not commercially available for the moment, and this experiment cannot be carried out at present.

(29) Rosenkranz, B.; Bettmer, J.; Busher, W.; Breer, C.; Cammann, K. *Appl. Organomet. Chem.* **1997**, *11*, 721–725.

**5. Potential Chemical Processes Controlling MMHg Artifact Formation from Inorganic Mercury.** The further step will be to try to elucidate the potential mechanisms controlling MMHg formation from inorganic mercury. Taking into account the important role of carbon and sulfur in the behavior of mercury in the environment, the total organic carbon and sulfur content of the reference materials has been determined, and its relationship with artificial MMHg formation has been studied.<sup>24</sup> Values are shown in Table 5, parts a and b. Data corresponding to mercury level and percent methylation yield calculated by dividing MMHg formed by inorganic mercury (Hg<sup>2+</sup> in solution + MMHg formed) are also included for the different derivatization conditions tested.

*Artifact MMHg Formation Pathway under Aqueous Derivatization.* Without CH<sub>2</sub>Cl<sub>2</sub> extraction (Table 5, part a) and, therefore, with high inorganic mercury levels present during the derivatization/organic extraction step, it can be observed that the methylation yield decreases when the inorganic mercury level increases. Similar results were found by Hammerschmidt and Fitzgerald.<sup>21</sup> Different factors could be responsible for that. First, the methylation yield is controlled by chemically available mercury corresponding only to the reactive fraction of total mercury. In this case, organic carbon may be an important factor. CRM sediments with high organic carbon content (6.40%) have the lower methylation yield (0.111 or 0.132%). These results can be related to several potential pathways. First, in the acid extract buffered to pH 4 before ethylation, inorganic mercury can be strongly associated with organic ligands leached from the sedi-



ment. This organic complexation may thus control the availability of inorganic mercury for further methylation pathways in the aqueous or organic phase. Second, as mentioned earlier, a possible exhaustion or quenching of the methylation mechanisms of unknown alkylating agents can also occur in the solution.<sup>21</sup> Finally, a demethylation reaction may also play a significant role in controlling MMHg concentration, which has not been clearly elucidated until now.

*Artifact MMHg Formation Pathway under Organic Derivatization.* In comparison, when  $\text{CH}_2\text{Cl}_2$  extraction is used before derivatization (Table 5, part b), the methylation yield increases with increasing mercury content. In addition, much higher methylation yields are obtained for IAEA 356 and CRM 580 certified reference sediments, ranging from 0.855 to 5.074% for both ethylation and Grignard derivatization (Table 5, part b). This yield enhancement is a trend opposite to that previously observed without  $\text{CH}_2\text{Cl}_2$  extraction. Solvent extraction, thus, allows decreasing inorganic mercury content in solution, but also causes higher reactivity for direct methylation of inorganic mercury or transalkylation of  $\text{Et}_2\text{Hg}$  after derivatization.<sup>29</sup>

In this case, the organic extraction step is processed in a strong acidic medium (i.e., concentrated  $\text{HNO}_3$  and  $\text{HCl}$ ), allowing most of the mercury species to occur in labile and reactive form (chloride species). Thus, most of the inorganic mercury transferred to the  $\text{CH}_2\text{Cl}_2$  solution is probably more reactive, and its ethylation is not affected by organic matter. In addition, different inorganic mercury extraction and artifact methylation yields were obtained for IAEA 356 and CRM 580, although they have similar inorganic mercury levels in solution (Table 5, part b). IAEA 356 has a high sulfur content (0.88%) in comparison with the other materials (0.25 and 0.24%). This amount of sulfur in marine sediments is due to important sulfide concentrations. This sulfide fraction is solubilized in a strong acidic medium and may be easily extracted by an organic solvent. Once in the organic phase, sulfur may behave as a quencher for further artifact MMHg formation and transalkylation. Consequently, the higher the amount of sulfides in the sediments, the lower the yield of methylation will be.

Additionally, organic compounds such as humic, fulvic and organic acid are potentially precipitated at such low pH and can be efficiently transferred into the organic phase. The enhancement of the organic extraction of such potential methylating agents may, thus, improve the reaction yield for artificial MMHg formation.

This hypothesis is supported by the highest methylation yield obtained for organic-carbon-rich CRMs (i.e., IAEA 356 and CRM 580), as compared to IAEA 405, exhibiting low organic carbon content (1.75%) (see Table 5, part b).

## CONCLUSIONS

A procedure for SID-MS analysis of MMHg in sediments has been developed by using open-focused microwave extraction, ethylation, and CGC-ICP-MS detection. Although highly precise, the isotope dilution procedure is not free of interference, and artificial MMHg formation was detected. Systematic experiments allowed determination of the sources and definition of conditions for controlling the artifact formation during analytical determinations. From these experiments, it seems evident that MMHg artifacts do not occur during the spiking or extraction step, but mainly, during the final derivatization and extraction and that the inorganic mercury amount in this last step is the determining factor. Transalkylation reactions in the final organic phase became the most plausible mechanisms, since the very different ethylation methods tested give comparable methylation yields. However, this cannot be confirmed for the moment.

Organic carbon and sulfur levels are both recognized as indicators of the sediment biogeochemistry and, thus, may significantly help in understanding unintentional chemical formation of MMHg artifacts. The analysis of the results obtained for the different sediment reference materials indicates that carbon and sulfur levels could play a critical role on MMHg formation. However, very limited data are available at this point (only three reference materials with two different levels of sulfur and carbon), showing the urgent need for additional studies.

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## **Chapitre C – I**

**Détermination de la bioaccumulation des métaux et organo-métaux  
par les groupes trophiques de la macrofaune benthique de l'estuaire  
de l'Adour (Golfe de Gascogne)**



## **Détermination de la bioaccumulation des métaux et organo-métaux par les groupes trophiques de la macrofaune benthique de l'estuaire de l'Adour (Golfe de Gascogne)**

Les organismes benthiques représentent un maillon important de la chaîne trophique dans les environnements aquatiques. Ils sont aussi d'excellents indicateurs pour évaluer les changements environnementaux et l'impact écologique des polluants. Les sédiments sont connus pour être un piège des contaminants métalliques et organométalliques et les organismes benthiques y sont directement exposés. L'étude de la bioaccumulation et de la bioamplification des métaux et organométaux par ces premiers groupes trophiques est donc d'un réel intérêt afin d'appréhender le transfert de ces polluants vers les groupes trophiques supérieurs.

Néanmoins, cette étude nécessite un important développement de techniques que ce soit pour l'échantillonnage ou pour l'analyse. En effet, afin d'étudier la bioaccumulation voire la bioamplification par les organismes benthiques, il est d'abord nécessaire de réaliser un échantillonnage précis représentatif de l'écosystème étudié. Enfin, d'un point de vue analytique, la détermination des concentrations en métaux et organométaux est d'autant plus délicate à réaliser que la masse d'échantillon disponible est faible et que les concentrations sont proches des limites de détection.

Dans ce travail, une étude multidisciplinaire entre chimistes et biologistes est conduite afin d'étudier la bioaccumulation des métaux et organométaux dans la macrofaune benthique de l'estuaire de l'Adour. Une méthode intégrée d'échantillonnage et d'analyse a été développée. L'échantillonnage est réalisé de manière à classer les individus par groupes trophiques (suspensivores, dépositivores et prédateurs). L'optimisation d'une extraction micro-ondes permet l'analyse sur le même extrait des métaux et organométaux permettant ainsi la détermination sur de très faibles masses d'échantillon. Enfin, l'analyse simultanée des composés du mercure et des butylétains est réalisée grâce à l'utilisation de la dilution isotopique.

Cette méthode a permis de déterminer la contamination et le transfert trophique des métaux et organométaux de la population benthique de l'estuaire de l'Adour. La biodiversité des organismes benthiques semble être liée à la contamination des sédiments. La bioaccumulation est dépendante du type de polluants métalliques et du groupe trophique. Les dépositivores sont généralement plus impactés par une contamination directe du fait de leur mode d'alimentation. La biomagnification parmi les groupes trophiques benthiques est démontrée uniquement pour le MMHg.

## Determination of metal and organometal trophic bioaccumulation in the benthic macrofauna of the Adour estuary coastal zone (SW France, Bay of Biscay)

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### ABSTRACT

A multidisciplinary approach has been carried out in order to investigate metal and organometal bioaccumulation in macrobenthic populations. A complete method coupling a sampling strategy and classification of benthic organisms with a performant analytical procedure for the analysis of both metal and organometal has been developed. A single sample preparation method using a TMAH extraction and species specific isotope dilution make possible the analysis of metal and organometals in the same extract which is specially interesting for very low mass samples. Low detection limits has been obtained in the range of 12-250 pg g<sup>-1</sup> for mercury and butyltin compounds and 0.4-50 ng g<sup>-1</sup> for metals with good precision (1-10% RSD) even for very low mass sample (0.02 g). This method allows for monitoring the contamination and the trophic transfer of metal and organometal as well as the biodiversity and the trophic structure of the macrofauna. This method has been applied to the monitoring of metal and organometal bioaccumulation in the macrobenthic population of the Adour Estuary (south west, France). Bioaccumulation has been determined in relation with the feeding guild of benthic organisms. The results demonstrate that bioaccumulation is dependent of the type of metallic contaminant and higher bioaccumulation is generally observed for deposit feeders directly impacted by sediment contamination. Bio-magnification along the trophic levels was highlighted for MMHg but no significant trend was observed for the other.

**KEYWORDS:** metal, organometal, bioaccumulation, trophic level, benthic macrofauna.

## 1- INTRODUCTION

There is a serious concern throughout the world about heavy metal pollution and their impact on living organisms. Trace metal deposits in aquatic systems tend to accumulate in sediments and water where they may threaten benthic biota and higher trophic fauna <sup>1</sup>. It has long been recognized that metals in the marine environment have a particular significance in ecotoxicology since they are highly persistent <sup>2</sup>. Monitoring pollution and predicting toxic metal effects on marine fauna are essential <sup>3</sup>. Macrobenthic communities are excellent indicators for biotic integrity and are suitable for evaluating environmental changes and environmental impacts due to contaminants <sup>4</sup>. Unfortunately metal uptake by organisms and trophic transfer in the food chain are often not reported <sup>5,6</sup>. Field studies report metal concentrations in benthic organisms and the potential toxicity effect in particular organisms; and the trophic transfer is generally studied in artificial ecosystems <sup>7</sup>. If criteria for bioaccumulation and bio-magnification of metals in aquatic organisms are based on the availability of metals and on the trophic transfer, it should first be demonstrated that benthic organisms play an important role in the food web and in which compartment metals are taken up by these fauna. The relationship between metal accumulation and the feeding behavior of the benthic organisms is an important point in terms of bioavailability of different metals in the aquatic environment and trophic transfer to the food web.

The sources, fate and bioaccumulation of mercury, especially as monomethylmercury (MMHg) have received increasing attention recently (US EPA 1997) due to the impact of fish consumption on human health <sup>8</sup>. Others metals and organo-metals are also concerned by regulations, such as organotin compounds which are known as highly toxic <sup>9</sup> and cadmium, which the anthropogenic pressure is also important due to waste incineration <sup>10</sup>.

Knowledge of the concentrations of heavy metals in biological tissues is desirable, in particularly in benthic organisms, to understand the bioconcentration in the food web. Sampling strategy and analytical method are very important and few studies have reported complete methods. An accurate measurement of metals in benthic populations needs the coupling of an efficient sampling method and a precise analytical method with low detection limits. Indeed, sampling of the benthic population should represent as true as possible the real repartition of the different species and the analytical method should be sufficiently accurate and precise to measure low concentrations in very low mass samples.

Total metals contents and speciation were generally performed by different methods. The first step of the sample preparation is the extraction. Normally, the extraction procedure for speciation analysis is not the same as the procedure for total metal determination due to the necessity of gentle extraction conditions to the repartition of the different species is not modified. For total metal determination, the use of concentrated acid is generally combined with microwave irradiation to obtain a complete dissolution of the sample <sup>11</sup>. Recently, alkaline reagent (Tetramethyl ammonium hydroxide, TMAH) has been successfully used to leach inorganic constituents from

biological samples <sup>12-14</sup>. Pozebon et al. <sup>12</sup> reported the determination of several elements by electrothermal vaporization inductively coupled plasma mass spectrometry (ETV ICPMS) in certified biological tissues (bovine liver NBS 1577a and dog fish muscle DORM 1). Although satisfactory results were obtained, it was noted that impurities in the TMAH solution limited performances. For speciation analysis, gentle solvents are used, such as TMAH for mercury <sup>15,16</sup> and acetic acid for organotin compounds <sup>17</sup>. In addition, the use of speciated isotope dilution has been applied recently for speciation analysis <sup>15,16,18</sup> and has been considered as a definitive method. This technique offers great potential in terms of accuracy and precision since quantitative recoveries during the sample preparation are not necessary and rearrangement reactions are easily detected.

In this study we propose a complete method including sampling strategy and an analytical procedure for the determination of trace metals and the speciation of mercury and butyltin in macrobenthic populations. This method allows for monitoring of the contamination and the trophic transfer of metals and organometals easily and rapidly with a single TMAH extraction for both trace element analysis and speciation analysis using species specific isotope dilution. This method has been applied to the monitoring of metals and organo-metals bioaccumulation in the benthic macrofauna trophic levels of the Adour estuary (France).

## 2- EXPERIMENTAL

### 2.1- Sampling strategy

#### 2.1.1- Sampling stations

Samples were collected in the Adour Estuary (South France) during late spring between 14<sup>th</sup> and 20<sup>th</sup> June 2001 during a cruise aboard the French research vessel “Côte de la Manche”. During this campaign (METADOUR 1), sediment and macrofauna samples were taken using a 0.1m<sup>2</sup> Smith-McIntyre grab. Water columns were also sampled during this cruise for both metal and organometal partitioning and microbial investigation using acid-clean Go-Flo sampling bottles. Results on the latter are presented elsewhere <sup>19</sup>.

Located in the south-west of France, the Adour estuary is flowing into the Gulf of Biscay (Atlantic Ocean). The whole mixing zone of this estuary (0-25 km) approximates a uniform channel, isolated from its original intertidal and salt marsh areas by large human dyking. Human modifications of the natural features of this estuary, mainly for urban and industrial developments in the lower part and agricultural extension in the upper part, has generated an important speed current <sup>20</sup>, improving the dominant part of the fresh waters compared to the saline waters in the water volume mixing. This results in a very low residence time for both waters and particles entering the estuary, suggesting a dominant transfer to the ocean during medium to high flow conditions. The estuary is collecting freshwaters from a whole drainage basin with a cumulated area of 16.380 km<sup>2</sup> and a total population of less than one million inhabitants, mainly located in several medium size cities arranged along the main rivers continuum. The annual average water flow entering the estuary is close to 300 m<sup>3</sup>.s<sup>-1</sup> (mean observed during the last 20 years) and can vary from up to 2800 m<sup>3</sup>.s<sup>-1</sup> during brief flood events to 50 m<sup>3</sup>.s<sup>-1</sup> during summer months (from June to September).

Four representative stations are presented in Figure 1. Station A is located in the ocean at around 10 km from the estuary mouth close to the shelf break, station B is located on the continental shelf, station C is located at the estuary outflow and station D is located inside the lower part of the estuary close to the harbor and industrial zones.

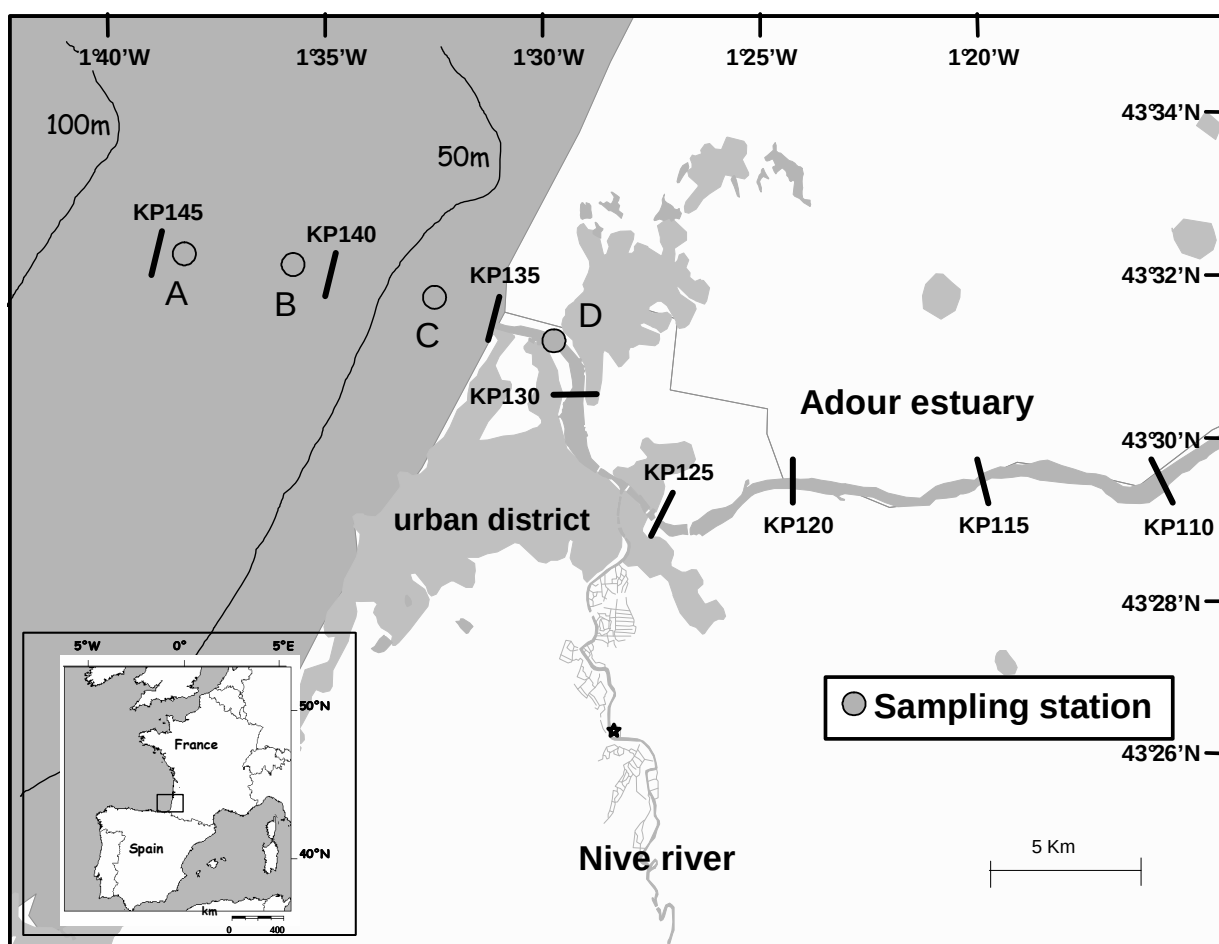
#### 2.1.2- Sampling procedure

Samples were taken using a 0.1m<sup>2</sup> Smith-McIntyre grab, five replicates being sampled at each station. Samples were immediately sieved on a 1mm sieve mesh size and residue was stored in a cool place, without fixing. Samples were sorted for macro fauna within 24h. For every replicate, all invertebrate specimens were

identified according to the species, numbered and then stored in a pre-clean pillbox (i.e. one pillbox for one species per station).

Species were classified into feeding groups following Gaston et al.<sup>21</sup> and Bonsdorff et al.<sup>22</sup>. All macro fauna species collected in the study were included in the trophic structure analysis. Measured parameters for macrobenthic communities include mean abundance (total number of individuals per sampling station), total and mean number of sampled species at every station, Shannon diversity index and Pielou regularity index.

In order to relate general quality or “health” of the communities with metal concentration, a biotic index for soft bottom benthos was applied. It is derived from the relative proportions of ecological groups, which are related to the degree of sensitivity or tolerance to environmental stress gradients<sup>23,24</sup>. This index ranges from 0 to 6, lower values under 1.2 reflecting unpolluted communities while values between 1.2 and 6.0 reflect moderately to highly polluted communities.



**Figure 1:** Sampling sites in the Adour Estuary (south-west. France) – A) ocean. B) continental shelf. C) estuary outflow. D) estuary.

## 2.2- Analytical procedure

### 2.2.1- Instrumentation and reagents

For total metal concentrations, an Elan 6000 ICPMS (Perkin Elmer) was used with a Scott spray chamber. For speciation analysis of mercury and tin, a gas chromatograph (HP 6850) equipped with a capillary column was connected to the ICPMS *via* a Silcosteel (Restek) transfer line described previously in detail <sup>18</sup>. GC ICPMS conditions for the simultaneous speciation of mercury and tin compounds were described also previously <sup>15</sup>. An open focused vessel microwave oven Prolabo A301 (Fontenay-sous-Bois, France) was used for the extractions of solid samples.

All reagents used were analytical grade and ultrapure water was obtained from a MilliQ system (Millipore). Multielemental solution (accutrace II) was used. Inorganic antimony, tin and thallium were obtained from Spex Certiprep (Metuchen, NJ, USA). Natural tributyltin chloride (TBTCl) and enriched <sup>117</sup>TBTCl (<sup>117</sup>TBTCl, 90.5 µg.mL<sup>-1</sup>) were obtained from LGC Limited (Teddington, UK). Stock solutions of MBT and DBT (1000 mg L<sup>-1</sup>) of natural isotopic composition were prepared by dissolving BuSnCl<sub>3</sub> and Bu<sub>2</sub>SnCl<sub>2</sub> (Aldrich) in methanol. Stock solutions of inorganic mercury (IHg) and MMHg (1000 mg L<sup>-1</sup>) of natural isotopic composition were prepared by dissolving mercury (II) chloride (Strem Chemicals) in 1% HCl and methylmercury chloride (Strem Chemicals) in methanol, respectively. Working standard solutions were prepared daily by appropriate dilution of the stock standard solutions in 1% HCl and stored in the fridge. <sup>201</sup>HgO was obtained from Oak Ridge National Laboratory (USA). The protocol of MMHg synthesis is described in previous work <sup>25</sup>. The concentrations of the enriched TBT and MMHg solutions were calculated by reverse isotope dilution mass spectrometry (RIDMS) and the isotopic compositions were also determined.

Ultrapur Tetramethyl ammonium hydroxide (TMAH) 25% w/w was obtained from Fluka (Buchs, Switzerland) and methanol from MERCK (Darmstadt, Germany). Sodium tetraethylborate (NaBEt<sub>4</sub>), purity higher than 99%, was obtained from Strem Chemicals (Newburyport, USA). 1% (w/w) NaBEt<sub>4</sub> solutions were daily prepared and kept in the dark.

Reference materials BCR 710 (oyster tissue) and BCR 278R were obtained from the Institute for Reference Materials and Measurements (IRMM) (Geel, Belgium) and were used for the validation of the method.

### 2.2.2- Sample analysis

*Sample preparation* - Samples were frozen till the sample preparation. Then they were placed in a freeze dried system during 48 h to ensure the complete removal of moisture. Each sample was then crushed using an agate mortar and stored in a clean tube at -4 °C until the spiking and extraction procedure.

Extraction procedure for simultaneous analysis of mercury and butyltin compounds was previously described <sup>16</sup>. Briefly, 5 mL of TMAH was added to the sample and submitted to microwave irradiation (3 min., 40W). The extract was centrifuged for 5 min at 2500 rpm and the supernatant was transferred to a clean tube with a Teflon cap and stored at 4°C. 0.4 mL was diluted in 10 mL for the total metals analysis and 2 mL was submitted to ethylation for speciation analysis. Previously optimized ethylation procedure was used <sup>16</sup>. Concentrated ammonium hydroxide and 5 mL of acetic acid / sodium acetate buffer (0.1 M) are added to 2 mL of the extract to set the pH at 5. 1 mL of iso octane and 1 mL of 1% (w/w) sodium tetraethylborate are added. The tube is immediately capped and hand shaken for 5 min. The organic phase is finally transferred to an injection vial and stored at - 18°C until measurement.

Species specific isotope dilution (SSIDMS) is based on the addition of a precise amount of an isotopically labeled form of the analyte to the sample. In previous work <sup>16</sup>, the spiking procedure has been described. Briefly, sample is accurately weighed in a microwave extraction vessel, spiked with known amounts of enriched <sup>117</sup>TBTCl and <sup>201</sup>MMHg solutions and directly submitted to extraction. Error on the isotope ratio in the final determination was minimized by adding a particular amount of enriched standard to give a final isotope ratio close to unity.

For very low mass samples, a preconcentration is necessary to detect organometallic species. After ethylation, the vial containing the organic phase is placed on an evaporation ramp. The iso-octane was evaporated under a gentle stream of argon until approximately 50 µL of solution, necessary for the injection in the GC ICPMS, remained. By weighing organic phase before and after the preconcentration step the preconcentration factor for each sample was calculated.

*Metal analysis* - The multi element determinations were performed using an ICPMS with typical operating conditions. The instrument tuning was performed daily using a 10 µg.L<sup>-1</sup> of Mg, Ce, Ba, Pb, Rh solution in order to optimize the instrument in terms of sensitivity, resolution, oxides and doubly charged ions levels. Detection at masses 51, 53, 59, 60, 65, 66, 75, 107, 114, 208 was chosen on the basis of their abundances and the absence of interferences. Each isotope was followed with an integration of 100 ms. The analysis of the TMAH extracts was performed by standard additions after a 25 dilution or a 250 dilution for the most concentrated elements. Blanks of the whole sample preparation procedure were also determined to check any contaminations and to calculate detection limits.

*Speciation analysis* -Optimized operating conditions used for the GC separation and the ICPMS detection for the simultaneous speciation of MMHg and TBT by species specific isotope dilution have been described elsewhere <sup>16</sup>. Briefly the GC temperature program used is a linear gradient between 60 °C and 250 °C with a rate of 60 °C.min<sup>-1</sup>. Injection was performed with splitless mode in the injector heated at 250 °C. The carrier gas flow



(Helium) was 25 mL.min<sup>-1</sup> and the make up gas flow (Argon) for the transfer line was 300 mL.min<sup>-1</sup>. The transfer line was connected to the ICPMS by a cyclonic chamber allowing the simultaneous nebulisation of standard solutions (thallium and antimony) for the mass bias correction. Detection was performed at masses 117, 120, 201, 202 with 30 ms integration time for each isotope which allows an adequate definition of the chromatographic peaks; and 121, 123, 203, 205 with 5 ms integration time for the mass bias correction.

MMHg and TBT were analyzed by SSIDMS whereas IHg, MBT and DBT were analyzed by standard additions. Each sample was injected three times and the chromatographic peak integration was performed using the Origin software. Isotope ratios were always measured as peak area ratios and corrected by mass bias factor. Blanks were also determined to check for any contamination.

### 3- RESULTS AND DISCUSSION

#### 3.1- Analytical performances and validation of the method

##### 3.1.1- Analytical performances

*Metal analysis* - The detection limits (LODs) for the analyte after a TMAH dissolution are shown in Table 1. The LOD are calculated from three times the standard deviation of five determinations of TMAH blanks. The LODs fall in the range of a few  $\text{ng.g}^{-1}$  except for Zn where the LOD is close to  $2 \mu\text{g.g}^{-1}$  (certainly due to the poor quality of MilliQ water). The reproducibility is also achieved by the relative standard deviation calculated on five determinations of a  $10 \mu\text{g.L}^{-1}$  standard in 1% TMAH which, is the concentration of the TMAH sample extracts. The precision on the measurements are presented in Table 1. RSD% range between 1.2 % for Cd and 10.1 % for Ag. Low LODs and good precision values permits to achieve the analysis of trace metals after a TMAH extraction with excellent efficiency.

| Element                                    | V    | Cr   | Co  | Ni  | Cu   | Zn   | As  | Ag   | Cd   | Pb  |
|--------------------------------------------|------|------|-----|-----|------|------|-----|------|------|-----|
| Detection limits<br>( $\text{ng.g}^{-1}$ ) | 20.8 | 50.5 | 1.5 | 2.8 | 10.1 | 1950 | 6.9 | 0.4  | 19.6 | 9.0 |
| RSD (%) 10<br>$\mu\text{g.L}^{-1}$ n=5     | 3.3  | 3.4  | 3.1 | 2.8 | 7.1  | 2.6  | 2.8 | 10.1 | 1.2  | 4.7 |

**Table 1:** Analytical performances for total metal determination by ICPMS

*Speciation analysis* - Performances of the analytical method for mercury and butyltin compounds are presented in Table 2. Absolute detection limits are calculated by three times the standard deviation of 5 injections of TMAH blanks. Very low values were obtained ranging between 22 and 60 fg. Analytical detection limits were also evaluated considering a sample mass of 0.5 g; values are very low (below  $50 \text{ pg.g}^{-1}$ ). In addition, LODs of the whole procedure were calculated for very low sample mass (0.02 g) which is the typical sample mass expected for the major part of real macrobenthic samples. Preconcentration factors were generally between 3 and 5 times and allowed detection limits of less than  $250 \text{ pg.g}^{-1}$ . The reproducibility of the method was also evaluated by injecting 5 times a 10 pg standard containing the different species. RSD of between 1.3 and 1.9 % were obtained. The very low LODs and the excellent precision on the measurements allow us to obtain a very accurate and precise method for the speciation of mercury and butyltin compounds in biological tissues, even for very low sample mass.

| Species                                                                                              | MMHg | IHg | MBT | DBT | TBT |
|------------------------------------------------------------------------------------------------------|------|-----|-----|-----|-----|
| Absolute detection limits (fg)                                                                       | 15   | 40  | 60  | 35  | 30  |
| Analytical detection limits (pg.g <sup>-1</sup> )<br>(sample mass 0.5 g, preconcentration factor 0)  | 12   | 33  | 50  | 29  | 25  |
| Analytical detection limits (pg.g <sup>-1</sup> )<br>(sample mass 0.02 g, preconcentration factor 5) | 63   | 167 | 250 | 146 | 125 |
| RSD (%) (10 pg n=5)                                                                                  | 1.6  | 1.3 | 1.6 | 1.8 | 1.9 |

**Table 2 :** Analytical performances for simultaneous speciation of tin and mercury by CGC-ICPMS

### 3.1.2- Validation of the TMAH microwave extraction

*Metal analysis* - TMAH is an efficient reagent for hydrolyzing biological tissue samples, degrading proteins, lipids and sugars. The potential problem with this procedure is interference from matrix components, so calibration using standard additions is necessary. Previous work has shown that solubilization efficiency was improved by using an ultrasonic bath <sup>14</sup>, microwave digestion <sup>15</sup> or concentrated TMAH <sup>15</sup>. In our case, we have used concentrated TMAH (25%) coupled with microwave irradiation. Table 3 presents results for a certified reference material digestion by two different methods. Results for the extraction using TMAH with open microwave digestion are compared to values found using a classic acid extraction in a closed microwave system which is considered as a reference method (method EPA 3052 <sup>26</sup>). Good agreement with certified values is found for Cu, Zn, As, Cd and Pb for both extraction techniques. For non certified values (ie. V, Ni, Ag), concentrations found with a TMAH extraction are in agreement with values found with an acid extraction except for Ni. Only 64% of Ni is recovered compared to the quantity found with an acid extraction.

*Speciation analysis* - Validation of the method was performed using the CRM 710, an oyster tissue. MMHg and TBT were determined using species specific isotope dilution. IHg, MBT and DBT were determined by external calibration. The results are given in Table 4. Results for MMHg and TBT agree with the certified values with an excellent precision of less than 3%. For MBT and DBT, the results are also in agreement with the reference values. For IHg, the value is lower than the indicative value. The same result was observed for MMHg determined by external calibration (53.3 ng.g<sup>-1</sup>). Lower recoveries for mercury species could be explained by a non optimal ethylation at pH 5 and matrix interferences due to organic matter. These low recoveries can be easily corrected by isotope dilution for MMHg or by standard addition for IHg.

| Element | Certified value<br>mean±SD (µg.kg <sup>-1</sup> ) | Extraction HNO <sub>3</sub> /H <sub>2</sub> O <sub>2</sub><br>mean±SD (µg.kg <sup>-1</sup> ) | Extraction TMAH<br>mean±SD (µg.kg <sup>-1</sup> ) |
|---------|---------------------------------------------------|----------------------------------------------------------------------------------------------|---------------------------------------------------|
| V       |                                                   | 0.70±0.07                                                                                    | 0.81±0.13                                         |
| Co      |                                                   | ND                                                                                           | 0.34±0.04                                         |
| Ni      |                                                   | 1.11±0.12                                                                                    | 0.78±0.09                                         |
| Cu      | 9.45±0.13                                         | 9.35±0.87                                                                                    | 8.62±0.75                                         |
| Zn      | 83.1±1.7                                          | 76.2±6.9                                                                                     | 90.3±7.2                                          |
| As      | 6.07±0.13                                         | 5.74±0.49                                                                                    | 5.39±0.24                                         |
| Ag      |                                                   | 0.27±0.02                                                                                    | 0.28±0.07                                         |
| Cd      | 0.348±0.007                                       | 0.32±0.02                                                                                    | 0.31±0.04                                         |
| Pb      | 2.00±0.04                                         | 2.01±0.09                                                                                    | 1.83±0.20                                         |

**Table 3** : Validation of the method with the CRM 278R for trace metal determination.

| Compound                                             | MMHg                             | Hg total                       | MBT                             | DBT                             | TBT                              |
|------------------------------------------------------|----------------------------------|--------------------------------|---------------------------------|---------------------------------|----------------------------------|
| Certified value<br>ng.g <sup>-1</sup> ± SD<br>(RSD%) | 115.0±9.0<br>(7.8%)              | 260±50*<br>(19,2%)             | 50.0±14.0*<br>(28.0%)           | 82.0±15.0<br>(18.3%)            | 133.0±19.0<br>(14.3%)            |
| Found value ng.g <sup>-1</sup><br>± SD<br>(RSD%)     | 114.0±3.2 <sup>a</sup><br>(2.8%) | 197,8±8,3 <sup>b</sup><br>(5%) | 50.3±2.6 <sup>b</sup><br>(5.2%) | 93.8±2.3 <sup>b</sup><br>(2.4%) | 148.4±3.7 <sup>a</sup><br>(2.5%) |

(\*) indicative values (a) Determination by SSIDMS (b) Determination by standard addition

**Table 4** : Validation of the method with the CRM 710 for the simultaneous speciation of mercury and butyltin compounds.

### 3.3- Benthic macrofauna diversity and metals and organometals bioaccumulation

#### 3.3.1- Biodiversity

A total of 209 individuals belonging to 79 different species were sampled during the study. Most of the species belonged to polychaetes (37%), crustaceans (29%) and molluscs (23%), while in terms of abundance, crustaceans, molluscs and polychaetes were dominating with respectively 37, 34 and 26 % of the abundance. Most species represented were the mollusc bivalves *Mysella bidentata* and *Macrura sultorum*, the crustaceans cumacea *Diastylis rugosa* and *Dyastiloides biplicata* and the polychaetes *Nephtys caeca* and *Nereis rava*.

Benthic communities showed clear segregation between open ocean stations A and B and more estuarine stations C and D. Stations A and B showed higher species richness with means of 12 and 17.4 species per sample while diversity of station C and D was much lower (see Table 5). Conversely, abundance was significantly higher at station C (83 ind. per 0.1 m<sup>2</sup> samples) than on the other sampling stations (22 to 32 ind. per 0.1 m<sup>2</sup> sample). Shannon diversity index was high in the outer part of the estuary, with values above 3.2 on stations A and B and was much lower in the inner part, with values below 1.8. The same pattern was observed in term of evenness (Pielou index, Table 5). The benthic index also showed the clear difference between outer stations and inner estuarine stations, the former being in normal conditions and the latter being moderately polluted, according to the calculated indices.

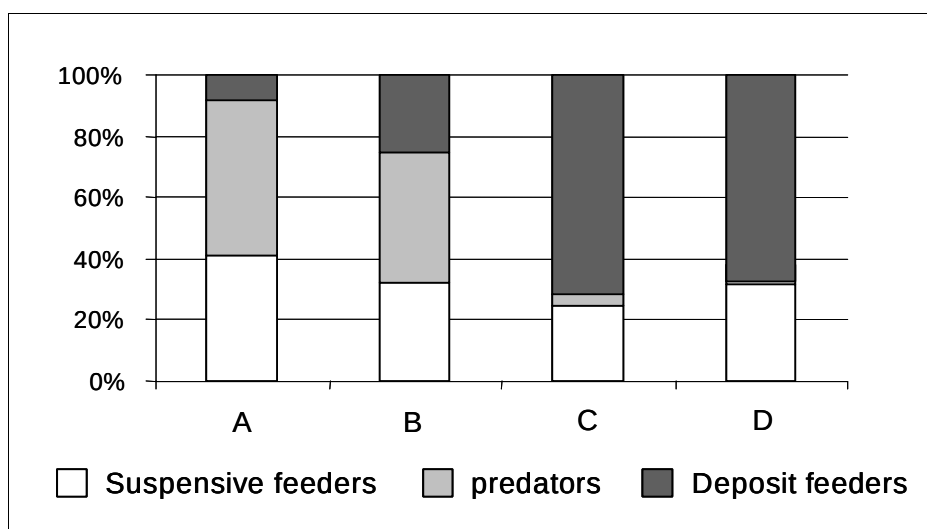
| Parameter                       | STATION A       | STATION B       | STATION C       | STATION D       |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Total number of species         | 36              | 45              | 16              | 11              |
| Mean Number Species ( $\pm$ SD) | 12.0 $\pm$ 2.9  | 17.4 $\pm$ 2.5  | 7.4 $\pm$ 2.7   | 4.2 $\pm$ 1.1   |
| Mean Abundance ( $\pm$ SD)      | 22.2 $\pm$ 5.1  | 32.6 $\pm$ 7.7  | 83.2 $\pm$ 37.1 | 31.0 $\pm$ 24.8 |
| H'                              | 3,2116          | 3,7694          | 1,7824          | 1,6122          |
| J                               | 0,9034          | 0,9184          | 0,6076          | 0,7926          |
| Benthic Index                   | 0.84 $\pm$ 0.21 | 0.91 $\pm$ 0.14 | 2.68 $\pm$ 0.20 | 3.67 $\pm$ 0.58 |

**Table 5:** Biological parameters for 4 stations of the Adour Estuary

Another way of investigating benthic community structure is using feeding types (guilds) by comparison of trophic dominance and structure within the community. Data were split into three categories based on feeding guild:

- suspensive feeders, animals feeding on suspended organic particles
- predator, animal which consume living animals
- deposit feeders, animal gathering organic particles from the sediment

Figure 2 presents the repartition of these different feeding groups of the communities within the sampling stations. Macrobenthos data show that community composition differs for the sampling stations in the Adour Estuary with an important presence of predator of open sea station whereas in the estuarine part, predators represent a minor group.

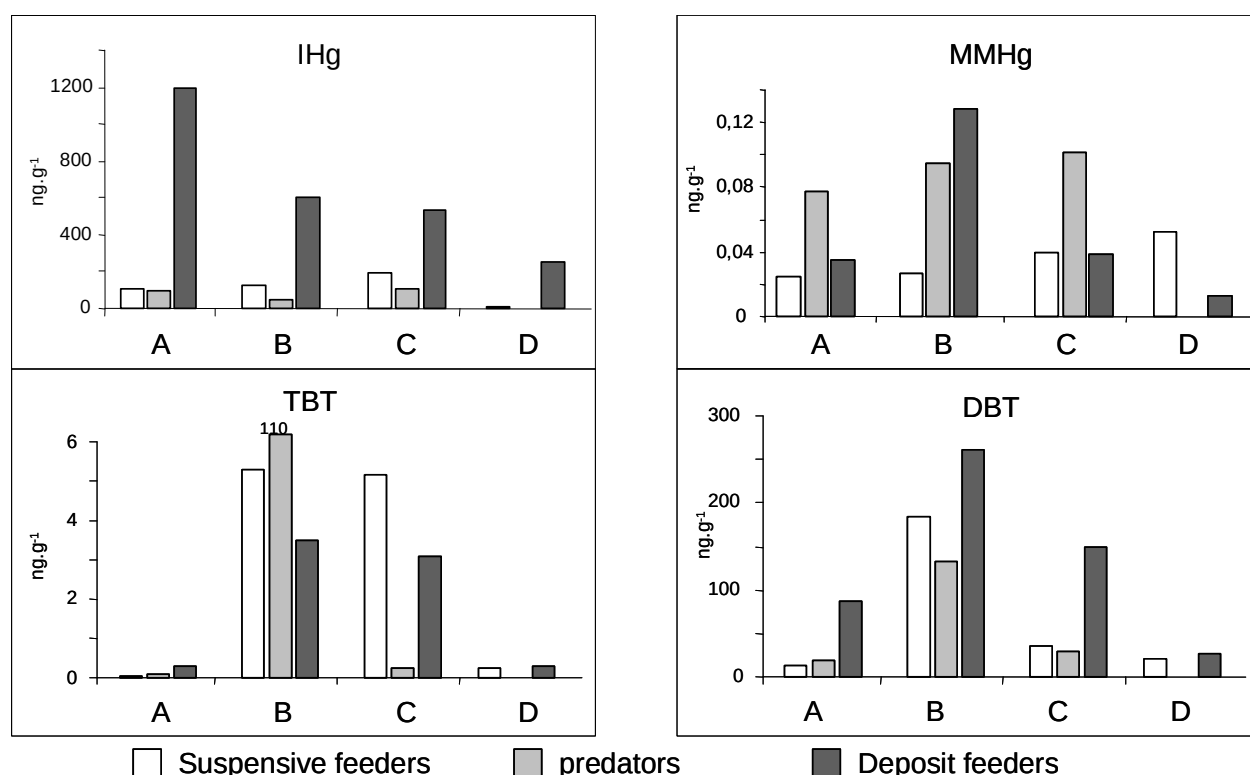


**Figure 2 :** Biodiversity in the Adour Estuary.

Each sample was submitted to analysis of metal and organometal and the mean concentrations were calculated for each of the three trophic groups in order to assess possible bioaccumulation and biomagnification along the food chain. Some organisms have been found in very few numbers. When the dried biomass didn't exceed 10 mg, the analysis couldn't be performed. Only species presented in larger biomass (more than 10 mg) were taken into account in this study.

### 3.3.2- Trace metal and organometal content

Figure 3 presents the concentrations of MMHg, IHg, DBT and TBT in the three trophic groups according the sampling stations. The concentrations of MMHg in these tissues are relatively low (less than  $0.12 \text{ ng.g}^{-1}$ , with a mean of  $0.06 \text{ ng.g}^{-1}$ ) compared to the value set by EPA guidelines <sup>8</sup> ( $1 \text{ ng.g}^{-1}$ ). Inorganic mercury concentrations are also low with a maximum of  $1200 \text{ ng.g}^{-1}$  for deposit feeders of the station A and a mean value of  $297 \text{ ng.g}^{-1}$ . In addition, an increase of IHg concentrations can be observed from the estuary to the ocean. Benthic organisms living in the ocean are much more contaminated than those living in the estuary. This result should be compared with mercury concentrations in water, sediment and suspended matter for the different stations to investigate a possible correlation between concentrations in the different environmental compartments. Table 6 presents metal and organometal concentrations in sediments as well as organic carbon contents. As it has been demonstrated previously <sup>27</sup>, inorganic mercury concentration in sediments is correlated with organic matter content. In the case of the Adour Estuary, Stoichev et al. <sup>27</sup> have shown that higher mercury concentrations were found for coastal sediments due to the transportation of fine organic particles from the inner estuary to the coastal zone or due to contamination from polluted particles from the Spanish coast.

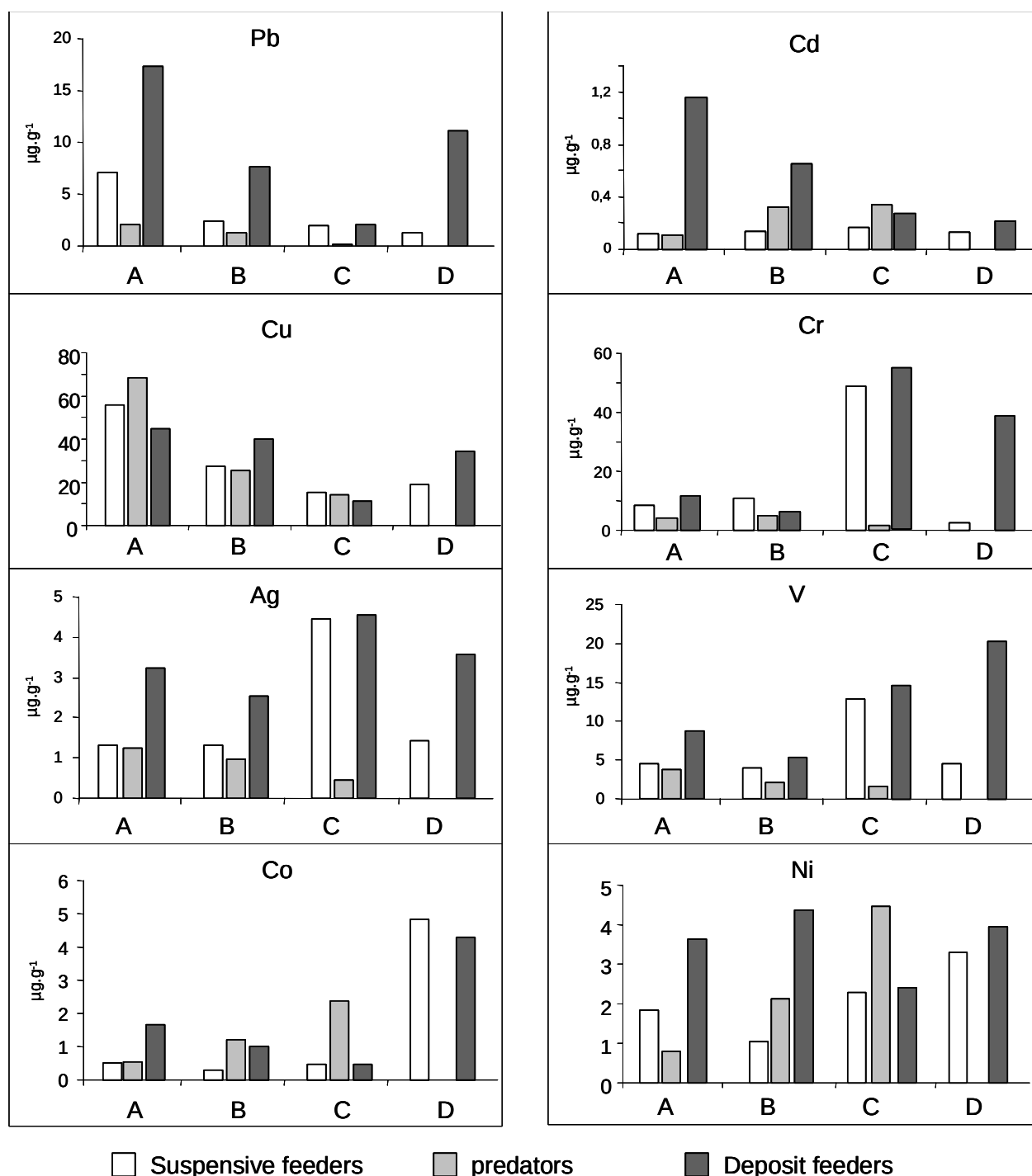


**Figure 3 :** Mercury and tin species concentrations in the four sampling sites for three trophic groups.

For butyltin compounds, important concentration variations can be observed between the different stations. Station B seems to be highly contaminated by butyltin compounds with a TBT concentration of 110  $\text{ng.g}^{-1}$  for predators whereas for the other stations TBT concentrations are comprised between 0.03 and 5.3  $\text{ng.g}^{-1}$ . This result is associated with a high concentration of TBT in sediments for the station B (8.95  $\mu\text{g.g}^{-1}$ ) (Table 6). Regarding DBT concentrations, station B again seems contaminated with higher concentrations, both for macrobenthic population and for sediment relative to the other stations. High butyltin compounds concentrations could be due to a rejection of sediments dredged from the harbor containing these compounds due to their use in antifouling paints.

Trace metal concentrations in macrobenthic organisms obtained in this study are compiled in Figure 4. As trace metals body concentrations of aquatic invertebrates living in the same habitat may well vary greatly from one taxa to another, and even within closely related species in the same genus<sup>28-31</sup>, limited interest has been made on trophic guilds macro-invertebrates and their metals in their environment. Our data show that there is no obvious general trend, both for the different metals studied and between the various feeding guilds. Highest Pb and Cd metal concentrations were generally observed in tissues of deposit feeders and for marine station A, whereas highest Cr, V and Ag metal concentrations were found for estuarine stations C and D. Moreover, as found for inorganic mercury, both Cd in deposit feeders, Cu and Pb in suspensive feeders and Ag, Cu, Pb and V in predators experience an increase in concentrations as the stations move further from the estuary and into the

ocean. The reverse pattern (ie. decreasing metal concentrations) is observed for Co, Cd and Ni in predators from estuarine station C (no predators were found at station D inside the estuary) to marine station A.



**Figure 4 :** Trace metals concentrations in the four sampling sites for three trophic groups.

Furthermore, there is no clear relationship between metal concentrations in deposit feeders and levels in sediments, except for Pb and Co. Nevertheless, comparing our sediments and body data to freshwater sediments and macro-invertebrates feeding guilds, there are indications that they fall into the lower part of the relationship given by Goodyear and McNeill <sup>6</sup> for deposit feeders and predators. Considering suspensive feeder tissues, high



Cr, V and Ag concentrations were observed at station C, which is characterized by the lowest organic carbon, fine grain size and low metal content. This could suggest that at this site, suspended particulate matter with a high metal load are transiting but are not ending in the sedimentary pool.

| Compound                   | STATION A | STATION B | STATION C | STATION D |
|----------------------------|-----------|-----------|-----------|-----------|
| Org C (%)                  | 0.5       | 0.3       | 0.1       | 1.8       |
| MMHg (ng.g <sup>-1</sup> ) | 0.01      | 0.23      | 0.03      | 0.01      |
| IHg (ng.g <sup>-1</sup> )  | 464       | 102       | 25        | 477       |
| DBT (µg.g <sup>-1</sup> )  | 0.76      | 2.23      | 0.43      | 1.07      |
| TBT (µg.g <sup>-1</sup> )  | 0.72      | 8.95      | 0.09      | 1.91      |
| Pb (µg.g <sup>-1</sup> )   | 35.3      | 23.2      | 11.3      | 30.3      |
| Cd (µg.g <sup>-1</sup> )   | 0.11      | 0.14      | 0.11      | 0.39      |
| Cu (µg.g <sup>-1</sup> )   | 13.3      | 10.1      | 4.7       | 26.3      |
| Cr (µg.g <sup>-1</sup> )   | 32.0      | 40.2      | 20.2      | 48.9      |
| Co (µg.g <sup>-1</sup> )   | 7.5       | 6.8       | 4.1       | 10.4      |
| Ni (µg.g <sup>-1</sup> )   | 82.5      | 27.8      | 31.2      | 53.6      |

**Table 6** : Concentrations of metals and organometals in sediments for 4 stations of the Adour Estuary.

Previous work in the Bidasoa estuary (Bay of Biscay), on metal bioaccumulation by the bivalve *Scrobicularia plana* have demonstrated this species as efficient in reflecting the contamination of surficial sediment with metals<sup>32</sup>. Comparing metal concentrations in benthic organisms of the Adour estuary, especially the deposit feeders, values found in clams (*Scrobicularia plana*) of the Bidasoa estuary are in the similar concentrations ranges.

### 3.3.3- Bioaccumulation factors

As the major part of pollutants can be accumulated into the sediment, they can be bioaccumulated by benthic organisms. One method to measure and to predict the bioavailability of sediment-associated contaminants to estuarine benthos is the use of bioaccumulation factors. Several methods are available to assess bioaccumulation factor but bioavailability is affected by sediment characteristics such as levels of organic carbon, acid volatile sulphide, iron, and manganese and aluminium oxides. For mercury compounds, Lawrence and Mason<sup>33</sup> have shown the importance of organic matter in controlling mercury species solubility and hence bioavailability from sediments. Moreover organic matter is the dominant fraction containing mercury. The biota-sediment accumulation factor (BSAF) can be thus determined by this formula:

$$BSAF = \frac{C_{biota}}{C_{sed} \div C_{OM}}$$

where  $C_{biota}$  is the metal concentration in biota ( $\text{ng.g}^{-1}$ ),  $C_{sed}$  is the metal concentration in sediment ( $\text{ng.g}^{-1}$ ) and  $C_{OM}$  is the organic matter content in sediment (%). If BSAF is higher than 1, bioaccumulation of the compound can be considered.

Table 7 presents bioaccumulation factors for inorganic mercury for the different feeding groups and for each station. Factors are within the same range (between 0.03 to 2.14) but are systematically higher for deposit feeders. Calculated BSAF for deposit feeders are found equal or higher to 1 showing a notable bioaccumulation process of IHg for deposit feeders for all the investigated stations.

|                   | STATION A | STATION B | STATION C | STATION D |
|-------------------|-----------|-----------|-----------|-----------|
| Suspensive feeder | 0.11      | 0.38      | 0.76      | 0.03      |
| Predator          | 0.11      | 0.14      | 0.42      | -         |
| Deposit feeder    | 1.29      | 1.76      | 2.14      | 1.01      |

**Table 7 :** Biota-sediment accumulation factor (BSAF) for inorganic mercury.

Among the different trace metals investigated in the biota and in the sediments, there is evidence of bioaccumulation processes only for few metals (Cu, Cd and Cr). For Cu and Cd, bioaccumulation occurs for stations A, B and C but not for the inner estuary site, in almost all feeding guilds and in deposit feeders. In addition, Cu and Cd bioaccumulation factor are increasing seaward, BSAF varying from 0.2 to 2.1 for Cu and from 0.2 to 5 for Cd. In the case of Cr, bioaccumulation is only observed in deposit and suspensive feeders and at station C exclusively.

#### 3.3.4- Relationship between metal concentrations and feeding guilds

*Mercury species* - The highest MMHg concentrations are found for predators, except for station D, where no predators were found. This result suggests and confirms that MMHg is subject to biomagnification in the trophic food chain. MMHg bioaccumulates through the levels of the aquatic food chain and bioaccumulation in benthic food chain is highlighted. Regarding the concentrations of inorganic mercury, the highest concentrations are found for the deposit feeders. Concentrations are 4 to 10 times higher than those found for suspensivores and predators. Lawrence and Mason <sup>33</sup> have described a higher bioaccumulation for MMHg than for IHg for amphipods in Lacava Bay. This is correlated with organic matter contents in sediments which control IHg and

MMHg concentrations. IHg is more strongly bound to particulate organic matter than MMHg, so IHg is more available for species where the principal food source is sediment.

*Butyltin compounds* – Concerning TBT accumulation, no general trend can be given for the benthic organisms. The major finding reside in the fact that for a contaminated station like station B, all the three groups seems to be impacted by the high concentration of TBT in sediment. For DBT, deposit feeders exhibit the higher concentration explaining a direct bioaccumulation by particles assimilation but no biomagnification can be highlighted. Fent <sup>34</sup> has reviewed the bioaccumulation and bioamplification studies on organotin compounds in marine invertebrates. The conclusion was that TBT is accumulated to significant levels with only minor elimination but biomagnification in aquatic systems does not seem to occur, or only to a minor extent.

*Metals* – Pb concentrations gradient can be established between the feeding guilds. Concentrations decrease by the order deposit feeders>suspensive feeders>predators suggesting no biomagnification for this metal in the studied area. The same result was found by Timmerman et al. <sup>34</sup> who found that carnivore species contain lower Pb concentrations than other guilds. However, Goodyear and McNeill <sup>6</sup> have shown that Pb biomagnification did occur in some circumstances in freshwater invertebrates. Regarding the set of data for copper, it is not possible to determine a Cu concentration gradient between the feeding guilds as the mean values for the three groups are too similar. Previously, Goodyear and McNeill <sup>6</sup> illustrated that macro-invertebrates bioaccumulate Cu without a significant difference between guilds. Consequently, Cu also appears not to be subjected to biomagnification. This can be explained by the fact that many invertebrates are able to regulate at least partially the bioaccumulation of Cu <sup>31</sup>. Concerning Cd, concentrations in the different feeding guilds follow the gradient deposit feeders>predators > or = filterers for the marine stations, whereas no significant differences are found for the two estuarine stations. There is also no biomagnification along the food chain for Cd, although some invertebrate species are able to bioaccumulate Cd to a large degree, as ingested Cd is stored in the body without excretion <sup>36-38</sup>.

### 3.3.5- Metal and organometal contents and benthic community health

Bioaccumulation of heavy metals by aquatic organisms is a reasonably well documented field of research (see Goodyear and McNeill review <sup>6</sup>). Compiling the available literature on heavy metal bioaccumulation by freshwater macro-invertebrates, Goodyear and McNeill <sup>6</sup> found a unique relationship between metal concentrations in the environment (sediments and water) and in individual feeding guilds, indicating the relative importance of different sources of metals to different feeding types. However, as different organisms accumulate trace metals to different concentrations in their tissues, even those living in the same habitat <sup>27,28</sup>, large differences can be recorded from

one environment to another as well as in similar environmental condition (with respect to metal concentration) depending on the specific organism involved <sup>39,40</sup>. This large variability is clearly documented on the log/log plots from Goodyear and McNeill <sup>6</sup> as accumulated trace metal body concentration of feeding guilds can be both high and low for one defined environmental condition, varying about one order of magnitude compared to a two to three order of magnitude change between weakly or largely contaminated environmental conditions. This is not surprising as for similar environmental metal levels; feeding guilds can be composed of different invertebrate types, each of them having their own accumulation pattern (the metal is used for an essential metabolic purpose, excreted, or stored in the body) depending on the physiology of the invertebrate <sup>37</sup>. Results obtained for the Adour estuary illustrated these observations because as tissue metal levels of the different feeding guilds show significant variability, metal concentrations of sediments do not change drastically. It can be noted that relative comparison of feeding guilds between stations should take into account the differences in species composition and abundance, as benthic communities showed clear segregation between near shelf stations A and B and more estuarine stations C and D. In addition, because our data fall into the lower end of the Goodyear and McNeill relationship's, it can be assessed that the studied estuary is not really contaminated by trace metals, mercury and tin compounds.

A final point to be discussed concerns the potential for metals to be transferred along the food chain. Although most invertebrates accumulate most metals in proportion to ambient availabilities, both in the medium and in food, there are several factors that affect metal accumulated in a prey species to have the potential to be taken up and accumulated or not by a predator. These factors are in particular, the trace accumulation pattern of the predator himself <sup>38,41</sup> and the availabilities of metals present in their diet <sup>42</sup>. In the case of the Adour estuary, we found no strong evidence of biomagnification along the food chain suggesting that the transfer of trace metals is controlled by both the quantity of metal accumulated in the prey and the physiological detoxification processes at each trophic level.

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## **Chapitre C – II**

**Biogéochimie du mercure à l'interface eau-sédiment de l'étang de Thau – Mesures des potentiels de méthylation du mercure dans les sédiments et la colonne d'eau et devenir du méthylmercure**



## **Biogéochimie du mercure à l'interface eau-sédiment de l'étang de Thau – Mesures des potentiels de méthylation du mercure dans les sédiments et la colonne d'eau et devenir du méthylmercure**

La toxicité et l'impact écologique du MMHg sont manifestes et sa concentration dans les systèmes aquatiques est contrôlée par des mécanismes de formation, de dégradation et d'échanges entre les différents compartiments. La méthylation du mercure est notamment un processus clé dans le cycle biogéochimique du mercure et peut avoir lieu à la fois dans les sédiments et dans la colonne d'eau. La dégradation du MMHg par déméthylation va alors permettre la limitation des concentrations en MMHg.

A l'heure actuelle, ces réactions réversibles, que sont la méthylation et la déméthylation du mercure, et donc la formation nette de MMHg sont relativement peu connus. Plusieurs facteurs biotiques ou abiotiques peuvent contrôler ces réactions mais l'identification et l'importance de chacun ne sont pas clairement décrites. Pour des environnements côtiers, le devenir du MMHg représente un réel intérêt afin de déterminer son impact sur les organismes aquatiques susceptibles de bioaccumuler le MMHg et ainsi que sur la santé lors de la consommation de ces derniers.

L'objectif de ce travail est de déterminer les taux de méthylation et de déméthylation régulant la concentration en MMHg dans les sédiments et la colonne d'eau de l'étang de Thau où l'activité ostréicole représente un intérêt économique important. Des expérimentations in situ sont développées grâce à l'utilisation de traceurs isotopiques stables pour les différents compartiments. Les méthodes d'incubation sont évaluées en terme de sensibilité et de justesse pour tracer les processus environnementaux. Les paramètres biogéochimiques et biologiques à l'interface eau-sédiment sont caractérisés afin d'étudier les facteurs contrôlant les processus de transformations du mercure.

Les résultats montrent que l'outil isotopique permet de suivre avec justesse et précision les différentes transformations du mercure dans les sédiments et la colonne d'eau. Les activités biologiques (microbiologique, phytoplanctonique), qui jouent un rôle important dans ce type d'écosystème côtier, semblent être liées à la méthylation du mercure dans la colonne d'eau. Par comparaison des différents flux de transformations et de transferts à l'interface eau-sédiment, il semble que les processus de transformations sont plus importants que les échanges entre l'eau et les sédiments. Enfin, la déméthylation dans les sédiments apparaît comme un processus important régulant les concentrations en MMHg dans l'étang de Thau.

## Biogeochemistry of mercury at the sediment water interface in the Thau lagoon. 2. Measurements of mercury methylation potential in sediment and water and fate of methylmercury

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### ABSTRACT

Methylation rates of mercury in surface sediments and water column of the Thau Lagoon (France) have been investigated by using *in situ* incubation experiments in order to determine the fate of methylmercury (MeHg) in such coastal environment under spring conditions. Isotopically enriched species have been used as chemical tracers and allowed the direct determination of specific methylation and demethylation yields. Each experimental method (cores experiments and water experiments) has been carefully evaluated in terms of sensitivity and reproducibility of the transformations rates and has been demonstrated as a powerful method to investigate transformation processes.

Although mercury methylation in surface sediment is a major process, significant MeHg formation in the water column has been measured for the first time in a coastal environment. In spring conditions, methylation yields are found higher in the water column (6.3%) than in sediments (0.8-1.3%). Area integrated rates for the experimental site demonstrate however that MeHg is mostly produced in surface sediment with a formation rate of 12 nmol m<sup>-2</sup> day<sup>-1</sup> compared to 1.8 nmol m<sup>-2</sup> day<sup>-1</sup> in the water column. Biological characteristics of the incubated samples have been also monitored indicating the involvement of sulphate reducing bacteria and plankton for Hg methylation in sediment and water column, respectively. For the investigated site, the results obtained have allowed to compare potential transformation and transfer pathways under spring experimental condition.

**KEYWORDS:** Mercury, stable isotopes, methylation, demethylation, sediment, water column, biotic processes

## 1- INTRODUCTION

Methylmercury (MeHg) is a metastable transient specie in sediment and water. Its concentration in aquatic systems is thus controlled by formation, degradation and exchange between compartments. The methylation of mercury (Hg) in water and sediments constitutes a key step in the cycling of Hg in aquatic systems. Methylation occurs predominantly in sediments and to a lesser extent in the water column (Jensen and Jernelov, 1969; Topping and Davis, 1981, Gilmour and Henry, 1991). But to date, methylation processes occurring in the water column have not yet been addressed. Moreover, mercury species are mobile and available to aquatic organisms for bioaccumulation and bio-amplification along the food chain (Stein et al., 1996). Because the mechanisms of methylmercury (MeHg) production appear to be different in sediment and water, an understanding of the source of methylmercury is required to predict the Hg cycling and the impact on the biota.

Methylation and demethylation are two important processes regulating the Hg cycle in aquatic environments (Compeau and Bartha, 1984; Ramlal et al., 1986; Pak and Bartha., 1998). It is important to note that since both methylation and demethylation occur, environmental MeHg concentrations reflect net methylation rather than actual rates of MeHg synthesis. In recent years, the use of radiotracers (Ramlal et al., 1986) and stable isotopes (Hintelmann and Evans, 1997, Hintelmann et al., 2000; Mauro et al., 2002, Rodriguez et al., 2004;) has made possible to distinguish between the two opposites processes of MeHg formation and decomposition.

Concerning sources of MeHg in natural environments, both biotic and abiotic methylation have been reported. Organisms from diverse taxonomic groups such as bacteria (Cleckner et al., 1999, Pongratz et al., 1999, King et al., 2001), algae (Pongratz et al., 1998; Mauro et al., 2002) have been shown to methylate mercury in laboratories studies. Microbial mercury methylation occurs in a variety of marine, estuarine and lacustrine sediments (Macalady et al., 2000, King et al., 2002). Several previous studies have indicated that sulfate reducing bacteria (SRB) are the primary mercury methylators in freshwater and estuarine sediments (Gilmour et al., 1992, Pak and Bartha, 1998; Macalady et al., 2000, Benoit et al., 2001). The abiotic pathway for mercury methylation in natural environment appears to be of minor importance (Weber, 1993, Gardfeldt et al., 2003). For coastal ecosystems, sediment has been shown as an important source of MeHg mediated by SRB activity. Potential Hg methylation takes place in the water compartment and can be induced by both chemical processes and biological activity of marine microorganisms. Bio-mediated pathways (i.e., biomethylation, bioredox reaction) have been proposed to play a significant role for mercury species transformations in sea water (Barkay et al., 2003). However in natural coastal sea water, too scarce investigations have been performed to clearly understand which chemical and biological pathways might be responsible for mercury species transformation, especially for methylated species.

The fate of MeHg also depend on the demethylation process. MeHg degradation is thought to be predominantly microbially mediated in water and sediment (Barkay et al., 2003). Numerous bacterial strains are capable of demethylating MeHg including aerobic and anaerobic species but demethylation appears to be predominantly

accomplished by aerobic organisms. Photolytic decomposition appears to be the only significant abiotic decomposition mechanisms in water. MeHg can be photolytically decomposed in surface water (Sellers et al., 1996; Gardfeldt et al., 2001) but the overall impact on the aquatic Hg cycle is still unclear and the end products of MeHg degradation have not been clearly identified.

Moreover, both methylmercury and inorganic mercury may be reduced to volatile  $\text{Hg}^0$  through biological and chemical reactions (Stein et al., 1996, Barkay et al., 2003). Volatilization from subtidal sediments appears unimportant relative to methylation and demethylation rates (Rodriguez et al., 2004) with the exception of intertidal sediments exposed to solar radiations (Canario and Vale, 2004). However, loss of  $\text{Hg}^0$  from the water column may be faster than methylation/demethylation pathways.

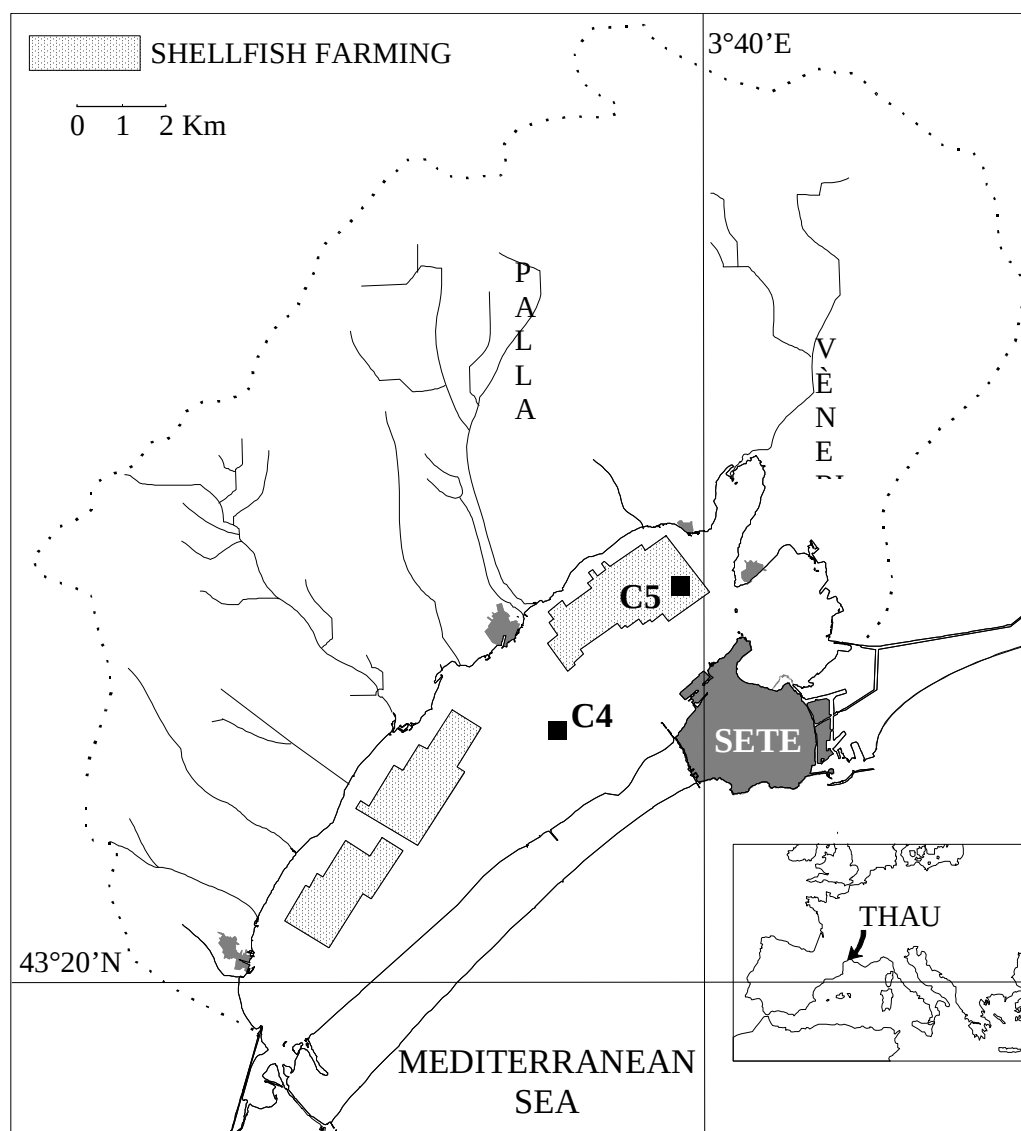
Controlling factors and mechanisms, involved in Hg methylation/demethylation processes and responsible for MeHg concentrations in coastal environment are as yet insufficiently understood. For these reasons, we developed and conducted *in situ* mercury methylation and demethylation experiments in both sediment and water column of an enclosed microtidal coastal bay (Thau lagoon, Mediterranean Sea). Incubation methods were evaluated in terms of sensitivity and precision.

In the framework of the French MICROBENT/PNEC interdisciplinary project, the characterization of the major geochemical and biological parameters at the sediment-water interface was also performed. A companion paper (Muresan et al., 2005) presents the distribution and the speciation of Hg in the solid phase and pore water of the sediment. Mercury fluxes at the water-sediment interface of the Thau Lagoon are also presented by Point et al. (2005).

## 2. MATERIALS AND METHODS

### 2.1. Sampling location

Sediments and water samples were collected in the Thau Lagoon (South East, France). The Thau Lagoon located on the French Mediterranean coast is a shallow enclosed bay sheltered with two narrow sea mouths. The lagoon is of notable economic importance for the Languedoc region because of the large-scale shellfish farming (oyster and mussel production is around 15,000 t.y<sup>-1</sup>) which occupy up to 20% of this 75 km<sup>2</sup> water body. Sediments were collected the 10<sup>th</sup> of April 2002 and the 22<sup>th</sup> of January 2004 on two different sampling sites (see Figure 1): station C4 (7.4m depth: 43°24'029N, 3°36'701E) and station C5 (8.0m depth: 43°25'990N, 3°39'656E) located respectively outside and inside the shellfish farming zone and both characterized by fine muddy sediment. Surface water samples were collected the 13<sup>th</sup> of May 2003 at station C4.



**Figure 1.** Location of sampling sites for water and sediments in the Thau Lagoon.

## 2.2. Sediment sampling and processing

Sediment cores were carefully sampled by divers in order to maintain the integrity of the sediment-water interface. Cores were collected in Plexiglas tubes (30mm ID × 30cm length). Plexiglas tubes and sealed underwater with rubber caps. Plexiglas tubes were previously pierced every 0.5 cm in the length and sealed with silicon allowing injections of 100µl of  $^{199}\text{HgCl}_2$  solution of 10 mg Hg l<sup>-1</sup> in each hole.  $^{199}\text{HgCl}_2$  was prepared by dissolving  $^{199}\text{HgO}$  (Oak Ridge National Laboratory, USA) in HCl. To measure methylation potentials for each 0.5 cm sediment section, cores were spiked every 0.5 cm on the first 5 cm with  $^{199}\text{HgCl}_2$ . Three replicate cores were collected: one reference core was stored directly after collection in a freezer at -20°C. The two remaining cores were incubated for 24 hours at *in situ* temperature in darkness. After incubation, cores were stored in freezer until next handling. In the laboratory, frozen cores were sliced every 0.5 cm, dry-frozen under vacuum and kept frozen until analysis.

## 2.3- Sampling and water incubations

Surface water was collected at the sub-surface (0.5 m) using 5L Teflon coated Go-Flo sampler (General Oceanic, Miami). The sample was transferred directly to previously acid cleaned PFA bottles (Nalgene, USA). Solutions of  $^{199}\text{HgCl}_2$  and  $\text{Me}^{201}\text{HgCl}$  were added to the bulk samples in order to obtain initial concentrations of 12.5 pmol l<sup>-1</sup> and 1.0 pmol l<sup>-1</sup> respectively.  $\text{Me}^{201}\text{HgCl}$  synthesis from  $^{201}\text{HgO}$  (Oak Ridge National Laboratory, USA) has been described previously by Rodriguez et al. (2002). Incubations were performed in triplicate and incubated directly in the Thau lagoon over a complete diurnal cycle (24 hours dawn to dawn), either exposed to sunlight or in the dark. Additionally, control assays were performed on filtered water samples spiked with the same amount of enriched stable isotope mercury species. To remove suspended matter/plankton, water was filtered through a 0.45µm PVDF membrane (Millipore). Finally, filtered and unfiltered waters were also incubated without spike addition to evaluate the behavior of the naturally occurring mercury species. At the end of the incubation period, aliquots of incubated bulk water samples were filtered in order to achieve mercury species partitioning between dissolved and particulate phases. All incubations were stopped by adding high purity HCl (1% v/v) and stored at +4°C in the dark until analysis. Analyses were performed within one month after field sampling and incubation experiments.

## 2.4- Hg and MeHg analysis

For inorganic mercury and methyl mercury analysis, sediments were treated according to the procedure described previously by Rodriguez et al. (2003). Briefly, 0.5 g of dried sediment homogenate was digested with 10 ml of nitric acid 6N in a microwave field (Prolabo A301, 40 W, 3 min.). After the digestion, the extract was centrifuged to remove solid particles and ethylated with sodium tetraethyl borate (Strem, USA). Speciation analyses were performed using capillary gas chromatography (Focus GC, Thermo Element) with inductively coupled plasma mass spectrometry (ICPMS X7, Thermo Element).

Mercury speciation analysis of the water samples were performed using propylation GC-ICPMS described by Monperrus et al. (2004). Briefly, concentrated ammonium hydroxide and 5 ml of acetic acid / sodium acetate buffer (0.1M) are added to 100 ml of the water sample to set the pH at 4. 0.2 ml of iso-octane and 1 ml of 0.5% (w/v) sodium tetrapropylborate are added. The flask is immediately capped and vigorously hand shaken for 5 min. The organic phase is finally transferred to an injection vial and stored at  $-18^{\circ}\text{C}$  until measurement. GC-ICPMS parameters have been described elsewhere (Rodriguez et al., 2002). Each sample was injected three times and the chromatographic peak integration was performed using chromatographic software. Isotopes ratios were always measured as peak area ratios and corrected by mass bias factor. Blanks were also determined to check for any contamination. Determination of the concentrations of each mercury species for each isotope (199, 201, 202) is carried out to evaluate methylation and demethylation yields.

### 2.5- Calculations of methylation/demethylation

Gross rates of mercury methylation/demethylation were determined by adding trace quantities of isotopically enriched  $^{199}\text{HgCl}_2$  and  $\text{Me}^{201}\text{HgCl}$  to intact sediment cores or water incubation bottles. The amount of methylated mercury deriving from the enriched isotope 199 during the incubation can be calculated by using the equation described previously by Hintelmann et al. (1995). To improve the precision on yield measurement, recovery of the spiked and transformed species after incubation were also investigated. Methylation yield is calculated by dividing the amount of  $\text{Me}^{199}\text{Hg}$  formed by the  $^{199}\text{Hg}(\text{II})$  recovered after the incubation period. The amount of demethylated methylmercury from the enriched spike is calculated in a similar way following the 201 isotope. Demethylation yield is calculated by dividing the quantity of  $^{201}\text{Hg}(\text{II})$  formed by the quantity of  $\text{Me}^{201}\text{Hg}$  added.

Measured yields of methylation and demethylation are assumed to define demethylation and methylation potentials in the considered system. Because the spiked mercury species are equally or more available than the natural Hg species, measured yields represent a maximum yield that can be obtained under natural conditions. In addition, speciation of the newly introduced Hg compounds equilibrates readily with the ambient species allowing the method to well predict environmental processes (Hintelmann et al., 2000).

### 2.6. Other parameters

Sulfate reduction rates were determined in triplicate cores. Sulfate reduction was measured by injecting 10  $\mu\text{l}$  of carrier free  $^{35}\text{SO}_4^{2-}$  (370 KBq) at 0.5 cm depth intervals. The cores were incubated for 6 hours at *in situ* temperature in darkness. Following incubations, sediments were sliced into 1cm thick sections and sulfate reduction rates were determined using by the single step Chromium reduction method of Fossing and Jorgensen (1989). Specific activities of  $^{35}\text{S}^{2-}$  and  $^{35}\text{SO}_4^{2-}$  were determined in by liquid scintillation counting (Beckman LS6500 liquid scintillation counter). Sulfate concentrations were determined as described by Tabatabai et al. (1974) and the sulfate reduction rates calculated according the method of Fossing and Jorgensen (1989).

For primary production measurements, 3 polycarbonate bottles (260-ml) were filled with bulk water. 2  $\mu\text{Ci}$  of  $^{14}\text{C}$ - $\text{NaHCO}_3$  tracer solution was added to each experimental bottle and the incubation was done simultaneously as the Hg incubation experiments. After incubation, samples were collected on 25 mm GF/F and filters were immediately rinsed with filtered seawater, taking the recommendations of Goldman and Dennet (1985) into account.  $^{14}\text{C}$  particulate uptake was determined using a liquid scintillation counter Packard model TRI-CARB 1600-TR with a  $^{14}\text{C}$  internal standard.

Silicic acid uptake was measured at the same time and in the same in situ conditions. 250 ml sample, in polycarbonate bottles, were spiked with 250000 dpm (830 Bq) of  $^{32}\text{Si}$  tracer (52 000 Bq  $\mu\text{g}^{-1}$  Si, Los Alamos National Laboratory). At the end of the incubation period, each sample was gently vacuum-filtered through a 0.6  $\mu\text{m}$  polycarbonate membrane filter (Nuclepore) and rinsed with filtered seawater. The filter was then placed with HF to dissolve biogenic silica. The amount of  $^{32}\text{Si}$  retained on the filter was determined by liquid scintillation counting, as described by Leynaert et al. (1996). The biogenic silica production rate (PBSi, in  $\text{nmol l}^{-1} \text{h}^{-1}$ ) is the fraction between the initially dissolved  $^{32}\text{Si}$  activity added to the sample, and the  $^{32}\text{Si}$  taken up by phytoplankton and counted on the filter at the end of the incubation, divided by the incubation time.

Samples for phytoplankton species composition were preserved with an acid lugol solution. Species were identified and counted by microscopic examination on an inverted microscope (Utermöhl, 1931)

## 2.7 Quality assurance / quality control

All equipment used for sub-sampling, sectioning, filtration, storage and analysis of sediment and water samples was carefully cleaned using successive acid bath ( $\text{HNO}_3$  and  $\text{HCl}$ ), rinsed with ultrapure MQ water (Millipore) and conditioned under a laminar flow hood (Class 100). Procedural blanks were performed throughout the sampling, the sample preparation and the analysis.

Analytical method for sediments was validated using a certified reference material (IAEA 405) for total Hg and MeHg. Recoveries for total Hg agree very well with the certified value with a mean value of  $820 \pm 50 \text{ ng g}^{-1}$  (certified value  $810 \pm 40 \text{ ng g}^{-1}$ ). But for MeHg, previous work in our lab has shown recovery of MeHg from IAEA 405 biased due to the artifactual formation of MeHg during the sample preparation (Rodriguez et al., 2002). Certified value had been successfully achieved by using a solvent pre-extraction with  $\text{CH}_2\text{Cl}_2$  in order to remove interfering  $\text{Hg(II)}$  from the extract. However, as mercury concentration ranges and chemical characteristics of the sampled sediments in the Thau Lagoon differ from IAEA 405, quality control of our analytical method has been checked. Addition of  $^{199}\text{Hg(II)}$  on the unspiked sediment extracts, has shown no artifactual formation of  $^{199}\text{MeHg}$  during analysis of sediments from the Thau lagoon even without using solvent extraction.



### 3. RESULTS AND DISCUSSION

#### 3.1-Cores experiments

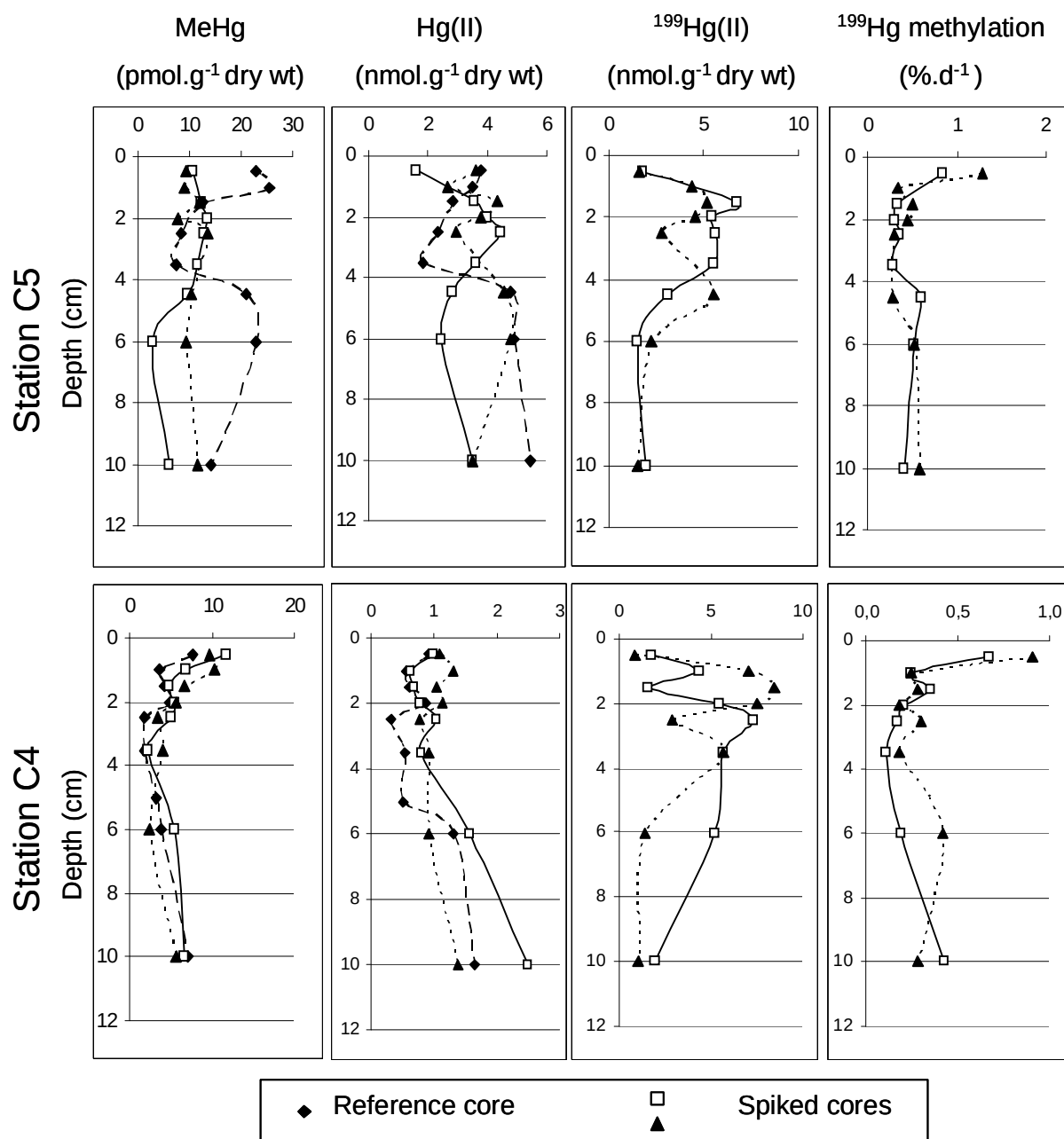
##### 3.1-1. *Evaluation of the cores experiments*

*Spatial variability* – In order to integrate representative results from the core incubations, it should be taken into account that sediment cores were collected at different locations within a defined sampling site (about 10m<sup>2</sup>). This introduces a natural level of variability which should be quantified for each sampling depending on the sediment characteristics (grain size, organic matter, benthic organisms). All experiments presented here were done in triplicate cores to evaluate this field variability.

Spatial variability of Hg species with depth, based on the relative standard deviation of the triplicate cores collected in April 2002, is shown in Figure 2. For station C5, very important variability is observed between the 3 cores. Hg species concentrations exhibit mean variability for the whole depth profile of 26 and 43% for Hg(II) and MeHg, respectively. For station C4, mean variability of 26 and 31% are found for Hg(II) and MeHg, respectively. Higher variability for MeHg at station C5 is probably linked to heterogeneities due to the occurrence of numerous shell debris in the sediment (station C5 located within the shellfish farming zone). In addition, bioturbation can not account for such higher variability in C5 since similar sediment reworking was reported at both sites (Duport et al., 2005). This spatial variability is however reasonable in order to evaluate the environmental fate of mercury.

*Spike distribution and recovery* – After incubation, <sup>199</sup>Hg(II) and Me<sup>199</sup>Hg formed were determined for each core sections. Repartition of the added <sup>199</sup>Hg(II) after incubation (Fig. 2) shows a redistribution of the spike within the core. In comparison of theoretically spiked <sup>199</sup>Hg(II) (about 5 nmol g<sup>-1</sup> to the depth of 5 cm) and the experimentally measured <sup>199</sup>Hg(II) in the sediment cores, no significant loss of spike is observed. However, <sup>199</sup>Hg(II) spiked is not perfectly recovered along the core compared to theoretically spiked concentration at each depth section (0.5 cm). Its repartition along the core is also different between the replicate cores. The differences between the theoretically spiked <sup>199</sup>Hg(II) and the experimentally measured one may be explained by redistribution of Hg species by bioirrigation and to a lesser extent by diffusion during incubation. This remobilization is thus dependent on the type of benthic organisms presented (gallery-diffusers, conveyors...) which also influences fluxes at the sediment-water interface (Point et al., 2005). Remobilization is also dependent on the sediment porosity and the presence of fixation sites for inorganic mercury like sulfides and organic ligands. A diffusion along the corer tube walls is also possible as well as a diffusion to the overlaying water from superficial layers by capillarity. However, no circulation of interstitial water was observed during incubation and only negligible amounts of <sup>199</sup>Hg(II) were measured in overlaying waters. Due to this potential mobility of the <sup>199</sup>Hg(II) spiked within the core, methylation

potential is calculated by dividing the quantity of  $^{199}\text{MeHg}$  formed by the quantity of  $^{199}\text{Hg(II)}$  recovered at the end of the incubation and not by the quantity theoretically added.



**Figure 2.** Sediment profiles of Hg species, spiked  $^{199}\text{Hg(II)}$  and  $^{199}\text{Hg}$  methylation in April 2002 for triplicate cores for stations C5 and C4.

*Performances of the methylation rates* - Following  $\text{Me}^{199}\text{Hg}$  formed during the incubation period, a methylation rate can be calculated for each core section (Fig.2). Conversely to the variation of the spike repartition along the core, the methylation rates are surprisingly homogeneous between the two spiked cores. Depth profiles of methylation rates exhibit the same pattern between replicate cores for both stations. This indicates that even if the

repartition of the spike is slightly modified during the incubation period and is different between replicate cores, the determination of the methylation remains accurate taking into account the recovered spiked quantity for the calculations.

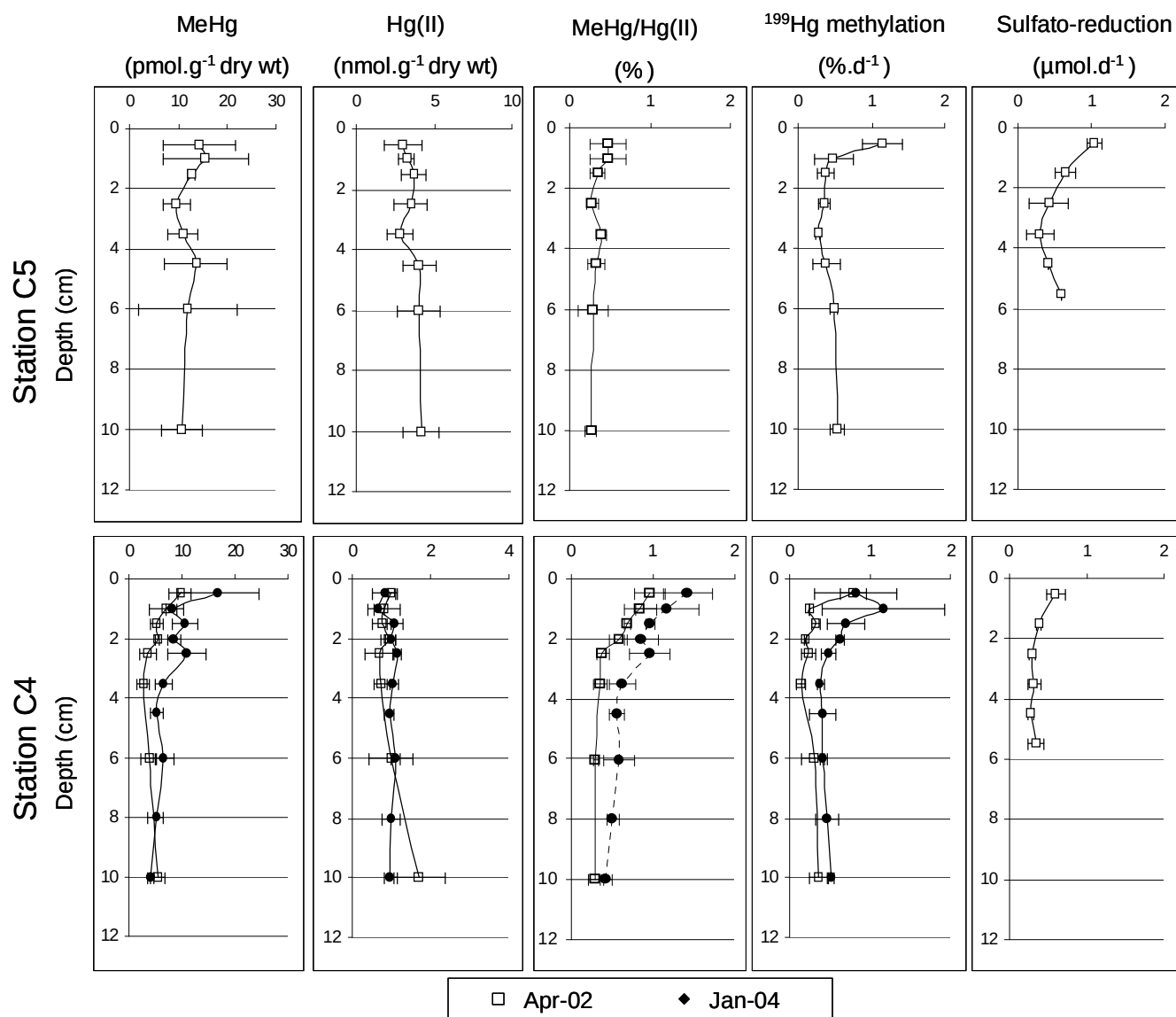
Detection limit of the newly formed MeHg is calculated according to equations previously presented by Hintelmann et al. (1997). The lowest measurable methylation yield with this analytical procedure is 0.02% which corresponds according to the spike amount ( $5 \text{ nmol g}^{-1}$ ) to  $1 \text{ pmol g}^{-1}$  of  $\text{Me}^{199}\text{Hg}$  formed. Precision on methylation potential is calculated from the standard deviation between the 2 cores and ranges between 1 and 50% with a mean value at 25% for both stations. Increasing the number of cores would help to improve precision on methylation yields. However, reproducibility of 25% is suitable especially for this very low concentration levels.

### **3.1.2- Sediment characteristic and Hg species natural concentrations**

Sediments in C5 and C4 consist of very fine particles (silt and clay) with a mean grain size between 10 and 20  $\mu\text{m}$  (Schmidt et al., 2005). The sediment accumulation rate, based on  $^{210}\text{Pb}$  activity, is about  $0.15 \text{ cm y}^{-1}$  in C5 and  $0.25 \text{ cm y}^{-1}$  in C4 (Schmidt et al., 2005). Surface sediments have similar porosity, slightly superior to 0.9 for both stations (Dedieu et al., 2005). The highest content of organic carbon was recorded in the top layer (0-1cm) with 4.35% in C5 and 2.99% in C4 (Mesnage et al., 2005). In addition, the presence of more labile organic matter has been observed in C5 compared to C4. Sediment  $\text{O}_2$  profiles with millimetre scale resolution showed the oxicline interface to be located within several millimetres from the sediment surface for both stations in April 2002, with 1-2mm and 3-4mm in C5 and C4 respectively (Dedieu et al., 2005). The upper sediment layer incubated for Hg methylation (0-0.5cm) is thus under oxic to suboxic conditions.

Mean values and standard deviations are calculated from the triplicate cores for ambient mercury species along the depth profile for station C5 and C4 as well as MeHg proportion (Fig. 3). For both sites, core profiles exhibit two clear characteristics. First ambient  $\text{Hg(II)}$  concentrations do not significantly vary with depth and averaged about  $1.0 \pm 0.4$  and  $3.6 \pm 0.5 \text{ nmol g}^{-1}$  dry weight for C4 and C5, respectively. Second, a maximum in MeHg concentrations is found at the sediment surface (0-0.5cm) followed by a decrease with depth especially for station C4. Spatial variability on MeHg concentrations for station C5 is too important to see any trend. However MeHg concentration levels for deeper sediment are higher at station C5 ( $11.5 \pm 1.5 \text{ pmol g}^{-1}$ ) than in C4 ( $4.3 \pm 1.2 \text{ pmol g}^{-1}$ ).

The concentration of Hg in coastal sediment is generally closely related to the amount of fine-grained particles, organic material and sulfides (Covelli et al., 1999, Stoichev et al., 2004).  $\text{Hg(II)}$  concentrations normalized to total organic carbon provided a ratio two times higher in C5 than in C4. Station C5 is potentially more exposed to pollutant inputs from the watershed. Nevertheless, metallic pollutants concentrations have not been found significantly higher for sediment in C5 (Point et al., 2005).



**Figure 3.** Sediment profiles of mean values (± standard deviation) of Hg species, <sup>199</sup>Hg methylation and sulfate reduction potentials for stations C5 and C4 in April 2002 (open symbol) and May 2003 (full symbol).

### 3.1.3- Hg methylation potential in sediment

*Relationship with sulfate reduction along the core* - Methylation potentials at station C5 and C4 are the greatest at the sediment surface and decrease with depth, similarly to natural MeHg concentrations profiles. Depth profiles of sulfate reduction rate and methylation potential in cores are also similar. The highest rates of sulfate reduction are found at the sediment surface then decreasing with depth (Fig.3). Sulfate reduction rates measured in April and integrated for the first 0-1 cm were 16.9 and 9.9 mmol SO<sub>4</sub><sup>2-</sup> m<sup>-2</sup> d<sup>-1</sup> in C5 and C4 respectively and are comparable with those determined in other organic carbon enriched coastal sediments (Welsh et al., 1996). The maximum methylation potential coincident with the maximum sulfate reduction activity illustrates the link between sulfate reduction bacterial activity and Hg methylation in sediments. Previous works have shown that in marine

sediments, net methylation rates are the highest in the transition zone within the oxycline. So called hypoxic to suboxic conditions are more conducive to the overall microbial activity and thus to the activity of SRB (Compeau and Bartha, 1984; Gilmour et al., 1998; Bloom et al., 1999).

At station C4, two series of experiment have been achieved in April 2002 and January 2004. During April 2002, higher methylation yields are recorded in the surface layer ( $0.79 \pm 0.17\%$ ) while during January 2004, the maximum is just below the surface (0.5-1cm) with  $1.32 \pm 0.5\%$ . Mercury methylation rates are generally greater during warmer season (Gilmour et al. 1998), because methylation processes are dominantly mediated by microbial activities (SRB). The surface water temperature measured in the Thau Lagoon was  $14.2^\circ\text{C}$  in April 2002 and  $8.8^\circ\text{C}$  in January 2004. Sulfate-reducing activity has not been yet determined in January 2004. However MeHg formation is known to be dependant not only on microbial temperature driven activity but also on mercury speciation and availability (Benoit et al., 2001). High SRB activity during warmer period may lead inorganic mercury to be less available for methylation due to its complexation with sulfidic compounds. Benoit et al. (2001) have previously shown that in sulfidic sediments, sulfides complexation of Hg inhibits its microbial uptake and methylation.

The stoichiometry of Hg methylation related to the sulfate reduction can be determined by dividing the methylation rate by the corresponding sulfate reduction rate. Similar molar ratios of  $5.5 \cdot 10^{-6}$  and  $5.6 \cdot 10^{-6}$  (mol/mol) have been estimated during the spring period for surface sediments from stations C5 and C4, respectively. This finding confirms the close link between sulfate reduction activity and Hg methylation and also suggests the potential use of sulfate reduction activity to evaluate Hg methylation in such environments.

*MeHg formation and abundance in surface sediment* – Surface sediment mercury species concentrations, MeHg proportion, methylation and sulfate reduction rates are summarized in Table 1 for the two first sediment layers (0-0.5 and 0.5-1 cm) since both water-sediment and oxic-anoxic interfaces appear as the privileged location for mercury methylation. The proportion of MeHg varies between the two sites with a proportion two times higher for station C4 ( $0.96 \pm 0.19\%$ ) than station C5 ( $0.47 \pm 0.22\%$ ). Conversely, methylation potential is higher for station C5 than station C4 with 1.32 and 0.79% respectively. Higher sulfate reduction activity at C5 may lead to higher methylation rates. Moreover, high organic carbon contents and the presence of more labile organic matter in C5 compared to C4 could explain the higher reactivity of sediment at C5.

In C4, a higher MeHg proportion is found although surface sediments exhibit a lower methylation potential compared to station C5. MeHg proportion calculations allow integrating processes over longer time scale whereas methylation potential is representative of the specific experimental period. Taking into account the accumulation rate ( $0.25 \text{ cm y}^{-1}$ ), the surface layer (0-0.5cm) may represent 2 years of MeHg turnover. Methylation is not the only process regulating the MeHg concentration in sediment. Demethylation of MeHg is also occurring simultaneously. For station C5, the methylation potential is higher but the demethylation is certainly an important

process limiting the MeHg proportion to increase (Ramlal et al., 1986). Our results suggest that demethylation is a significant process although demethylation potential has not yet been measured. Our results indicate also that methylation is an important source of MeHg in the sediment of the Thau lagoon and is strongly correlated with the sulfate reducing bacteria activity. Others parameters also control the Hg methylation in sediment such the sulfide concentration favoring Hg methylation (Muresan et al., 2005) by supporting the hypothesis of neutral Hg-S complexes controlling the bioavailability of Hg(II) (Benoit et al., 1999).

1

| Station | Date     | Depth (cm) | MeHg (pmol.g <sup>-1</sup> dry wt) | Hg(II) (nmol.g <sup>-1</sup> dry wt) | MeHg/Hg(II) (%) | Methylation rate (%.d <sup>-1</sup> ) | SR rate (mmol.m <sup>-2</sup> .d <sup>-1</sup> ) |
|---------|----------|------------|------------------------------------|--------------------------------------|-----------------|---------------------------------------|--------------------------------------------------|
| C5      | Apr 2002 | 0-0.5      | 14.3±7.4                           | 3.0±1.2                              | 0.47±0.22       | 1.32±0.03                             | 16.9±1.6                                         |
|         |          | 0.5-1      | 15.5±8.7                           | 3.2±0.5                              | 0.47±0.21       | 0.56±0.32                             | 10.6±2.2                                         |
| C4      | Apr 2002 | 0-0.5      | 9.7±2.0                            | 1.0±0.1                              | 0.96±0.19       | 0.79±0.17                             | 9.9±1.9                                          |
|         |          | 0.5-1      | 7.0±3.3                            | 0.8±0.4                              | 0.84±0.20       | 0.25±0.01                             | 6.3±0.4                                          |
|         | Jan 2004 | 0-0.5      | 11.9±1.0                           | 0.8±0.3                              | 1.42±0.30       | 1.03±0.23                             |                                                  |
|         |          | 0.5-1      | 8.0±1.0                            | 0.7±0.1                              | 1.17±0.38       | 1.32±0.54                             |                                                  |

**Table 1** : Mercury species concentrations, MeHg proportion, methylation and sulfate reducing rates for surface sediments.

## 3.2. Water experiments

### 3.2.1. Evaluation of the incubation method

In order to evaluate the representativity of our water incubation results, systematic experiments were performed in triplicate together with control assays. Incubation protocol has been adapted from the method generally used for primary production measurements using incubation of unfiltered water during a diurnal cycle dawn to dawn (Lohrenz et al., 1992). Controlled specific incubation experiments have been also performed in dark condition and with filtered water. The dark control, generally used as a control for non-photoautotrophic carbon fixation or adsorption, allows in addition to investigate photochemically induced methylation/demethylation processes. Incubation of filtered water has been performed under the same incubation conditions to discriminate abiotic processes from those mediated by plankton (i.e. phyto and bacterio-plankton).

Similarly to sediment incubations, detection limits and reproducibility of the incubation method can be evaluated. Detection limits for monitoring methylation and demethylation can be calculated by equations given previously by Hintelmann et al. (1997). The detection limit for observing any formation of new MeHg or Hg(II) is not only based on the detection limit of the GC-ICPMS system but on the precision on the isotopic ratios measured and the natural abundances of the stable isotope tracer. To determine methylation potential, the detection limit for calculations with isotopes 199 and 202 is about 0.02%. Taking into account the spike level (12.5 pmol l<sup>-1</sup>), this method allows to detect 0.002 pmol l<sup>-1</sup> of newly formed Me<sup>199</sup>Hg. For demethylation (isotopes 201 and 202), the

limit of detection of the demethylation yield is 3% and corresponds to a concentration of 0.03 pmol l<sup>-1</sup> of newly formed <sup>201</sup>Hg(II).

Precision of the whole incubation procedure taking into account sampling, incubation proceeding and analytical measurements have been evaluated for three independent incubation bottles for each incubation condition. Relative standard deviations were found to range between 3 and 60% depending on concentration levels. Mean values for our set of measurements was established at 25 and 21% for methylation and demethylation yields respectively. These performances allow thus to investigate mercury transformations in the water column.

### 3.2.2. Surface water characteristics and Hg species natural concentrations

The surface water sample collected in May 2003 at the C4 station was characterized by a salinity of 33.7 and a temperature of 17.1°C. Phytoplankton abundance in the collected sample was significantly high with 3.5 million of cells per liter. The sampling period was about one month after a spring phytoplankton bloom exhibiting *chlorophyll* a concentrations up to 3.1 µg l<sup>-1</sup> the 14<sup>th</sup> of April 2003 and 1.6 µg l<sup>-1</sup> the 12<sup>th</sup> of May 2003 (Ifremer, REPHY). This suggests that a major part of suspended matter was composed of phytoplankton cells. Phytoplankton species distribution in the collected sample was dominated by the presence of chryptophyceae (35%), dinoflagelates (22%), chlorophyceae (20%), and diatoms (20%). Quantification of primary production for this surface water using <sup>14</sup>C spikes, simultaneously as the 24 hours mercury incubation experiments, shows high carbon uptake rate at 14 µmol C l<sup>-1</sup> d<sup>-1</sup> confirming the important presence of photosynthetic plankton in the considered water sample. For the dark control incubation, low inorganic carbon assimilation of 0.2 µmol C l<sup>-1</sup> d<sup>-1</sup> was measured. In addition, a high assimilation of silicate (<sup>32</sup>Si) of 2 µmol Si l<sup>-1</sup> d<sup>-1</sup> and a resulting C/Si uptake ratio of 7 are characteristic of significant diatoms activity compared to other taxa. In such coastal surface water, the presence of heterotrophic organisms is also significant but no direct measurements of microbial activity or biomass have been performed.

For the investigated seawater sample, mercury speciation analysis was achieved for filtered and unfiltered water. Natural concentrations of Hg(II) were 3.24±0.12 and 4.09±0.17 pmol l<sup>-1</sup> for filtered and unfiltered water, respectively. Methylmercury concentrations were 0.25±0.05 and 0.41±0.07 pmol l<sup>-1</sup> for filtered and unfiltered water, respectively. Such concentrations are within the range of typical concentrations found for coastal sea water (Gilmour et Henry, 1991). Proportions of Hg associated with suspended matter represent 39% and 21% for MeHg and Hg(II) respectively for natural mercury species. MeHg presents thus a higher affinity with suspended matter than Hg(II).

Suspended matter play an important role in the transport and the reactivity of Hg(II) and MeHg in aquatic systems. Particulate Hg consists of Hg bound to mineral particles and detritic organic matter as well as biogenic particles such as bacterial aggregates, algae or phytoplankton (Hurley et al., 1994). In coastal environment, the higher affinity of Hg(II) to particles is generally observed when particulate matter are composed essentially by mineral and inert organic compounds. For the investigated water of the Thau Lagoon, collected just after a spring

bloom period (3.5 million of cells per liter), we suppose that a major part of suspended matter is composed of living organic matter (i.e. plankton). The higher affinity of MeHg with suspended matter could be explained by a greater affinity to biogenic particles whereas Hg(II) tends to bind more strongly to mineral particles and detrital organic matter. In addition, Mason et al. (1996) have demonstrated the higher assimilation of MeHg by diatom cells compared to Hg(II). These observations may explain the greater affinity of MeHg for biogenic particles than Hg(II) in the Thau Lagoon.

### 3.2.3. Methylation/demethylation potentials in surface water

*Methylation* - Net methylation yields can be calculated simultaneously by dividing the concentration of Me<sup>199</sup>Hg formed by the concentration of the added <sup>199</sup>Hg(II). Figure 4 presents the yield of Me<sup>199</sup>Hg formed after 24 hours of incubation for filtered and unfiltered seawater. Concentrations are given in Table 2. For filtered water, low concentrations of Me<sup>199</sup>Hg are formed from the spiked <sup>199</sup>Hg(II) with 0.15 and 0.09 pmol.l<sup>-1</sup> for the diurnal cycle incubation and the dark control respectively; corresponding to methylation yields lower than 1.2%. Incubations of unfiltered water exhibit a higher concentration of Me<sup>199</sup>Hg formed with 0.79 pmol.l<sup>-1</sup> corresponding to a methylation yield of 6.3% under light condition whereas for the dark control, only 1.7% of the spiked <sup>199</sup>Hg(II) is methylated. For this seawater sample, mercury methylation seems to be dependant of the presence of both sunlight radiation and particles (ie phyto- and bacterio- plankton cells). This result suggests a strong relationship between mercury methylation and plankton activity.

| <b>Diurnal cycle</b> | Fraction  | MeHg (pmol.l <sup>-1</sup> ) | Hg(II) (pmol.l <sup>-1</sup> ) | <sup>199</sup> MeHg formed (pmol.l <sup>-1</sup> ) | <sup>201</sup> Hg(II) formed (pmol.l <sup>-1</sup> ) |
|----------------------|-----------|------------------------------|--------------------------------|----------------------------------------------------|------------------------------------------------------|
| Filtered water       | Dissolved | 0.25±0.09                    | 3.68±0.14                      | 0.15±0.03                                          | 0.09±0.03                                            |
| Unfiltered water     | Bulk      | 0.40±0.05                    | 4.04±0.15                      | 0.79±0.03                                          | 0.13±0.01                                            |
|                      | Dissolved | 0.22±0.03                    | 3.20±0.57                      | 0.24±0.02                                          | 0.11±0.03                                            |
| <b>Dark control</b>  | Fraction  | MeHg (pmol.l <sup>-1</sup> ) | Hg(II) (pmol.l <sup>-1</sup> ) | <sup>199</sup> MeHg formed (pmol.l <sup>-1</sup> ) | <sup>201</sup> Hg(II) formed (pmol.l <sup>-1</sup> ) |
| Filtered water       | Dissolved | 0.25±0.03                    | 3.13±0.31                      | 0.09±0.02                                          | 0.03±0.02                                            |
| Unfiltered water     | Bulk      | 0.36±0.01                    | 3.90±0.28                      | 0.22±0.11                                          | 0.09±0.01                                            |
|                      | Dissolved | 0.21±0.07                    | 3.25±0.25                      | 0.08±0.04                                          | 0.07±0.02                                            |

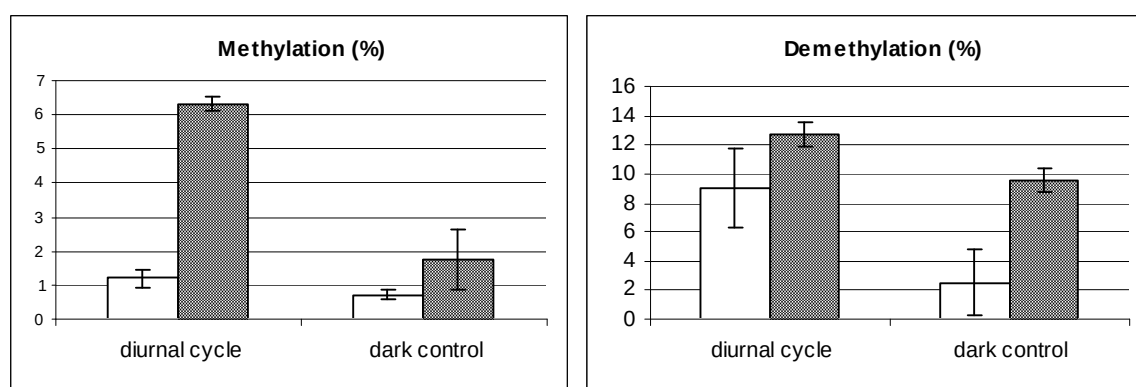
**Table 2** : Mercury species, <sup>199</sup>MeHg formed and <sup>201</sup>Hg(II) concentrations found for water incubations.



The results are the first suggesting in situ mercury methylation in the water column in the presence of microplankton species and under light induced processes confirming the hypothesis given by Topping and Davies (1981). The results can be compared with observations from previous studies on different types of photosynthetic organisms. Pongratz et al. (1998) have demonstrated the production of MeHg by polar macroalgae in sea water. Mauro et al. (2002) have confirmed that the mercury methylation in flood plain is linked to bacteria activities associated with macrophytes reaching significant methylation potentials (1.5-7.7%). However, these organisms are completely different than those found in the water of the Thau Lagoon.

As for sediment, the stoichiometry Hg methylation can be assessed by dividing the Hg methylation rate by the primary production rate. A molar ratio of  $5.6 \cdot 10^{-8}$  has been calculated and could be compared with further investigations (i.e. spatial and temporal variations).

**Demethylation** - Demethylation of methylmercury can be followed simultaneously in the same sample measuring newly formed  $^{201}\text{Hg(II)}$  concentrations during the incubation period. Figure 4 presents the transformation yield of the added  $\text{Me}^{201}\text{Hg}$  after incubation for filtered and unfiltered water, for a diurnal cycle and the dark control. Concentrations are indicated in Table 2. Significant  $\text{Me}^{201}\text{Hg}$  demethylation yields were found for the diurnal cycle experiment under both filtered and unfiltered conditions with 9.0 and 12.8 %, respectively. These yields correspond to equivalent concentrations of formed  $^{201}\text{Hg(II)}$ , with  $0.09 \pm 0.03$  and  $0.13 \pm 0.01$   $\text{pmol.l}^{-1}$  for incubations with or without particles, respectively. For the dark control, lower degradations of  $\text{Me}^{201}\text{Hg}$  spiked were observed exhibiting 2.5 and 9.6% of demethylation yields for filtered and unfiltered water respectively. These results suggest that demethylation is driven both by photochemical processes and biotic mechanisms. Although demethylation is partially photochemically induced, results of the dark control with a higher demethylation rate for unfiltered water compared to filtered water indicate that MeHg degradation is mainly mediated by plankton activities.



**Figure 4.** Methylation and demethylation yields of filtered (without dot) and unfiltered (with dots) water for a diurnal cycle and the dark control.

### **3.2.4. Role of sunlight radiations**

Both methylation and demethylation processes are found to be strongly dependent on sunlight radiations. However both transformations do not seem to be driven by the same type of mechanism. In the case of mercury methylation, higher methylation yields are found for water incubations during a diurnal cycle and with the presence of biogenic particles (i.e., phyto- and bacterio-plankton). Methylation potential seems thus to be dependent on the light condition and probably on photosynthetic activity. Quantification of primary production in these incubations under light condition using  $^{14}\text{C}$  spikes shows high primary production for the diurnal cycle incubation. A high assimilation of silicate, characteristic of diatoms activity, has been also found. These results suggest that methylated mercury may be produced in this seawater sample through both phytoplanktonic photobiological metabolism and bacterial activities associated with phytoplankton.

Symbiotic mechanisms between phytoplankton and bacteria are likely to favor mercury methylation (Cleckner et al., 1999). Under light condition, primary production activity enhances the release of labile dissolved organic compound. Microbial activity could then be stimulated by the assimilation of this source of carbon. Cleckner et al. (1999) have shown that methylation in periphyton in Everglades is also coupled to photosynthesis supporting the photosynthetic microbial sulfur cycle. However, to date no studies have investigated the photosynthetic metabolism of marine micro-organisms on Hg cycling.

In the case of demethylation of mercury, high demethylation yields are found for unfiltered water incubations both for the diurnal cycle incubation and the dark control. Demethylation yields observed under dark condition may be attributed to microbial demethylation. Moreover, in the absence of particles, higher demethylation yield for the diurnal cycle incubation compared to the dark control point out that demethylation can be photochemically induced as PFA incubation bottles allows to expose samples to both visible and UV-B radiations. This is consistent with previous results found by Sellers et al (1996), showing MeHg photodegradation in surface lake waters.

Volatilization processes were not investigated in this set of experiments. Gaseous species ( $\text{Hg}^0$ ,  $\text{Me}_2\text{Hg}$ ) were thus not determined. To assess their potential significance, mass balance between the quantities of recovered spiked and formed species compared to the theoretically added quantity have been performed. The mass balance shows a maximum loss of 10% that may correspond to the volatilization of mercury species.

### **3.2.5. Partitioning of Hg species**

Partition of Hg species between dissolved and particulate phases exhibits significant differences between ambient, spiked and formed species (Table 3).

For natural MeHg and spiked  $\text{Me}^{201}\text{Hg}$ ,  $54 \pm 8$  and  $64 \pm 8\%$  of the species are recovered in the dissolved fraction respectively; whereas only  $31 \pm 4\%$  of the formed  $\text{Me}^{199}\text{Hg}$  is found in the dissolved phase. These findings first

suggest that added MeHg imitates the ambient MeHg partitioning behavior. Secondly, the major part of MeHg formed is mainly recovered in the particulate phase, which corroborates methyl mercury association to phytoplankton and/or bacteria and thus specific intracellular turnover of biogenic MeHg.

In the case of Hg(II), repartition between dissolved and particulate fractions do not vary significantly between ambient, formed and spiked Hg(II). Distributions are similar with  $79\pm 8$ ,  $83\pm 7$  and  $95\pm 8\%$  present in the dissolved fraction for ambient, formed and spiked Hg(II) suggesting the good equilibration of spiked Hg(II) with the water sample.

| Ambient MeHg<br>(%)   | <sup>201</sup> MeHg added<br>(%)   | <sup>199</sup> MeHg formed<br>(%)   |
|-----------------------|------------------------------------|-------------------------------------|
| 54±8                  | 64±8                               | 31±4                                |
| Ambient Hg(II)<br>(%) | <sup>199</sup> Hg(II) added<br>(%) | <sup>201</sup> Hg(II) formed<br>(%) |
| 79±8                  | 95±8                               | 83±7                                |

**Table 3** : Proportion of mercury species in the dissolved fraction (%) for ambient, added and formed species at station C4 in May 2003 ( $\pm$  standard deviations, n=3).

### 3.3. Implications for the fate of MeHg in the Thau Lagoon (spring period)

The cycling of MeHg at the water-sediment interface can be emphasized from potential transformation rates in sediment and water and benthic fluxes measurement performed at station C4 (Point et al., 2005) (see Table 4). This station is located in the central part of the lagoon. MeHg formation rate in surface sediment has been measured in April 2002 at  $12 \text{ nmol m}^{-2} \text{ day}^{-1}$ , considering methylation potential at the surface sediment layer (0-0.5cm) area integrated. MeHg formation rate in the water column can be estimated integrating MeHg formation rate in sub-surface water in May 2003 to the whole depth profile (7.5 m). MeHg formation integrated rate in water is thus evaluated at  $1.8 \text{ nmol m}^{-2} \text{ day}^{-1}$ .

MeHg produced in both compartments can be then exchanged at the sediment-water interface. Benthic fluxes of MeHg from sediments of the Thau Lagoon were measured in May 2003 using benthic chambers and average  $0.3 \text{ nmol m}^{-2} \text{ day}^{-1}$  (Point et al., 2005). Measured fluxes are within the range of previous measured fluxes in coastal bays (Gill et al., 1999, Covelli et al., 1999). MeHg burial rate in the surface sediment layer can be estimated by multiplying the accumulation rate of  $0.25 \text{ cm yr}^{-1}$  based on <sup>210</sup>Pb activity (Schmidt et al., 2005) and the dry weight MeHg concentration in surface sediment (i.e. concentration in dried sediment corrected by the dry density  $0.5 \text{ g cm}^{-3}$ ). An average MeHg burial rate of  $0.04 \text{ nmol m}^{-2} \text{ day}^{-1}$  is thus obtained.

The tentative comparison shows that the main source of MeHg in the Thau Lagoon is the microbial methylation of mercury in sediment. MeHg mobility from sediment to the water column is linked to benthic processes (Point et

al., 2005) but represents only 17% of MeHg source in the water column compared to direct methylation occurring in the water compartment. Assuming that methylation rate in water was determined in May 2003 during a period of high biological turnover, MeHg formation rate is probably overestimated with respect to the annual average. Additional experimentations on the methylation potential in the water compartment have shown a rate of  $0.28 \text{ nmol m}^{-2} \text{ day}^{-1}$  during winter (Monperrus et al., in preparation), which still represents an important source of MeHg available for the pelagic food web.

At least, the fate of MeHg in sediment and water will also be affected by two others important processes: the bioaccumulation and the demethylation of MeHg.

The bioaccumulation rate by benthic organisms could also significantly influence MeHg concentration in sediments by acting as a sink. Based on sediment assimilation parameters given in Jorgensen et al (1991), ingestion rates for the benthic species sampled at station C4 (Thouzeau et al., 2005) can be assessed. Ingestion rate of  $1.8 \text{ nmol m}^{-2} \text{ day}^{-1}$  has been found for MeHg but corresponds only to the quantity of MeHg transiting within benthic organisms and thus may overestimate MeHg trapped by biota. A more suitable uptake rate, representing the quantity of MeHg really assimilated by benthic organisms, can be evaluated for the major benthic specie (*Tapes aureus*, suspensive feeder) (Thouzeau et al., 2005). The calculation is based on the MeHg concentrations measured in the tissue ( $0.1 \text{ nmol g}^{-1}$ ) (Monperrus et al., 2004), and the biomass found at station C4 ( $3.53 \text{ g m}^{-2}$ ) and with the assumption that they accumulate for 1 year. The accumulation rate of MeHg was estimated to be  $0.001 \text{ nmol m}^{-2} \text{ day}^{-1}$ .

Demethylation rate in sediment has not been determined with this set of experiments but might be a very important process governing MeHg concentrations in sediments. In fact, considering all the estimated rates for sources and sinks of MeHg in the Thau lagoon, a mass balance allows the estimation of the demethylation in the same order of magnitude as the methylation rate.

| Potential transformation rates ( $\text{nmol.m}^{-2}.\text{day}^{-1}$ ) |            |       |
|-------------------------------------------------------------------------|------------|-------|
| Potential methylation rate in sediment                                  | measured   | 12    |
| Potential methylation rate in water                                     | measured   | 1.8   |
| Potential demethylation rate in water                                   | measured   | 0.4   |
| Exchange rates ( $\text{nmol.m}^{-2}.\text{day}^{-1}$ )                 |            |       |
| Benthic efflux rate*                                                    | measured   | 0.3*  |
| Burial rate                                                             | calculated | 0.04  |
| Benthic organisms uptake rate                                           | calculated | 0.001 |

**Table 4 :** Area integrated potential transformation and exchange rates ( $\text{nmol.m}^{-2}.\text{day}^{-1}$ ) of MeHg at the sediment water interface of station C4 in the Thau lagoon under spring condition.

#### 4. SUMMARY

Based on the results presented in this work, several conclusions can be drawn on the mercury methylation experiments in sediment and water column:

- Experimental approaches, using isotopically enriched Hg species, have been used for coastal sediment and water compartments. Excellent reproducibility and very low detection limits for transformations yields allow to provide key information on Hg transformations.
- Methylation in the water column of coastal environment, mediated by biotic processes, has been for the first time highlighted and appears to play a key role in the cycling of MeHg in the Thau Lagoon during spring period.
- Transformations of mercury in the water column and sediment are of primary importance when compared to water-sediment benthic fluxes.
- High accuracy and precision on transformation and transfer rates allow the possibility to upgrade mass balance approaches and to develop conceptual model of Hg cycling in aquatic environments.

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## **Chapitre C – III**

### **Méthylation, déméthylation et réduction du mercure dans des eaux de surface côtières et marines de la Méditerranée**

## Méthylation, déméthylation et réduction du mercure dans des eaux de surface côtières et marines de la Méditerranée

Les eaux de surface, et plus particulièrement la zone euphotique, représente un compartiment important pour le cycle biogéochimique du mercure en terme d'échanges avec l'atmosphère et de lieu privilégié pour les réactions de transformations des espèces chimiques du mercure. Ces eaux sont aussi le lieu privilégié pour le développement des populations bactériennes et phytoplanctoniques ainsi que pour le transfert trophique vers les organismes pélagiques supérieurs. Plusieurs études ont révélé des concentrations anormalement élevées en MMHg pour des poissons de la Méditerranée où les concentrations en mercure total dans l'eau sont généralement faibles voire identiques à d'autres océans. Différentes réponses à cette « anomalie » ont été proposées comme la formation importante de MMHg par les bactéries mais à l'heure actuelle les sources de MMHg en Méditerranée ne sont pas clairement définies.

Plusieurs mécanismes rentrent en jeu pour la quantité nette de MMHg disponible pour la bioaccumulation. Ces réactions (méthylation, déméthylation et réduction) sont régies par des facteurs biotiques ou abiotiques. Les réactions photochimiques représentent un potentiel important pour les eaux de surface ainsi que les réactions gouvernées par les activités bacterio- ou phyto-planctoniques. Néanmoins, peu de réponses sur l'existence et la proportion de chacun de ces mécanismes sont données pour des eaux de surface marines.

L'objectif de ce travail est de mettre au point des techniques d'expérimentations in situ à l'aide de traceurs isotopiques stables afin de déterminer les potentiels de méthylation, déméthylation et réduction du mercure ainsi que le transfert du MMHg vers le phytoplancton. Cette étude est réalisée pour des eaux de surface côtières et océaniques pour différentes saisons. La recherche des facteurs prépondérants influençant ces réactions a aussi été mise en œuvre en modifiant les paramètres d'incubations tels que la lumière et la présence de phytoplancton

Cette étude permet pour la première fois d'établir des taux de transformations des espèces de mercure dans les eaux de surface de la Méditerranée. L'utilisation de traceurs isotopiques permet la détection de très faibles taux de transformations même à des niveaux de concentrations très bas. La méthylation du Hg est fortement corrélée à l'activité des micro-organismes (bactérie et phytoplancton). Les réactions photochimiques semblent majoritairement gouverner la déméthylation et la réduction mais des processus biotiques secondaires existent. Ces résultats mettent en évidence la méthylation du IHg dans la zone euphotique ainsi qu'un transfert du MMHg vers le phytoplancton ce qui établit une nouvelle source potentielle de MMHg dans les environnements côtiers et marins.

## Mercury methylation, demethylation and reduction rates in coastal and marine surface waters of the Mediterranean Sea

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### ABSTRACT

In situ experiments using isotopically labeled mercury species (<sup>199</sup>Hg(II) and Me<sup>201</sup>Hg) were used to investigate mercury transformations mechanisms (methylation, demethylation, reduction) in coastal and marine surface waters of the Mediterranean Sea. We have assessed the relative contribution of photochemical versus biological processes to Hg transformation mechanisms as well as the mercury species uptake by micro-organisms. Coastal and marine euphotic zone appear as a significant reactor for Hg transformations. Hg methylation is mainly influence by pelagic micro-organisms (phytoplankton and bacterioplankton). This evidences a new potential MeHg source in the marine water column, in particular in oligotrophic deep sea basin in which biogeochemistry is mostly governed by heterotrophic activity. For coastal and marine surface waters, although MeHg is mainly photochemically degraded, demethylation yields observed under dark condition may be attributed to microbial demethylation. Photoreduction and photochemical reactions are the major mechanisms involved in DGM production for surface waters but bacterial or phytoplanktonic reduction of Hg(II) cannot be excluded. At the deep euphotic zone, photochemical processes are minimal due to the attenuation of sunlight radiations promoting the biotic processes. DGM production and demethylation mechanisms are thus probably reduced whereas Hg methylation is enhanced by autotrophic and heterotrophic processes. Investigations on mercury species uptake evidence the strong affinity of MeHg for micro-organisms which is of importance as they represent the first trophic level in the pelagic food web.

**KEYWORDS:**..mercury, methylation, demethylation, euphotic zone, biotic processe,Mediterranean Sea.

## 1- INTRODUCTION

Although very high mercury concentrations have been previously measured in pelagic fish, such as tuna (Storelli et al. 2002), only few investigations have been carried out to describe the level and the cycle of mercury in the Mediterranean Sea (Cossa et al. 1997, Pirrone et al. 2003, Horvat et al. 2003). Recent study (Storelli et al. 2003) show that nearly 80% of the albacore and 60% of the bluefin tuna muscle tissues exceed the maximum level for total mercury fixed by the European Commission Decision ( $0.5 \text{ mg.kg}^{-1}$  wet weight). An interesting feature of mercury biogeochemistry in the Mediterranean Sea is that several fish species from this area show higher methylmercury (MeHg) concentrations in their tissues than same fish species in the Atlantic Ocean (FAO 1986). In opposition, Hg species concentrations in the open waters of both oceans are similar or even higher in the Atlantic Ocean for total Hg (Mason and Sullivan 1999, Cossa et al. 1997).

These inconsistent results addressed questions about factors which govern the enrichment of mercury in Mediterranean fish. Anthropogenic influences from cinnabar deposits and volcanoes was considered but it has been suggested that the higher mercury levels noted in many larger pelagic fish species in the Mediterranean Sea are not related to anthropogenic inputs. Bacci (1989) suggested that the higher temperature of the deep Mediterranean water might promote the bacterial formation of MeHg in the water and sediment, which may result in higher mercury bioaccumulation in fish tissue. Cossa et al. (1994) have detected high levels of dimethylmercury ( $\text{Me}_2\text{Hg}$ ) in Mediterranean water at the oxygen depletion zone and attributed it to bacterial formation. Low oxygen concentration connecting with the particles accumulation zone may enhance the microbial activity. High ratios of methylated mercury forms compared to total mercury were also found in the water column of the Mediterranean Sea (Tessier et al. 2004). However, Hg measurements in the Mediterranean Sea at deep sea sites are not yet well documented, especially for speciation. Although results converge to mercury methylation mediated by bacteria and stimulated in the Mediterranean Sea by high water temperature, no proof of this high methylation potential was provided. The “Mediterranean mercury anomaly” is thus still unclear and further investigations are required to better understand the MeHg sources. Hg transformations in marine environment were generally extrapolated from natural concentrations with the assumption of a steady state (Mason et al. 1999, Cossa et al. 1997). No in situ experimental measurements have been performed to understand biogeochemical mechanisms of mercury in sea water.

Surface sea water, especially the euphotic zone, is an important compartment for the global Hg biogeochemical cycle. The air-water interface may favor Hg inputs and transfer to the atmosphere and intense oxido-reduction reactions (Mason et al. 2001). The euphotic layer represent also a key zone for the carbon cycling as maximal bacterial and phytoplanktonic production occurs in the surface layers of the Mediterranean Sea (Van Wambeke et al. 2000, Tanaka 2002). Repartition of bacterioplankton and phytoplankton in the Med Sea is mainly governed by climate changes and present variability with higher proportions of phytoplankton during

bloom periods and the predominance of heterotrophic bacteria during post bloom periods (Moran et al. 2001). These micro-organisms also represent the first trophic levels in the marine pelagic food web and may represent the main source for Hg bioaccumulation.

The net amount of biologically available MeHg is a function of the concentrations and processes that alter the bioavailability of MeHg or its precursor, divalent mercury. Its concentration in aquatic systems is controlled by formation, degradation and exchanges between compartments. Mercury methylation and demethylation are two important processes regulating the Hg cycle in aquatic environments (Compeau and Bartha 1984; Gilmour et al. 1992, Pak and Bartha 1998) and can be driven by both biological activities and abiotic mechanisms.

Potential Hg methylation takes place in the water compartment and can be induced by both chemical processes and biological activity of marine microorganisms. Bio-mediated pathways (i.e., biomethylation, bioredox reaction) have been proposed to play a significant role for mercury species transformations in sea water (Barkay et al., 2003). Organisms from diverse taxonomic groups as bacteria (King et al., 2000; Cleckner et al. 1999, Pongratz et al., 1999), macro algae (Pongratz et al., 1998) have been shown to methylate mercury in laboratories studies. The abiotic pathway for mercury methylation is possible in natural environment but appears to be of minor importance (Weber, 1993).

The fate of MeHg also depends on the demethylation process. MeHg degradation is thought to be predominantly microbially mediated in water (Barkay et al., 2003). Numerous micro-organisms are capable of demethylating MeHg including aerobic and anaerobic species but demethylation appears to be predominantly accomplished by aerobic organisms (Mason et al. 1995). Photocatalytic decomposition remains the only significant abiotic decomposition mechanisms in surface waters exposed to sunlight (Sellers et al., 1996)

At least, as inorganic mercury is the substrate for methylation, its reduction into elemental mercury and the sea to air exchanges are also important processes regulating MeHg levels in surface waters. The production of dissolved gaseous mercury (DGM) is mostly photomediated and surface sea water thus represents a propitious site for reduction and volatilization of mercury. Biological mercury reduction by bacteria has been also invoked in the Pacific Ocean (Mason and Fitzgerald 1993) and estuarine environments (Mason et al. 1995, Siciliano et al. 2002). Phototrophic organisms, such as phytoplankton, have also been found to reduce mercury (Poulain et al. 2004).

However in natural sea waters and more precisely in the Mediterranean waters, too scarce investigations have been performed to clearly understand the mercury methylation potential as well as chemical and biological pathways responsible for mercury species transformation. The purpose of the present article is to further investigate methylation, demethylation and reduction potentials of mercury in surface sea water of the Mediterranean Sea as well as the uptake of MeHg by phytoplankton. These transformations and transfer were evaluated both for coastal and marine surface waters for different seasons using isotopically enriched tracers. The significance and the reliability of the calculated transformations rates were evaluated taking into account the



natural mercury species concentrations. The in situ experimental results are discussed towards the relevance of major controlling factors such as sunlight radiations and biological activities.

## 2- EXPERIMENTAL SECTION

### 2-1- Study sites

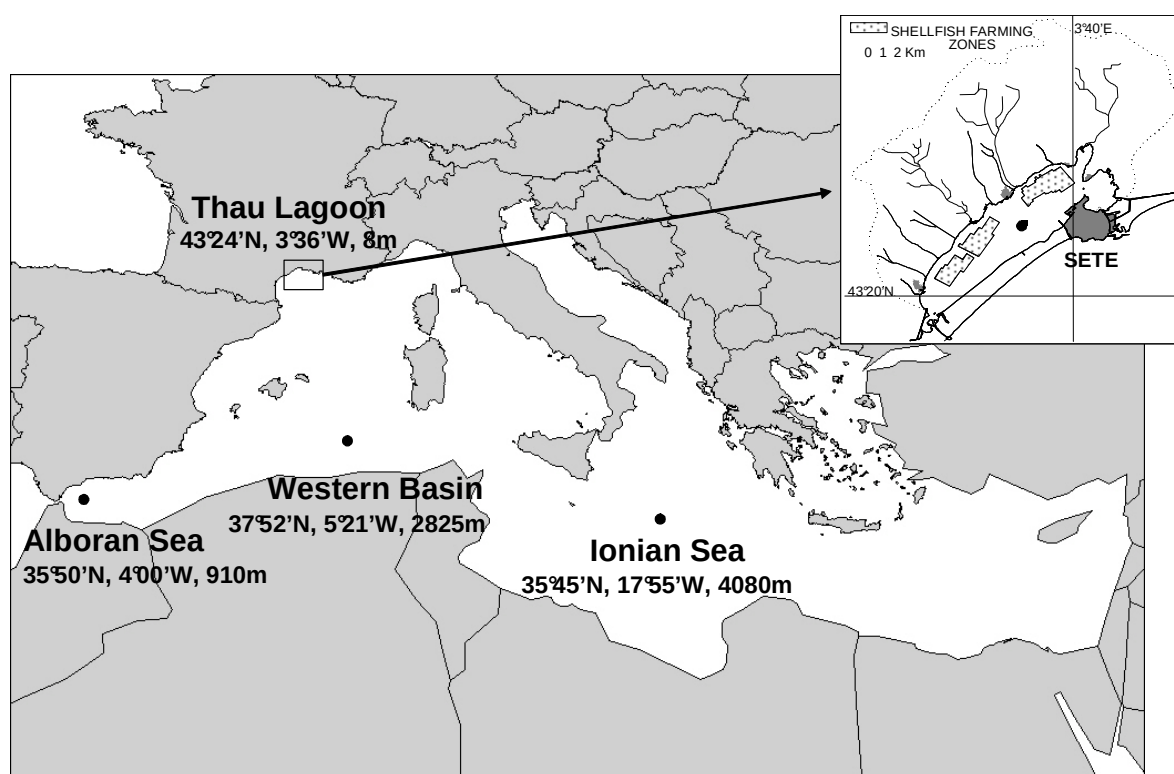
Water incubations were performed for surface water from a coastal sampling site and open sea sites of the Mediterranean Sea presented in Figure 1.

*Coastal site* – Coastal water samples were collected in the Thau Lagoon (South East, France). The Thau Lagoon located on the French Mediterranean coast (Gulf of Lion) is a shallow enclosed bay sheltered with two narrow sea mouths. The lagoon is of notable economic importance for the Languedoc region because of the large-scale shellfish farming (oyster and mussel production is around 15,000 t.y<sup>-1</sup>) which occupy up to 20% of this 75 km<sup>2</sup> water body. Surface water samples were collected in May and October 2003, January, April and July 2004 at a station located in the middle of the Lagoon (7.4m depth: 43°24'029N, 3°36'701E).

*Deep sea sites* – The Mediterranean Sea is an enclosed basin connected to the Atlantic Ocean by the narrow strait of Gibraltar and connected to the Black Sea by the Dardanelles/Marmara Sea/Bosphorus system. It is made up of two sub-basins, the western and the eastern Mediterranean connected by the Strait of Sicily. The circulation and hydrography of the Mediterranean Sea waters are driven by the net fresh water loss and heat loss to the atmosphere and the exchange of salinity and heat through the Strait of Gibraltar. Water incubations were performed for South Western basin (SWB), the Ionian Sea (IS) and the Alboran Sea (AS) during two oceanographic cruises on the Urania research vessel (CNR, Italy) during August 2003 and March 2004. The station located in the South Western basin (37°52'N, 5°21'W) is 2825 m depth and is an oligotrophic area of the Mediterranean Sea. The Ionian Sea is one of the most oligotrophic zones of the Mediterranean Sea and the sampling station was 4080 m depth (35°45'N, 17°55'W). The Alboran Sea (nearby the Gibraltar strait) is also characterised by a high primary production and the sampling station was 910 m depth (35°50'N, 4°00'W).

### 2-2- Sampling and analysis of aqueous and gaseous species

*Sampling* – Coastal surface water was collected at the sub-surface (0.5 m) using 5L Teflon coated Go-Flo sampler (General Oceanic, Miami). Marine water samples were collected using a stainless steel rosette equipped with 24 Niskin samplers and CTD probes providing a continuous monitoring of hydrological parameters (Sea-Bird Electronics Inc, USA). Sampling depths for marine waters were chosen in the photic zone at the maximum of chlorophyll (SWB: 60m in August, 65m in March, IS: 85m in August and March, AS: 60m in August). The sample was transferred directly to previously acid cleaned PFA bottles (Nalgene, USA) and submitted to incubation experiments. Niskin samplers were checked for contamination by intercomparison with Teflon coated Niskin.



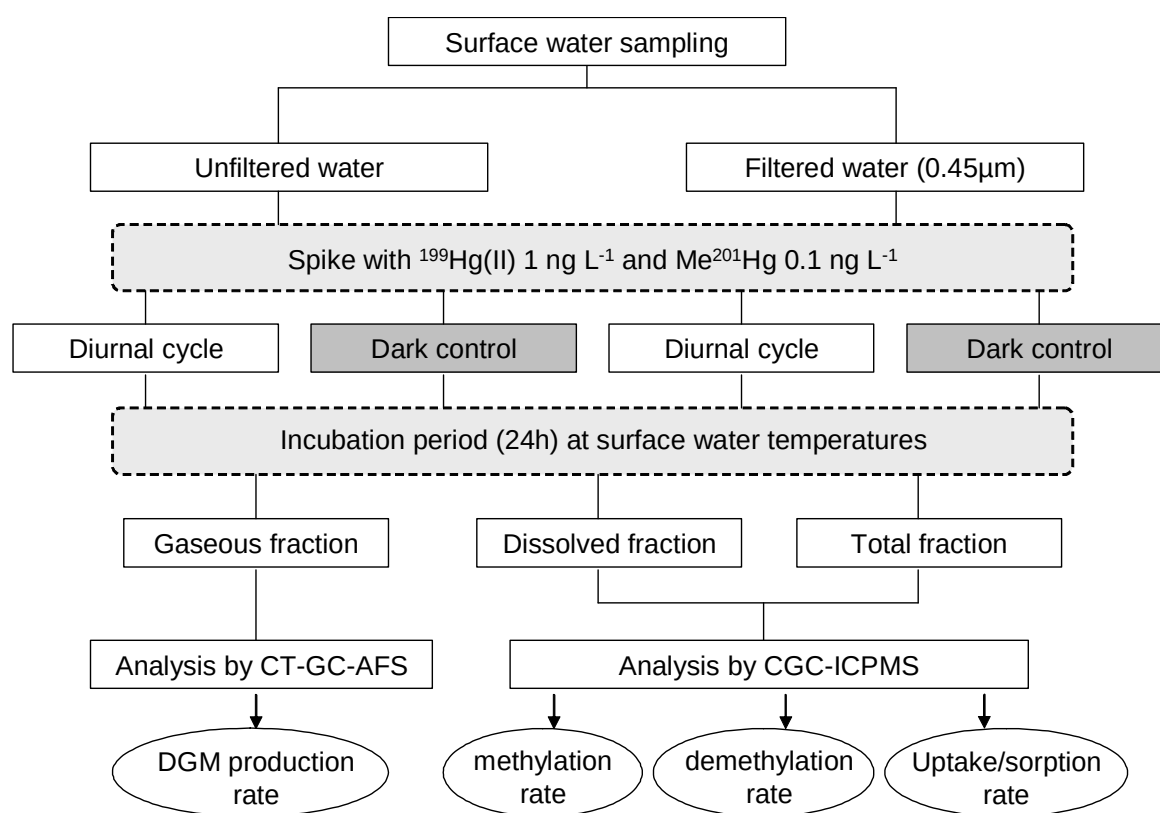
**Figure 1:** Sampling stations location in the Mediterranean Sea (2003-2004)

*Analysis of MeHg and Hg(II)* - Mercury speciation analysis of the water samples were performed using propylation GC-ICPMS previously described by [Monperrus et al. \(2004a\)](#). Briefly, concentrated ammonium hydroxide and 5 ml of acetic acid / sodium acetate buffer (0.1M) are added to 100 ml of the water sample to set the pH at 4. 0.2 ml of iso octane and 1 ml of 0.5% (w/v) sodium tetrapropylborate are added. The flask is immediately capped and vigorously hand shaken for 5 min. The organic phase is finally transferred to an injection vial and stored at  $-18^{\circ}\text{C}$  until measurement. GC-ICPMS parameters have been described elsewhere ([Monperrus et al. 2004a](#)). Isotopes ratios are always measured as peak area ratios and corrected by mass bias factor. Blanks were also determined to check for any contamination. Determination of the concentrations of each mercury species for each isotope (199, 201, 202) is carried out to evaluate methylation and demethylation yields.

*Analysis of gaseous species* - Measurements of gaseous Hg species ( $\text{Hg}^{\circ}$  and  $\text{Me}_2\text{Hg}$ ) were carried out in the field by cryofocussing technique hyphenated to atomic fluorescence spectrometer (AFS). Samples were thoroughly transferred to a purging vessel under Ar atmosphere, subsequently purged under He flow ( $15\text{ min}$ ,  $0.3\text{ l.min}^{-1}$ ) and analyzed with cryogenic trapping gas chromatography and quartz furnace AFS detection (CT-GC-AFS) ([Stoichev et al. 2003](#)). For the coastal station experiments, DGM measurements were performed with gold coated sand traps. DGM concentrations represent the sum of elemental mercury ( $\text{Hg}^{\circ}$ ) and dimethylmercury ( $\text{Me}_2\text{Hg}$ ).

### 2-3- Incubation experiments

Incubation experiments were performed within 2 hours after the sampling. Experimental design for water incubations has been described in a previous work (Monperrus et al., 2004b) and is detailed in figure 2. Briefly, solutions of  $^{199}\text{HgCl}_2$  and  $\text{Me}^{201}\text{HgCl}$  were added to the bulk samples in order to obtain initial concentrations in the range of 1 to 2  $\text{ng l}^{-1}$  and 0.1 to 0.2  $\text{ng l}^{-1}$  respectively. Incubations were performed in triplicate and incubated directly in the Thau lagoon shore waters (depth < 0.5m) or in temperate sea water baths on board flowed by pumped surface sea water, over a complete diurnal cycle (24 hours dawn to dawn), either exposed to sunlight or in the dark. Additionally, control assays were performed on filtered water samples spiked with the same amount of enriched stable isotope mercury species. To remove suspended matter/plankton, water was filtered through a  $0.45\mu\text{m}$  PVDF membrane (Millipore). At the end of the incubation period, aliquots of incubated bulk water samples were filtered in order to achieve mercury species partitioning between dissolved and particulate phases. All incubations were stopped by adding high purity HCl (1% v/v) and stored at  $+4^\circ\text{C}$  in the dark until analysis. Analyses for gaseous species were performed directly on site and analyses for MeHg and Hg(II) were performed within one month after field sampling and incubation experiments.



**Figure 2:** Flow chart of the experimental procedure for water incubations.

## 2-4- Methylation, demethylation and gaseous production rates

Gross rates of mercury methylation/demethylation were determined by adding trace quantities of isotopically enriched  $^{199}\text{HgCl}_2$  and  $\text{Me}^{201}\text{HgCl}$  to water incubation bottles. The amount of methylated mercury deriving from the enriched isotope 199 during the incubation can be calculated by using the equation described previously by [Hintelmann et al. \(1997\)](#). The amount of demethylated methylmercury from enriched spike is calculated in a similar way following the 201 isotope ([Monperrus et al. 2004b](#)).

Methylation rate is calculated by dividing the amount of  $\text{Me}^{199}\text{Hg}$  formed by the  $^{199}\text{Hg(II)}$  added and the incubation time. Demethylation yield is calculated by dividing the  $^{201}\text{Hg(II)}$  formed by the  $\text{Me}^{201}\text{Hg}$  added and the incubation time. Formation of dissolved gaseous species rate is calculated by dividing the sum of gaseous mercury species formed ( $\text{Hg}^\circ + \text{Me}_2\text{Hg}$ ) by the total mercury added ( $\text{Me}^{201}\text{Hg} + ^{199}\text{Hg(II)}$ ). Gaseous mercury species formed are deduced from measured concentrations after incubation of spiked sample and natural concentrations after incubation of non spiked sample.

Detection limits of the transformations rates were evaluated according equations given by [Hintelmann et al. 1997](#) and were previously described ([Monperrus et al. 2004b](#)). They were found to be 0.02, 3 and 0.5 %  $\text{d}^{-1}$  for the methylation, demethylation and reduction rates respectively. These very low detection limits allow us to determine with confidence transformation processes using this experimental procedure.

## 2-5- Biogeochemical parameters

*2-5-1- Irradiance, temperature, salinity, Chlorophyll, oxygen* – For marine samples, CTD multiparameters probes sampler provided a continuous measurements of temperature, salinity, oxygen and chlorophyll (Sea-Bird Electronics Inc, USA). For coastal water, temperature and salinity were measured on site using a temperature/conductivity probe (WTW). Irradiance measurements were measured for coastal and marine water incubations for the incubation period, using cumulative global radiation (weather station).

*2-5-2- Phytoplankton speciation* – The method for phytoplankton enumeration is fully described by [Utermöhl \(1931\)](#). Samples for phytoplankton species composition were preserved with an acid lugol solution (1%) and stored in the dark at 4°C until analysis. Species were identified and counted by microscopic examination on an inverted microscope ([Utermöhl, 1931](#)). Only qualitative data were validated for this study, providing the relative abundance of the different plankton species.

### 3- RESULTS

#### 3-1- Biogeochemical characteristics and natural mercury species concentrations of incubated samples

Hydrological parameters and natural mercury species concentrations of the incubated samples from both coastal and marine stations are presented in Table 1. Coastal and marine waters present completely different hydrological characteristics such as salinity and phytoplankton abundance and taxonomic groups.

Surface waters from the Thau Lagoon present high biomass density and can be considered homogeneous with the water column for such shallow system. Samples from the different season exhibit variations of salinity comprised between 34.4 and 41.4 and are characterized by an important presence of phytoplankton cells. Chlorophyll a concentrations range between 1.25 to 2.72  $\mu\text{g l}^{-1}$  during our experiment but can reach much more higher values during phytoplankton bloom events. In May 2003, phytoplankton species distribution in the collected sample was dominated by the presence of chryptophyceae (35%), dinoflagelates (22%), nanoplankton (20%), and diatoms (20%) (see Table 2).

|                    | Hydrological parameters |        |          |                                |                                  | MeHg                        |                          | Hg(II)                      |                          | DGM                         | Me <sub>2</sub> Hg          |
|--------------------|-------------------------|--------|----------|--------------------------------|----------------------------------|-----------------------------|--------------------------|-----------------------------|--------------------------|-----------------------------|-----------------------------|
|                    | Depth (m)               | T (°C) | Salinity | Chl a ( $\mu\text{g l}^{-1}$ ) | Radiation ( $\text{J cm}^{-2}$ ) | Conc ( $\text{ng l}^{-1}$ ) | Particulate fraction (%) | Conc ( $\text{ng l}^{-1}$ ) | Particulate fraction (%) | Conc ( $\text{ng l}^{-1}$ ) | Conc ( $\text{ng l}^{-1}$ ) |
| Thau-May 03        | 0.5                     | 17.1   | 34.4     | 1.25*                          |                                  | 0.107                       | 48                       | 0.82                        | 37                       | ND**                        | ND                          |
| Thau-Oct 03        | 0.5                     | 14.2   | 35.2     | 2.72*                          | 1351                             | 0.081                       | 32                       | 0.66                        | 39                       | 0.077                       | ND                          |
| Thau-Jan 04        | 0.5                     | 8.5    | 37.4     |                                | 690                              | 0.064                       | 26                       | 0.64                        | 19                       | 0.017                       | ND                          |
| Thau-Apr 04        | 0.5                     | 15.6   | 34.7     |                                | 2054                             | 0.075                       | 32                       | 0.89                        | 26                       | 0.019                       | ND                          |
| Thau-Jul 04        | 0.5                     | 24.4   | 41.4     |                                | 2824                             | 0.036                       | 33                       | 0.39                        | 26                       | 0.116                       | ND                          |
| Western S-Aug 03   | 60                      | 15.2   | 37.4     | 0.22                           | 1804                             | 0.072                       | 16                       | 0.28                        | 46                       | 0.055                       | 0.005                       |
| Western S-Mar 04   | 65                      | 14.0   | 37.8     | 1.19                           | 674                              | 0.040                       | ND                       | 0.15                        | ND                       | 0.021                       | 0.004                       |
| Ionian Sea-Aug 03  | 85                      | 15.3   | 38.5     | 0.06                           | 2939                             | 0.135                       | 30                       | 0.34                        | 9                        | 0.084                       | 0.020                       |
| Ionian Sea-Mar 04  | 85                      | 14.9   | 38.7     | 0.94                           | 738                              | 0.133                       | ND                       | 0.18                        | ND                       | 0.052                       | 0.013                       |
| Alboran Sea-Aug 03 | 60                      | 18.3   | 36.3     | 0.09                           | 1794                             | 0.043                       | 14                       | 0.58                        | 7                        | 0.043                       | 0.006                       |

\*Indicative value from Rephy (Ifremer), \*\*Not determined, DGM=Hg<sup>0</sup>+Me<sub>2</sub>Hg

**Table 1:** Hydrological parameters and natural mercury species concentrations for coastal and marine sites.

Conversely marine waters are investigated as much more oligotrophic systems with lower biomass and primary production. The waters for incubations experiments were sampled in the euphotic layer at the deep chlorophyll maximum. As expected, chlorophyll a concentrations are always found higher in March (0.94-1.19  $\mu\text{g l}^{-1}$ ) compared to August (0.06-0.22  $\mu\text{g l}^{-1}$ ). Proportions of phytoplankton taxonomic groups are similar for the different

marine stations with the predominance of nanoplankton, dinoflagellates, chryptophyceae and diatoms. The proportion of the nanoplankton, which comprised heterotrophic and autotrophic species, is higher during August compared to March for all the investigated stations. In general, eutrophic waters are characterized by a domination of primary producers biomass while the characteristic of oligotrophic systems is the dominance of bacterial biomass over phytoplankton biomass (Cho et Azam 1990). This is in accordance with previous investigations on microbial and phytoplanktonic production in the Mediterranean Sea (Van Wambeke et al. 2000, Moran et al. 2001, Tanaka et Rassoulzadegan 2002). Higher phytoplanktonic biomass in March is related to spring bloom period whereas lower values in August correspond to a post-bloom period. According to Van Wambeke et al. (2000), the microbial production is preponderant compared to primary production for the post-bloom periods. Moran et al. (2001) demonstrated that in the south western basin, nanoplankton is dominated by the abundance of heterotrophic bacteria with one order of magnitude higher than autotrophic organisms.

| Station             | Thau Lagoon | Western Basin |          | Ionian Sea |          | Alboran Sea |
|---------------------|-------------|---------------|----------|------------|----------|-------------|
| Date                | May 2003    | Aug 2003      | Mar 2004 | Aug 2003   | Mar 2004 | Aug 2003    |
| Diatoms (%)         | 20          | 19            | 16       | 12         | 47       | 2           |
| Dinoflagellates     | 22          | 19            | 32       | 28         | 49       | 36          |
| Cryptophyceae       | 35          | 7             | 11       | 24         | 3        | 23          |
| Nano (unidentified) | 20          | 55            | 35       | 34         | 9        | 37          |
| Ciliates            | 3           | 0             | 2        | 2          | 1        | 1           |
| Phaeocystis         | 0           | 0             | 5        | 0          | 0        | 0           |
| Dictyocha           | 0           | 0             | 0        | 0          | 0        | 0           |

**Table 2:** Relative abundances (%) of phytoplankton species for coastal and marine water samples.

Mercury species concentrations exhibit also different patterns for coastal and marine waters with lower total mercury concentrations and lower Hg particulate fractions for the marine waters. For coastal station, surface waters were sampled for different periods and mercury species concentrations vary with seasons. For MeHg and Hg(II), higher concentrations are generally found during spring and fall and lower concentrations are found during July. MeHg concentrations range from 0.036 to 0.107 ng l<sup>-1</sup> and IHg concentrations from 0.39 to 0.89 ng l<sup>-1</sup>. MeHg proportion compared to total mercury is generally constant ranging from 9 to 11%. Fraction of MeHg associated to particulate matter range from 26 to 48% whereas fraction of Hg(II) associated to particles is in the range of 19-39%. Lower proportions in the solid phase are found lower during January certainly due to lower phytoplankton abundance in winter.

Hg species concentrations of surface waters are relatively low, as has been found in other coastal bays (Mason et al. 1999, Bloom et al. 2004). In the Chesapeake Bay, Mason et al. (1999) have also demonstrated seasonal

variations with higher MeHg concentrations for surface waters in February compared to August. MeHg production in sediments by microbial activities is likely to be strongly dependent on temperature (Monperrus et al. 2004b). For such shallow system, benthic fluxes from the sediment to the water column are possible and thus may generate strong seasonal gradients in surface water (Point et al. 2004, Choe et al. 2004).

For gaseous species, only DGM were measured but Me<sub>2</sub>Hg is generally below the detection limit for coastal water (Coquery et al. 1997). Seasonal variations are observed for DGM with higher values during warm seasons which are generally reported for coastal surface waters (Baeyens and Leemarkers 1998).

Marine waters exhibit different patterns of mercury species concentrations compared to coastal waters with lower total mercury concentrations but higher MeHg proportions except for the Alboran Sea where concentrations and MeHg proportion are in the same range than for coastal water. Hg species concentrations for the whole water column of the investigated stations (Tessier et al. 2004), have showed homogenous concentrations of total Hg for the euphotic zone and MeHg concentration maxima at the deep chlorophyll maxima. Maxima of Hg<sup>0</sup> concentrations were generally found in the top of the euphotic zone whereas the bottom corresponded to the apparition of Me<sub>2</sub>Hg (Tessier et al., in prep.).

At the water depth used for our experiments, Hg(II) concentrations range from 0.15 to 0.58 ng l<sup>-1</sup> with lower values in spring season, for the deep basins (South Western basin and Ionian Sea). Conversely MeHg concentrations show high values comprised between 0.040 and 0.135 ng l<sup>-1</sup> and MeHg represent a very important part of total mercury with proportions ranging from 20 to 42% of the total mercury. The most oligotrophic station (Ionian Sea) exhibits the higher MeHg concentrations for both season as well as the higher MeHg proportion. These concentrations are similar to previously measured values in the Mediterranean Sea (Cossa et al. 1997, Horvat et al. 2003). Comparison of these data with those obtained in surface waters of other oceanic environments show a similarity with those obtained in the Pacific Ocean (Mason and Fitzgerald 1991) and in the Atlantic Ocean (Mason and Sullivan 1999).

For gaseous species, both Hg<sup>0</sup> and Me<sub>2</sub>Hg have been detected. DGM concentrations range from 0.021 to 0.084 ng l<sup>-1</sup> and Me<sub>2</sub>Hg from 0.004 to 0.020 ng l<sup>-1</sup> which is in the range of previous work on Mediterranean Sea water (Cossa et al. 1994). Both for DGM and Me<sub>2</sub>Hg, concentrations are lower in March 2003 than in August 2003. DGM concentration ranges are in a good agreement with results previously find in the Mediterranean Sea (Horvat et al. 2003) and in the western basin (Cossa et al. 1997)

### 3-2 Transformations rates for coastal and marine waters

The different calculated rates for methylation, demethylation and DGM production at different seasons and for coastal and marine waters are displayed in Table 3. These rates are given for incubation of unfiltered and filtered water for both diurnal incubation and dark control.



|                    | Unfiltered water          |                           |                           |                           |                           |                           | Filtered water            |                           |                           |                           |                           |                           |
|--------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
|                    | Diurnal incubation        |                           |                           | Dark control              |                           |                           | Diurnal incubation        |                           |                           | Dark control              |                           |                           |
|                    | M<br>(% d <sup>-1</sup> ) | D<br>(% d <sup>-1</sup> ) | G<br>(% d <sup>-1</sup> ) | M<br>(% d <sup>-1</sup> ) | D<br>(% d <sup>-1</sup> ) | G<br>(% d <sup>-1</sup> ) | M<br>(% d <sup>-1</sup> ) | D<br>(% d <sup>-1</sup> ) | G<br>(% d <sup>-1</sup> ) | M<br>(% d <sup>-1</sup> ) | D<br>(% d <sup>-1</sup> ) | G<br>(% d <sup>-1</sup> ) |
| Thau-May 03        | 6.3                       | 12.8                      |                           | 1.8                       | 9.6                       |                           | 1.2                       | 9.0                       |                           | 0.7                       | 2.5                       |                           |
| Thau-Oct 03        | 0.9                       | 24.5                      | 9.1                       | 3.1                       | 3.8                       | 2.4                       | 0.2                       | 13.9                      |                           | 0.4                       | 8.1                       |                           |
| Thau-Jan 04        | 0.8                       | 16.4                      | 7.9                       | 1.4                       | <1.5                      | 5.2                       | 0.4                       | 11.3                      |                           | 0.9                       | <1.5                      |                           |
| Thau-Apr 04        | 3.0                       | 17.9                      | 16.9                      | 1.2                       | 10.9                      | 9.1                       | 1.5                       | 5.0                       | 12.7                      | 0.6                       | 8.8                       | 8.3                       |
| Thau-Jul 04        | 2.7                       | 13.5                      | 16.8                      | 2.0                       | 5.4                       | 12.3                      | 1.7                       | 12.6                      | 16.3                      | 1.3                       | 1.4                       | 11.4                      |
| Western B-Aug 03   | 0.5                       | 17.1                      | 4.3                       | 1.1                       | 8.0                       | 3.0                       |                           |                           |                           |                           |                           |                           |
| Western D-Mar 04   | 0.4                       | 12.9                      | 3.2                       | 0.4                       | 6.4                       | 2.2                       | <0.02                     | 5.4                       | 1.1                       | <0.02                     | 6.4                       | 1.0                       |
| Ionian Sea-Aug 03  | 0.3                       | 19.1                      | 15.2                      | 0.6                       | 2.8                       | 7.9                       |                           |                           |                           |                           |                           |                           |
| Ionian Sea-Mar 04  | <0.02                     | 6.4                       | 4.1                       | <0.02                     | 3.3                       | 2.7                       | <0.02                     | 3.3                       | 2.4                       | <0.02                     | 6.4                       | 2.4                       |
| Alboran Sea-Aug 03 | 3.0                       | 15.4                      | 10.8                      | 3.8                       | 8.4                       | 5.6                       |                           |                           |                           |                           |                           |                           |

M= methylation rate, D= demethylation rate, G= DGM production rate

**Table 3:** Methylation, demethylation and DGM production for coastal and marine waters.

### 3-2-1- Methylation rates

Methylation rates ranged from 0 to 6.3 % d<sup>-1</sup> for all the investigated stations. Surface waters of the coastal station exhibited higher methylation potentials (0.8-6.3% d<sup>-1</sup>) than marine stations (0-0.5% d<sup>-1</sup>) whatever the season except for the surface water from the Alboran Sea where 3 % d<sup>-1</sup> of methylation rate was found.

The results on the methylation rates showed a strong dependence with seasonal conditions, especially for the coastal station where lower methylation rates were observed for the incubations performed in October 2003 and January 2004. Very high methylation rate was observed in May 2003 with 6.3% d<sup>-1</sup>. For the marine surface waters, slightly lower values were found during March compared to August for the western basin and the Ionian Sea. No Hg methylation was observed for the surface water from the Ionian Sea in March in conjunction with a paucity of nanoplankton.

Considering the results obtained for the dark controls, methylation rates showed a strong relationship with the occurrence of sunlight radiations. Methylation rates under dark conditions were similar or higher than thus found for diurnal incubation except for the Thau lagoon during warmer seasons (May, April and July). For these seasonal coastal conditions, exhibiting high temperatures, important cumulative radiations and higher abundance of phytoplankton, the high methylation rates were systematically lower under dark condition than those found under light condition.

The methylation rates, determined for incubations of filtered water, demonstrated lower values compared to those determined for incubations of bulk water in all cases. Incubations without particles lead to methylation yields ranging from 0 to 1.7 % d<sup>-1</sup> with similar values for the dark controls of filtered water (0-1.3 % d<sup>-1</sup>).

### 3-2-2- Demethylation rates

The demethylation rates determined for coastal and marine waters were in the same range (6.4-24.5 % d<sup>-1</sup>). Seasonal variations of the intensity of MeHg demethylation was observed for the surface waters from the marine stations. The rates were systematically higher during August compared to the rates found during March. For coastal station, no trend was observed between demethylation rates and seasons.

Under dark conditions, the demethylation rates were found systematically lower compared to the results found for diurnal incubation with values ranging from 0 to 10.9 % d<sup>-1</sup>. An important fraction of the MeHg demethylation was thus directly controlled by light induced processes, i.e. photochemical reactions or phytoplankton activities.

Removing the particles from the water sample before incubation, lead to lower demethylation rates demonstrated both for coastal and marine stations. These results suggest a partial involvement of micro-organisms associated to suspended matter (i.e. bacterio- and phyto-plankton) for MeHg demethylation. The contribution of photosensitive micro-organisms might be greater since dark conditions inhibited demethylation processes.

### 3-2-3- DGM production rates

With regards to DGM production, rates were in the same range for coastal or marine waters incubations with values ranging between 4.1 and 16.9 % d<sup>-1</sup>. The results demonstrated a strong dependence to the seasons with relationships with both temperatures and cumulative radiations. Higher DGM production rates were found during summer (July-August) whereas lower rates were found during winter (January-March). The results for dark control incubations demonstrated the important contribution of sunlight radiations in DGM formation with lower rates in all cases. However significant demethylation rates were observed under dark conditions suggesting a contribution of others processes not photolytically induced.

The presence of particulate matter in the water did not appear as a major factor in DGM production as DGM production rates for filtered waters were similar to those found with bulk waters both for light and dark conditions.

DGM production includes the formation of both Hg<sup>0</sup> and Me<sub>2</sub>Hg and using cryogenic trapping for marine waters has allowed to determine the formation of both gaseous Hg species. Table 4 presents the formation rates of Hg<sup>0</sup> and Me<sub>2</sub>Hg for incubations of marine surface waters for the 2 investigated seasons and under the different incubation conditions.

Considering these results, it is possible to assess the significant formation of Me<sub>2</sub>Hg during these water incubations. The Me<sub>2</sub>Hg formation rates were relatively low during diurnal incubations with values ranging from 0 to 0.4 % d<sup>-1</sup>. The Me<sub>2</sub>Hg formation was enhanced under dark conditions with rates ranging from 0.5 to 1.5 % d<sup>-1</sup>.

Similar results were found for incubations of filtered waters with higher rates under dark conditions. This suggests that Me<sub>2</sub>Hg formation is mainly controlled by abiotic reactions enhanced under dark conditions.

|                   | Unfiltered water                                     |                                                         |                                                      |                                                         | Filtered water                                       |                                                         |                                                      |                                                         |
|-------------------|------------------------------------------------------|---------------------------------------------------------|------------------------------------------------------|---------------------------------------------------------|------------------------------------------------------|---------------------------------------------------------|------------------------------------------------------|---------------------------------------------------------|
|                   | Diurnal incubation                                   |                                                         | Dark control                                         |                                                         | Diurnal incubation                                   |                                                         | Dark control                                         |                                                         |
|                   | Hg <sup>0</sup><br>formation<br>(% d <sup>-1</sup> ) | Me <sub>2</sub> Hg<br>formation<br>(% d <sup>-1</sup> ) | Hg <sup>0</sup><br>formation<br>(% d <sup>-1</sup> ) | Me <sub>2</sub> Hg<br>formation<br>(% d <sup>-1</sup> ) | Hg <sup>0</sup><br>formation<br>(% d <sup>-1</sup> ) | Me <sub>2</sub> Hg<br>formation<br>(% d <sup>-1</sup> ) | Hg <sup>0</sup><br>formation<br>(% d <sup>-1</sup> ) | Me <sub>2</sub> Hg<br>formation<br>(% d <sup>-1</sup> ) |
| Western B-Aug 03  | 4.1                                                  | 0.24                                                    | 2.7                                                  | 0.32                                                    |                                                      |                                                         |                                                      |                                                         |
| Western B-Mar 04  | 2.9                                                  | 0.31                                                    | 1.5                                                  | 0.70                                                    | 0.8                                                  | 0.30                                                    | 0.2                                                  | 0.76                                                    |
| Ionian Sea-Aug 03 | 14.8                                                 | 0.40                                                    | 6.4                                                  | 1.50                                                    |                                                      |                                                         |                                                      |                                                         |
| Ionian Sea-Mar 04 | 4.1                                                  | 0                                                       | 2.2                                                  | 0.50                                                    | 2.4                                                  | 0                                                       | 2.4                                                  | 0                                                       |

**Table 4:** Production of dissolved gaseous mercury species in marine waters

### 3-3- Uptake/sorption rates of MeHg and Hg(II)

Suspended matter play an important role in the transport and the reactivity of Hg(II) and MeHg in aquatic systems. Particulate Hg consists of Hg bound to mineral particles and detritic organic matter and Hg assimilation by biogenic particles such as bacterial aggregates, algae or phytoplankton. Hg species partitions between dissolved and particulate fractions were determined for the investigated waters. Results of uptake/sorption rates for MeHg and Hg(II) are presented in table 5 for diurnal incubations and dark controls.

| Uptake/sorption (%) | MeHg               |              | Hg(II)             |              |
|---------------------|--------------------|--------------|--------------------|--------------|
|                     | Diurnal incubation | Dark control | Diurnal incubation | Dark control |
| Thau-May 03         | 36                 | 58           | 5                  | 13           |
| Thau-Oct 03         | 40                 | 41           | 15                 | 25           |
| Thau-Jan 04         | 37                 | 53           | 8                  | 24           |
| Thau-Apr 04         | 40                 | 66           | 8                  | 15           |
| Thau-Jul 04         | 34                 | 55           | 20                 | 22           |
| Western S-Aug 03    | 17                 | 25           | 18                 | 27           |
| Western S-Mar 04    | 5                  | 4            | 4                  | 4            |
| Ionian Sea-Aug 03   | 23                 | 28           | 4                  | 6            |
| Ionian Sea-Mar 04   | 44                 | 48           | 48                 | 50           |
| Alboran Sea-Aug 03  | 9                  | 11           | 9                  | 15           |

**Table 5:** Uptake/sorption rates for MeHg and Hg(II) for coastal and marine waters.

First, the results demonstrated systematic higher rates for water incubations under dark conditions compared to diurnal incubations for both species and for all the investigated stations. Second, mercury species partitions demonstrated different results for the coastal water compared to the marine waters. Particulate fractions for both MeHg and Hg(II) were systematically lower for the marine surface waters except for the water from the Alboran Sea.

For the coastal waters, the partitions observed for MeHg differed from the Hg(II) partitions. MeHg was generally more associated to suspended matter (uptake/sorption rates ranging from 34 to 40 %) compared to Hg(II) with particulate fraction ranging from 5 to 20%.

## 4- DISCUSSION

### 4-1- Controlling factors for mercury transformations and uptake/sorption

Methylation/demethylation and uptake mechanisms of Hg species in surface sea waters are known to be important factors in regulating the fate of MeHg and its bioavailability for biota and bioaccumulation along the marine food web. Mercury methylation, demethylation and reduction could be both biotically and abiotically mediated but most studies provide only a partial comprehension of the mechanisms involved. Using our experimental assay with light and dark incubations of unfiltered and filtered waters, we test the hypothesis that Hg methylation, demethylation and reduction mechanisms in surface waters are controlled by photochemical and/or biotic processes

#### 4-1-1- Methylation

In the light of our results, MeHg formation rates seem to be mainly influenced by the water temperature and by the presence of nanoplankton both for coastal and marine surface waters. This result indicates a significant proportion of the major contribution of biotic processes for Hg methylation. For coastal surface waters, warm periods are more conducive for Hg methylation with the presence of sunlight radiations. This high MeHg production can be associated to primary production during bloom period. Both phytoplanktonic photobiological metabolism (autotroph) and bacterial activities associated with phytoplankton production (heterotroph) are thus suggested to be linked with Hg methylation. For surface marine waters, higher Hg methylation rates obtained in August and under dark condition suggest that main mechanisms are mediated by heterotrophic activity. Indeed, during post bloom period, heterotrophic production is dominant in the euphotic zone of oligotrophic areas of the Mediterranean Sea ([Moran et al. 2001](#)). The higher relative abundance of nanoplankton, which include probably

more heterotrophic than autotrophic organisms, during August, corroborates a microbial Hg methylation mechanism in the euphotic layer.

Nor experimental result for Hg methylation in the water column of coastal and marine systems has been already established neither the influence on Hg methylation of micro-organisms living in the water column such as bacterio- and phyto-plankton. However several authors suggest that the low oxygen zones in the marine waters are most conducive to the formation of MeHg and Me<sub>2</sub>Hg (Mason and Fitzgerald 1991, Cossa et al. 1994). These zones correspond to significant microbial activity which corroborates with a biotic methylation mediated by bacteria. It is well known that microbial mercury methylation occurs in a variety of marine, estuarine and lacustrine sediments (King et al., 2000, Macalady et al., 2000). Several previous studies have indicated that sulfate reducing bacteria are the primary mercury methylators in freshwater and estuarine sediments (Gilmour et al., 1992; Benoit et al., 2001; Pak and Bartha., 1998; Macalady et al., 2000). However, as yet, few investigations have assessed the interaction between micro-organisms living the water column of marine environments and Hg methylation especially in the marine euphotic zone.

However Hg methylation occurs, to a lesser extent, in filtered water both for diurnal incubations and dark controls. Dimethylation occurs also in filtered water with higher rates under dark conditions. These conditions (absence of particles) appear also generate a higher Hg methylation rates during warm periods characterized by high primary production with certainly an important release of biogenic exudates to the dissolved fraction.

This part of Hg methylation might be caused by abiotic mechanisms, by the action of biogenic substances or by filter passing bacteria. Parkam et al. (1994) have proposed that the activity of exo-enzymes, present in filtered and unfiltered water, may also catalyze the formation of MeHg. Abiotic biogenic products of active metabolism are known to methylate Hg such as methylamine (e.g. methylcobalamine) and halogenated organic compounds (e.g. iodomethane) (Craig 1986, Weber 1993). Other abiotic processes have been proposed for Hg methylation, e.g. methylation catalyzed by metals ions (Fe<sup>2+</sup>, Fe<sup>3+</sup>) in the presence of humic substances (Lee et al. 1985, Gardfeldt et al. 2003).

Finally, microbial activities may also have a significant role for the methylation and dimethylation in filtered water as some bacteria may pass through the filter (0.45µm). With investigations in the Western Mediterranean Sea, Haller et al. (1999) have demonstrated that the amount of 0.2µm filterable bacteria varies from 4 to 11% of the total bacterial number in such oligotrophic systems. So a microbial mediated methylation can not be excluded for the filtered surface sea waters.

#### 4-1-2- Demethylation

The results of our experimental incubations show that an important part of the MeHg demethylation is mostly driven by sunlight radiations as demethylation rates decreased severely under dark conditions both for

filtered and unfiltered water. The presence of particulate matter (mineral and biogenic) also enhanced greatly the demethylation process especially for the coastal waters rich in living biomass. For marine waters, higher demethylation rates for dark controls are found in August when the microbial activity is though predominant.

Several abiotic and biotic MeHg demethylation processes can possibly explain maxima demethylation rates for bulk waters under light condition. First, UV-visible radiations may enhance abiotic demethylation (Inoko, 1981). The MeHg photolysis can generate the cleavage of the Hg-C bond by forming Hg(I) radical (Gardfeldt et al. 2001). Further studies have shown that this reaction is enhanced in the presence of organic compounds (Gardfeldt et al. 2001, Sellers et al. 1996). In the case of lakes, Sellers et al. (1996) have demonstrated that photodecomposition of MeHg is an important process compared to biotic demethylation.

Although demethylation is partially photochemically induced, results of the dark controls with high demethylation rates for unfiltered water indicate that MeHg degradation is also mediated by biotic processes either by bacterio- or phyto-plankton. Previous investigations have demonstrated that MeHg degradation can be microbially mediated in water and sediment (Barkay et al., 2003). Numerous bacterial strains are capable of demethylating MeHg including aerobic and anaerobic species. MeHg demethylation is accomplished by bacteria possessing the *merB* gene that encode for the organomercurial-lyase enzyme, which cleaves MeHg forming CH<sub>4</sub> and Hg(II) as end-products (Barkay et al. 2003, Robinson and Tuovinen 1984). In our case, for coastal and marine surface waters, although MeHg is mainly photochemically degraded, demethylation yields observed under dark condition may be attributed to microbial demethylation.

#### 4-1-3- Reduction

Our results in coastal area are similar to that observed in marine areas for which DGM production and temperature are closely linked. Higher DGM production rates are found during warmer periods. Photochemical processes appear to be the major contributing factor with systematic lower production under dark conditions. However, dark incubations provide significant DGM production rates similar for filtered and unfiltered water suggesting non-photochemical abiotic processes. Dissolved gaseous mercury appears thus to be formed by a combination of photochemical, biological and non biological processes.

First it is well known that for surface waters, DGM production is mainly associated to the photoreduction of Hg(II) (Nriagu. 1994). The fundamental role of sunlight in the formation of DGM is proved by the existence of a daily and seasonal behavior of the DGM concentrations in seawater (Kim and Fitzgerald 1988, Amyot et al. 1997, Cossa et al. 1997, Mason et al. 2001). The photoreduction may result from direct photolysis of Hg(II) complexes to Hg<sup>0</sup>. The photo-reduction may also result from photochemical reactions involving humic substances (Matthiessen 1998) or transition metals (Siciliano et al. 2003, Zhang and Lindberg 2001). In our study, for these oxic surface waters, photochemical mechanisms are certainly involved in the DGM production.

Several abiotic processes involving biogenic substances can also be considered to explain the Hg reduction in coastal and marine waters. Biogenic substances released by micro-organisms may play an important role in the DGM formation. Laboratory experiments of cultures of a marine diatom have clearly demonstrated that biogenic compounds are enhancing the DGM production both under light and dark conditions (Lanzillotta et al. 2004).

In previous works, besides abiotic formations of DGM, the biotic reduction of Hg(II) have been suggested (Mason et al. 1995, Barkay et al. 2003, Siciliano et al. 2002). Mason et al. (1995) have reported DGM production by microorganisms in seawater and freshwater with formation rates ranging from 0.5 to 10% d<sup>-1</sup>. They have demonstrated that eukaryotic phytoplankton are capable of reducing Hg(II) but bacteria are the more likely reducers. Bacteria can generate Hg<sup>0</sup> with the use of the bacterial mercuric reductase found for bacteria exhibiting the presence of the *merA* gene. Siciliano et al. (2002) correlated the microbial reductase activity with DGM concentrations within the water column of a lake.

Although the bacterial or phytoplanktonic reduction of Hg(II) cannot be excluded in the production of DGM, microbial reductase activity could not explain the entire DGM production rates for surface water because the lowest DGM production rates are recorded for filtered water under dark conditions. Photoreduction and photochemical reactions are certainly the major mechanisms involved in surface waters. At the deep euphotic zone, sunlight radiations are greatly attenuated. DGM production rate is probably reduced and mainly associated to biotic processes.

#### 4-1-4- Uptake/sorption

The results demonstrate the lower occurrence of uptake/sorption sites for oligotrophic waters characterized by low phytoplankton abundance compared to coastal waters. The waters from the Alboran Sea were differentiated by higher uptake/sorption rates which could be related to the higher relative proportion of diatoms and dinoflagellates representing the larger species of phytoplankton.

The results indicate a strong affinity of MeHg for micro organism cells. This is in agreement with previous results which conclude that Hg(II) tends to bind more strongly to mineral particles and detrital organic matter, whereas MeHg is more strongly associated with biogenic particles (Hurley et al., 1994). Mason et al. (1995) have also demonstrated the passive uptake of Hg chloride complexes as the principal accumulation route of both MeHg and Hg(II) in phytoplankton. Our results agree with their further conclusions on the more efficient uptake of MeHg by a coastal diatom (62%) compared to Hg(II) (15%) (Mason et al. 1996). Previous study on MeHg uptake rate by eukaryote algae also indicates a strong association of MeHg with cell membranes (Miles et al. 2001). In addition, a study on the MeHg interactions with phospholipids membranes has demonstrated the strong affinity and a tendency to accumulate into lipid layers (Girault et al. 1997). The strong affinity of MeHg for micro-organisms is of importance as they represent the first trophic level in the pelagic food web.

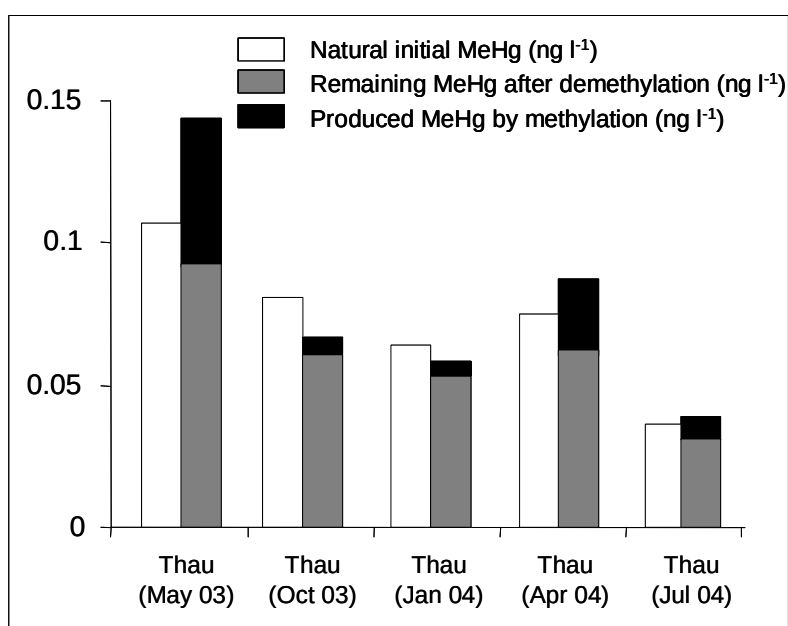
## 4-2- Significance of the measured transformation rates

Transformations and uptake/sorption rates were determined for coastal and marine waters during different seasons using additions of isotopically enriched species. Differences have been demonstrated using experimental results for each transformation process as a function of seasons, radiations, phytoplankton abundance and speciation. In order to find out the significance of such results, tentative budget for predicted species concentrations using experimental transformation rates for a diurnal incubation were compared to natural initial species concentrations.

### 4-2-1- Hg species transformation rates for coastal surface waters

The significance of methylation/demethylation rates was evaluated by comparing ambient MeHg concentrations with predicted MeHg concentrations changes over a diurnal cycle. Predicted MeHg concentrations derives from the MeHg concentrations taking into account the MeHg remaining after demethylation (i.e.  $[\text{MeHg}]_{\text{initial}} - ([\text{MeHg}]_{\text{initial}} \times \text{demethylation rate})$ ) and the produced MeHg by methylation (i.e.  $[\text{Hg(II)}]_{\text{initial}} \times \text{methylation rate}$ ).

The results for the coastal station incubations are presented in figure 3 for the different seasons. With regards to the results, the estimated MeHg concentrations are comparable to the natural MeHg concentrations exhibiting similar values and seasonal variations. These results suggest that our determination of transformations rates is coherent with ambient concentrations and represent realistically processes occurring in the environment.



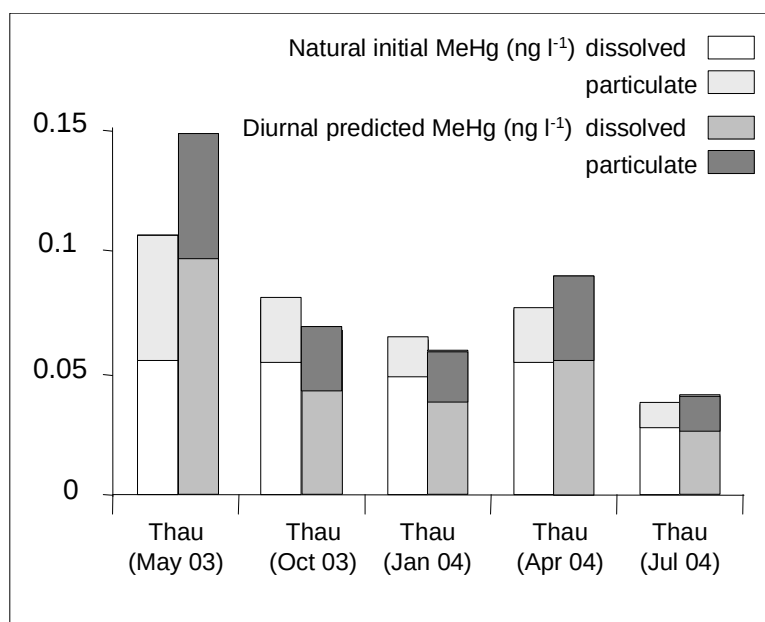
**Figure 3:** Natural and predicted MeHg concentrations for the coastal waters.



In the case of high methylation rates during warm periods (i.e. in May, April, July), 24h-predicted concentrations are found higher than initial ambient concentrations whereas, for low methylation rates, 4h-predicted MeHg concentrations were found always lower than the natural concentration.

Higher estimated MeHg values may highlight a net MeHg production in the water as the demethylation process appears to not compensate the methylation process. On the contrary, lower estimated MeHg concentrations lead to net demethylation rates generating a diminution of MeHg. This is observed with lower ambient MeHg concentrations during October and January corresponding to lower estimated MeHg concentrations and thus an active MeHg degradation.

The significance of uptake/sorption rates determined with the partition of the added isotopically enriched species ( $^{199}\text{Hg}(\text{II})$  and  $\text{Me}^{201}\text{Hg}$ ) is also evaluated by comparison with natural mercury species partitions. Results for MeHg partition for both initial and estimated MeHg (i.e.  $[\text{MeHg}]_{\text{predicted}} \times \text{uptake/sorption rate}$ ) are presented in figure 4.



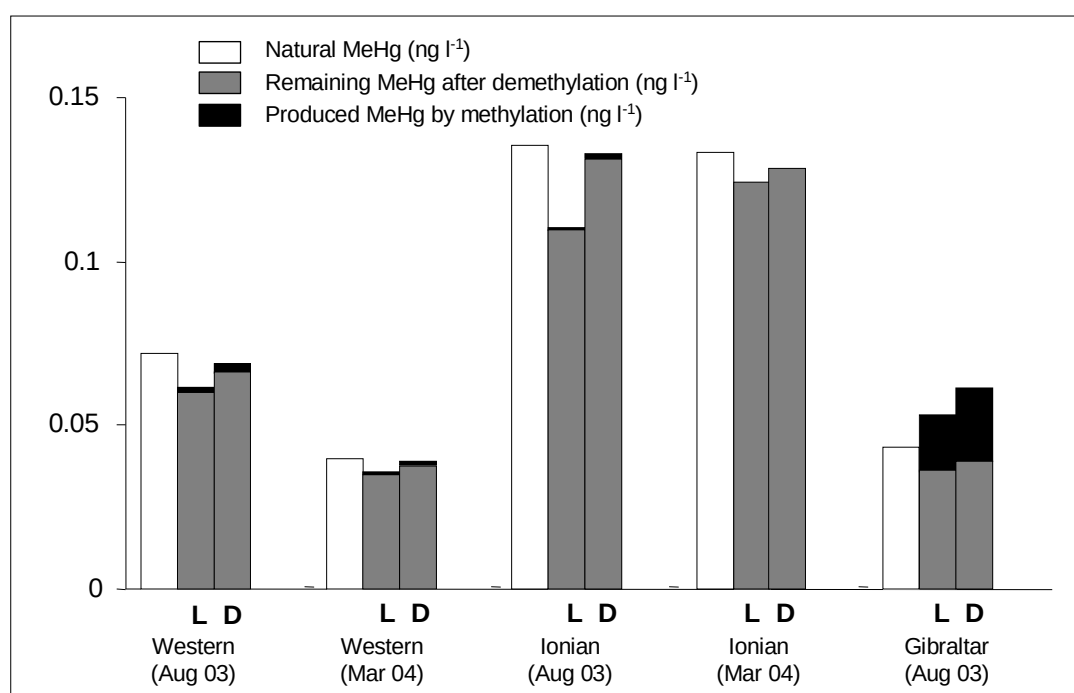
**Figure 4:** Initial and 24h-predicted MeHg partitions for the coastal waters.

As showed before, the calculated MeHg concentrations well represent the natural MeHg concentrations. With regards to the partitioning of MeHg between dissolved and particulate fraction, it also exists a good agreement between natural and modeled partitioning with similar seasonal variations. Similar comparison between natural and predicted partitioning for  $\text{Hg}(\text{II})$  can be done and results show that the  $^{199}\text{Hg}(\text{II})$  is generally less associated with particles compared to natural  $\text{Hg}(\text{II})$ . Uptake/sorption rates determined for the added  $\text{Me}^{201}\text{Hg}$  allow to provide realistic results and thus to evaluate MeHg uptake processes.  $^{199}\text{Hg}(\text{II})$  partitioning differences suggest a longer turnover or assimilation for  $\text{Hg}(\text{II})$  than for MeHg.

#### 4-2-2- Hg species transformation rates for marine surface waters

The significance of transformation rates is also evaluated for the incubations of marine waters. Results are presented in Figure 5 for each diurnal incubation experiment and associated dark control.

For marine waters, the Alboran Sea is the only station where a net MeHg production is predicted. For oligotrophic deep sea stations and for both seasons, no net MeHg formation is established taking into account reversible reaction rates (methylation/demethylation) for diurnal incubations. However high natural MeHg concentrations for these surface waters may suggest a net MeHg production under other conditions which should be more comparable with dark control incubations. As shown in figure 5 for the predicted MeHg concentrations for dark control (D), MeHg production is enhanced under dark conditions whereas demethylation is inhibited. This contributes to a more important MeHg formation under natural light conditions found at the sampling depths (60-85m) where light is largely attenuated.



**Figure 5:** Natural and calculated MeHg concentrations for the surface marine waters (L: diurnal incubation, D: dark control)

## 5- CONCLUSION

Overall, this study is the first assessment of mercury species dynamics in both coastal and marine surface waters of the Mediterranean Sea using in situ incubations with isotopically labeled mercury species. Mercury transformations in marine waters have been for the first time highlighted and appear to play a key role in the cycling of MeHg in marine environments. These results evidence methylation, demethylation and reduction mechanisms involving various biogeochemical pathways such as sunlight induced processes and both autotrophic and heterotrophic micro-organisms activities. Finally, this study demonstrates a new potential source of MeHg in the marine environment directly associated with quantitative transfer to the first trophic level of the marine food web.

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## **Chapitre C – IV**

**Etude de la méthylation du mercure et de la séquestration par des souches spécifiques de bactéries sulfato-réductrices isolées d'un sédiment estuarien par l'utilisation de traceurs isotopiques stables ( $^{199}\text{Hg}(\text{II})$ ,  $\text{Me}^{201}\text{Hg}$ )**



## **Etude de la méthylation du mercure et de la séquestration par des souches spécifiques de bactéries sulfato-réductrices isolées d'un sédiment estuarien par l'utilisation de traceurs isotopiques stables ( $^{199}\text{Hg}(\text{II})$ , $\text{Me}^{201}\text{Hg}$ )**

Comme nous l'avons vu précédemment (article C-II), les sédiments représentent un lieu privilégié pour les transformations du mercure. La méthylation du mercure semble être fortement liée à l'activité bactérienne et notamment à l'activité des bactéries sulfato-réductrices. Plusieurs études montrent leur rôle important pour la production de MMHg dans les sédiments augmentant ainsi le risque écologique pour les organismes aquatiques par des mécanismes de bioaccumulation.

La méthylation du mercure par les bactéries a été décrites dans plusieurs études mais ces travaux ont toujours été réalisés avec des incubations de quelques souches bactériennes commerciales. Ces études ont permis de mettre en évidence la méthylation bactérienne et de proposer des mécanismes intracellulaires générant cette méthylation. Cependant, dans l'environnement, la diversité des populations bactériennes est importante et à l'heure actuelle il est impossible de connaître précisément les espèces spécifiques de bactéries responsables de la méthylation du mercure. De plus, certaines bactéries sont aussi capables de déméthyliser le MMHg et donc de réduire la production nette de MMHg. Afin de connaître précisément la contribution des différentes espèces de bactéries responsables de la méthylation ou de la déméthylation, il est nécessaire d'étudier ces processus pour les différentes bactéries issues directement de l'environnement.

Dans cette étude, nous avons choisi d'étudier les potentiels de méthylation et de déméthylation de souches bactériennes isolées d'un sédiment estuarien. Des études préliminaires sur ce sédiment ont mis en évidence son potentiel de méthylation lié à l'activité de bactéries anaérobies. Le but de cette étude est d'isoler les différentes souches bactériennes présentes dans ce sédiment et de déterminer les potentiels de méthylation et de déméthylation pour chacune d'entre elles. L'utilisation d'espèces enrichies isotopiquement permet de déterminer simultanément les taux de ces processus réversibles tout en utilisant des niveaux de concentrations naturels.

Les résultats montrent que plusieurs souches bactériennes sont capables de méthyliser le mercure, toutes appartenant au groupe des bactéries sulfato-réductrices. Ils suggèrent que les processus de méthylation du mercure sont régulés par des métabolismes spécifiques à certaines souches et pas nécessairement à des groupes phylogénétiques. Ces bactéries méthylantes sont non seulement très résistantes au MMHg mais elles sont aussi capables de séquestrer efficacement les espèces de mercure par assimilation intracellulaire ou sorption sur les membranes cellulaires.

## Investigation of methylation and sequestration of mercury using labeled mercury species ( $^{199}\text{Hg}(\text{II})$ , $\text{Me}^{201}\text{Hg}$ ) by specific sulfate reducing bacteria strains isolated from an estuarine sediment

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### ABSTRACT

Mercury methylation by sulfate reducing bacteria (SRB) in aquatic sediments is a well established biogeochemical pathway. The specificity of SRB strains to methylate and further control the fate of monomethylmercury (MeHg) is however poorly understood as most experiments were dedicated to the bulk methylation rates using a few numbers of strains available from collections. In order to improve our knowledge on the specific methylation of mercury and MeHg fate in coastal sediments, anaerobic strains have been isolated from an estuarine sediment towards their resistance to inorganic mercury and subsequently incubated with isotopically labeled Hg species ( $^{199}\text{Hg}(\text{II})$ ,  $^{201}\text{MeHg}$ ) to determine any specific chemical transformation (methylation/demethylation) and solution partitioning (intra-/ extra-cellular medium). The results demonstrate that among the various anaerobic strains isolated from this sediment only some SRB, such as *Desulfovibrio* sp., *Desulfomicrobium* sp. have shown their potential to methylate Hg with specific methylation rates ranging from 3.5 to 14.8 %. $\text{d}^{-1}$ . None of them has been found to demethylate significantly spiked MeHg (i.e. demethylation rates < 20 %. $\text{d}^{-1}$ ). Methylating strains were neither associated to a specific SRB group nor to a genera, but show strong resistance to MeHg. In addition, methylating SRB strains exhibit a quantitative sequestration pathway accumulating both formed and spiked MeHg in the cell, while non methylating SRB only partially accumulate MeHg. These results demonstrate that Hg methylation pathway is primarily regulated by specific metabolism exclusively associated to SRB strains and not related to the phylogenetic characteristics of the SRB strains. Finally, SRB strains capable to methylate Hg are also specifically sequestering Hg species in the cellular medium suggesting that the fate of the produced MeHg must be closely associated to the biological turnover and benthic food web of such anaerobic bacterial communities.

**KEYWORDS:** mercury methylation, isolated bacteria, uptake, estuarine sediment, stable isotopes

## 1- INTRODUCTION

In estuarine sediments, processes of methylation and demethylation determine the fate of mercury species and thus its toxicity. Bacterial methylation is one of the major process which transforms inorganic mercury (Hg(II)) into toxic methylmercury (MeHg) which is easily bioaccumulated and bioamplified along the food web (Craig 1986). Several studies have shown that sulfate reducing bacteria (SRB) are important methylators of mercury in estuarine (Compeau and Bartha 1985) and freshwater (Gilmour et al. 1992) sediments. The best evidence for the link between sulfate reduction and mercury methylation was provided by inhibition studies (Compeau and Bartha 1985, King et al. 1999, Gilmour et al. 1998). Molybdate addition used as specific inhibitor of sulfate reduction activity has been found to completely inhibit biological mercury methylation. Others studies have shown that in pure culture, SRB grown in the absence of sulfate can generate MeHg only if they either exhibit fermentative metabolism (Benoit et al. 2001) or growth in cocultures with methanogens (Pak and Bartha 1998). Conversely high sulfate concentration medium promoting sulfide production through microbial sulfate reduction severely limits Hg methylation (Benoit et al. 1999). Pure culture of SRB may show high Hg methylation yields specifically when dealing with cultures of groups capable of utilizing acetate compared to those which cannot metabolize acetate. Acetate may stimulate methylation by a transmethylase induction in a variety of diverse SRB communities (Barkay et al. 2003). At present, most studies conclude for the occurrence of a link between biological Hg methylation and the presence of SRB. However, intracellular mechanisms promoting the methylation of Hg are still poorly understood. Previous studies assume that mercury must enter cells before its methylation. Hg methylation in *Desulfovibrio desulfuricans* strains has been found to take place via a side reaction of the acetyl-CoA pathway which would take place in the cytoplasm (Choi et al. 1994). A recent study on mercury methylation by SRBs, has shown that some of these strains can methylate Hg via a different route which would be independent from the acetyl-CoA pathway (Ekstrom et al. 2003).

Many of the SRB groups which realized the methylation in natural sediments have not been described in detail. Indeed, most studies on this topic were performed on cell cultures of bacteria strains which were not isolated from natural sediments showing evidence for MeHg production. The studies to understand bacterial methylation in pure culture experiments were using available SRB strains from culture collections such as *Desulfovibrio desulfuricans* LS (Jay et al. 2002, Pak and Bartha 1998, Ekstrom et al. 2003) and *Desulfobulbus propionicus* 1pr3 (Benoit et al. 1999). In natural environments, SRBs are represented by different groups which differ physiologically and phylogenically. These different SRB strains could present different potentials with respect to mercury methylation (King et al. 2001).

In the environment, the mercury methylation pathway has been found to be reversible. MeHg levels in the environment are thus tightly controlled by mercury demethylation which can occur by a reductive or an oxidative

pathway (Barkay et al. 2003). Mercury reduction is encoded by the microbial *mer* operon that is widespread among microorganisms (Barkay et al. 2003). Despite of the fact that mercury oxidation has been reported in anoxic sediments, the involvement of sulfate reducing bacteria remains to be demonstrated. In summary, mechanisms involved in mercury transformations are still not completely well established and unraveling the cycle remains a difficult task since both methylators and demethylators bacterial strains coexist in the same environmental conditions. This is especially true for coastal sediments in which oxic/anoxic oscillations may promote alternatively both Hg methylation and demethylation (Tseng et al, 2001).

In the Adour macrotidal estuary (Bay of Biscay, SW France), intensive environmental survey of both sediment and water compartments have evidenced the ubiquitous occurrence of MeHg (Stoichev et al. 2004, Point et al., 2004). In this study, specific surface sediments have been found to favor Hg methylation (Stoichev et al. 2004). More recently, incubations of these specific sediments have been conducted using sediment slurries and enriched isotopic tracers to determine the methylation and demethylation potentials with low Hg spike levels (Rodriguez et al. 2004). The use of double spikes of isotopically enriched species ( $^{199}\text{Hg}(\text{II})$ ,  $\text{Me}^{201}\text{Hg}$ ) allowed to simultaneously study the kinetics of both type of mechanisms. Under these conditions, Hg methylation has been demonstrated to occur mostly under anoxic and biotic conditions. Demethylation was also found to be an important process regulating the MeHg concentration in the sediment. Biological processes have thus been suggested to be of paramount importance for mercury transformations although the determination of specific strains responsible for the Hg transformation was not determined (Rodriguez et al. 2004).

In order to identify the bacterial strains involved in the methylation of Hg in this sediment, Hg methylation/demethylation reactions were studied using pure cultures isolated from the Adour estuary sediment. The Hg transformations were followed for all Hg resistant strains of the whole microbial community in pure culture experiments and using isotopically enriched mercury species at low spike levels ( $7 \mu\text{g L}^{-1} \text{ }^{199}\text{Hg}(\text{II})$ ,  $0.7 \mu\text{g L}^{-1} \text{ Me}^{201}\text{Hg}$ ). Transformations yields (methylation, demethylation and reduction) as well as uptake yields of the different mercury species by the isolated strains were investigated. These results have allowed us to propose metabolic pathways controlling the transformations and sequestration of mercury species by these bacteria.

## 2- MATERIALS AND METHOD

### 2-1- Isolation and identification of Hg resistant anaerobic bacterial strains

*Sampling site* - Bacterial strains used in this work were isolated from surface intertidal sediment from the Adour estuary. The Adour estuary is located on the Atlantic coast in the south west part of France (Bay of Biscay). Previous study on mercury species concentrations for surface sediments in the whole estuary (Stoichev et al. 2004) have shown this location as the best methylation potential with the highest MeHg to Hg(II) ratios concentrations. Slurries experiments on the same sediment have also demonstrated its high methylation potential (Rodriguez et al. 2004). Natural mercury species concentrations were found to be 240 ng g<sup>-1</sup> of total mercury and 2.2 ng g<sup>-1</sup> corresponding to 0.9% of MeHg compared to total mercury. The inoculum for the isolation procedures was the anoxic slurry described in Rodriguez et al. (2004).

*Isolation procedures* - The first isolation steps were carried out in natural filtered (0.2 µm) sampling site water supplemented with acetate-Na (10mM), lactate-Na (10 mM), glucose (2mM), NaHCO<sub>3</sub> (2.5 g.l<sup>-1</sup>) and Na<sub>2</sub>S.9H<sub>2</sub>O (0.05 g.l<sup>-1</sup>). The medium was prepared as described in Guyoneaud et al. (1996). Isolation procedures were either in deep agar dilution series (Pfennig and Trüper 1992) or in streak plates incubated in an anaerobic chamber. During all isolation procedures Hg(II) was added to reach final concentrations ranging from 1 to 10 mg.L<sup>-1</sup>. The final synthetic medium used for the last isolation steps and for maintenance and studies on the isolated strains contained (in g.l<sup>-1</sup>) : KH<sub>2</sub>PO<sub>4</sub>, 0.25 ; NH<sub>4</sub>Cl, 0.5 ; CaCl<sub>2</sub>.2H<sub>2</sub>O, 0.05 ; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.5; KCl, 0.5 ; NaNO<sub>3</sub>, 0.85 ; Na<sub>2</sub>SO<sub>4</sub>, 1.42 ; acetate-Na, 0.82 ; lactate-Na, 10 mM ; glucose, 0.25 M; SL12 B, 1 ml (Overmann et al. 1992). The medium was prepared according to Pfennig and Trüper (1992). After sterilization and cooling under N<sub>2</sub>/CO<sub>2</sub> mixture (9/1 in volume) 2.5 g of sterile NaHCO<sub>3</sub>, 0.05 g of sterile Na<sub>2</sub>S.9H<sub>2</sub>O and 1 ml of sterile vitamin V7 solution (Pfennig and Trüper 1992) were added per liter of medium. The pH was adjusted to 7.0-7.2 with HCl or NaOH solutions and distributed in sterile flasks closed with butyl stopper and flushed with N<sub>2</sub>/CO<sub>2</sub> gaz mixture (9/1 in volume). Purity of the isolated strains was controlled by microscopic observations of the cultures in a phase contrast Olympus BH2 microscope.

*Identification by partial 16S rDNA sequencing* - Isolation of genomic DNA, amplification of 16S rDNA by PCR, sequencing and phylogenetic analysis, were all carried out as described previously (Mouné et al. 2000; Guyoneaud et al. 2002). The primers used for the amplification were 8-Forward (5'-AGAGTTTGATCCTGGCTCAG) and 1489 reverse (5'-TACCTTGTTACGACTTCA). Sequencing was performed on an ABI PRISM 310 Sequencer (Applied Biosystems) using BigDye Terminator Cycle Sequencing Ready

reactions (Applied Biosystems) according to the manufacturer's instructions. DNA sequence analysis was performed via the infobiogen server (<http://www.infobiogen.fr>) by using FASTA, BLAST, ALIGNN, and CLUSTALW programs.

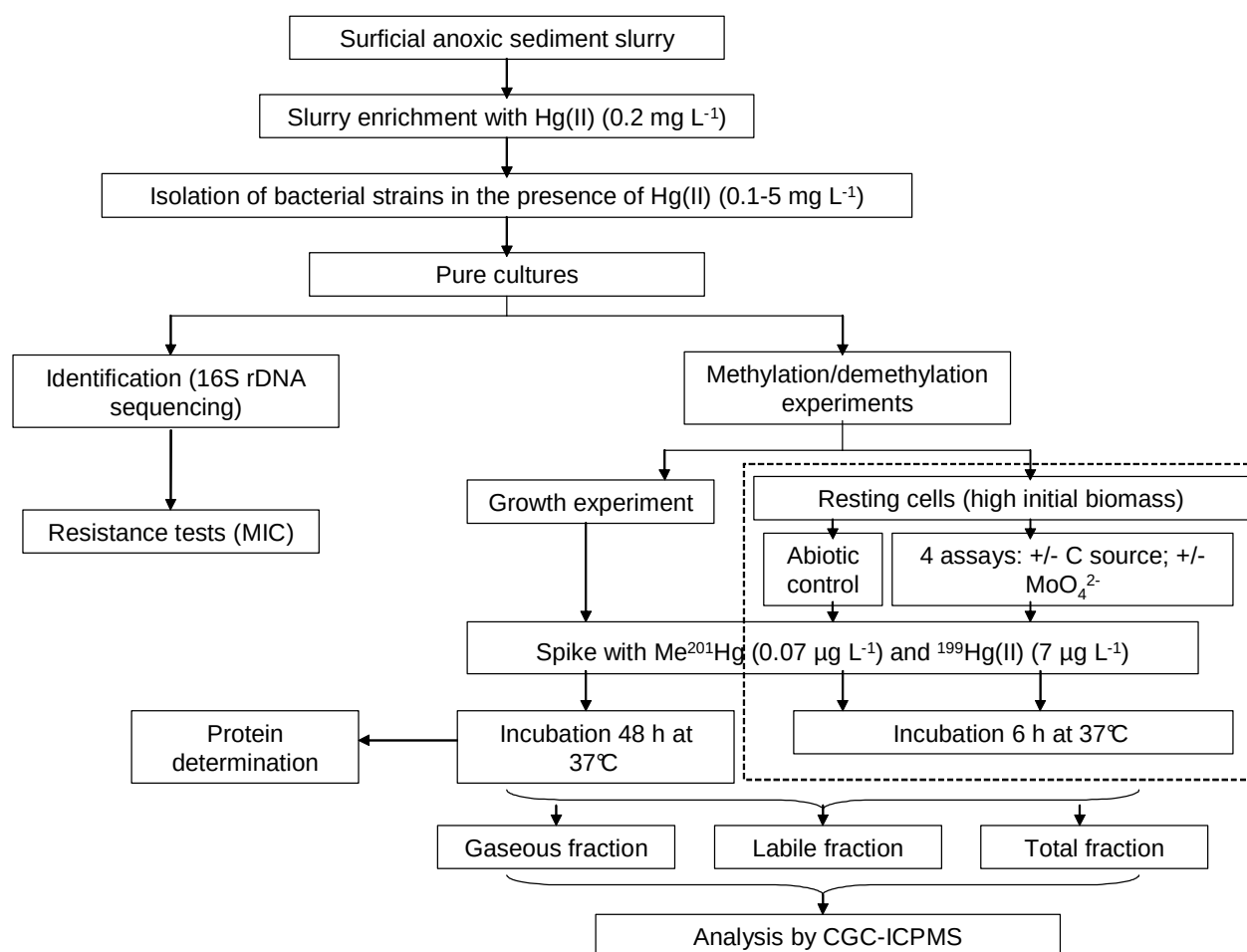
## 2-2- Determination of the minimal inhibiting concentrations (MIC)

All the experiments made to determine the MIC were realized in the synthetic medium in the absence of sulfide. A 1 ml aliquot of a 72-hours-old culture (strain ADR 04, ADR 14, ADR 20, ADR 21, ADR 22, and ADR 25) was inoculated in 9 ml of medium amended with different concentrations of Hg(II), Cu and Cd (2 to 128 mg.l<sup>-1</sup>) and MeHg (0.5 to 64 mg.l<sup>-1</sup>). Cultures without metallic spikes were used as controls. Bacterial strains were incubated for 15 days at 37°C and the growth was controlled by determining the Optical Density at 500 nm with a Bausch and Lomb Spectronic 20 spectrophotometer. MIC was defined as the lowest concentration of metallic spike that completely inhibited the growth after the incubations.

## 2-3- Methylation and demethylation experiments

All the experiments were realized here also in the synthetic medium in the absence of sulfide. The procedure for methylation/demethylation experiments is described in figure 1. The culture medium is distributed under sterilized conditions to acid cleaned vials, sealed with butyl stopper and purged with nitrogen in order to maintain anoxic conditions. The methylation and demethylation experiments were conducted to determine the production or the degradation of MeHg by growing cultures of isolated strains from the Adour estuary. Anaerobic vials containing 45 ml of medium were inoculated at 10% (v/v) with precultures. The obtained head space in the vials represented 15 ml. Two replicate culture bottles were prepared for each bacterial strain studied and spiked for a total concentration of 7 µg l<sup>-1</sup> with <sup>199</sup>Hg(II) and to 0.07 µg l<sup>-1</sup> with Me<sup>201</sup>Hg corresponding to the introduction of 315 ng and 3.15 ng per vial for each species respectively. Culture bottles were incubated at 37°C during 48 hours and then stored at +4°C until mercury speciation analysis. Just before analysis, the vials were maintained at room temperature (+20°C). Cell growth was monitored by protein measurement according to [Lowry et al. \(1951\)](#). Sterilized and uninoculated controls both with and without vitamin B12 were also incubated under the same conditions to control for abiotic methylation and demethylation.

In addition, cultures of ADR 21 and ADR 28 were inoculated into fresh medium to obtain a dense cellular suspension and then incubated at 37°C for 6 h. Cultures were then stored at +4°C until mercury speciation analysis. To examine the influence of sulfate reduction inhibition on Hg methylation for non growing cultures, same experiments were reproduced with addition of molybdate at 20 mM.



**Figure 1:** Flowchart of the isolation, identification and Hg transformations and resistance experiments of bacteria strains from the Adour estuary sediment.

## 2-4- Mercury speciation analysis

At the end of the incubation period, all formed and added mercury species were analyzed in the different fractions. Mercury species in pure culture experiments were determined in the head space volume, the labile fraction and the total content by CGC-ICPMS. Analytical setup and methodology for the CGC-ICPMS for mercury speciation analysis are described in detail elsewhere (Monperrus et al., 2004).

First, the gaseous species formed ( $\text{Hg}^0$  and  $\text{Me}_2\text{Hg}$ ) were determined in the head space volume and in the aqueous phase. Mercury species in the gaseous phase were analyzed by sampling directly the head space of the culture bottle with a 1 ml gas-tight syringe and direct injection to the CGC-ICPMS. Elemental mercury ( $\text{Hg}^0$ ) and dimethylmercury ( $\text{Me}_2\text{Hg}$ ) were determined with detection limits of 0.1 ng.L<sup>-1</sup> for both species under these processes of conditions. Dissolved gaseous elemental Hg in the liquid medium can then be determined using the

head space method taking into account the Henry's Law constant for the aqueous culture medium at room temperature (Sanemasa, 1975). Total gaseous elemental Hg was thus obtained adding both head space and dissolved fraction.

For the analysis of labile mercury species in the liquid media, 1 ml of the culture medium is sampled and directly submitted to derivatization. The derivatization of mercury species using ethylation has been described previously (Monperrus et al, 2003). Briefly 5 ml of acetic acid / sodium acetate buffer (0.1M) are added to set the pH at 4. 0.2 ml of iso-octane and 1 ml of 0.5% (w/v) sodium tetraethylborate are added for derivatization. After 5 minutes of vigorous agitation by hand, the organic phase is transferred to an injection vial and stored at  $-18^{\circ}\text{C}$  until CGC-ICPMS detection. The labile mercury species recovered by this method are then the free moieties occurring in the culture medium and therefore directly available for ethylation. These labile mercury species are thus assumed to originate from extracellular mercury species.

All mercury species in the liquid phase were analyzed using a microwave acid extraction prior to derivatization to solubilize the mercury species associated with refractory compounds or cells. The extraction procedure used for sediments described previously by Rodriguez et al. (2003) is adapted to pure culture samples. Briefly, 1 ml of the culture medium is digested with 2 ml of nitric acid 6N under a microwave field (Prolabo A301, 40 W, 3 min.). This method, using nitric acid as extractant, allows generally the solubilization of refractory sulfur and organic compounds (Tseng et al. 1997). The extract is then submitted to ethylation and CGC-ICPMS analysis. The detection limits obtained under this experimental protocol were  $8 \text{ ng l}^{-1}$  and  $4 \text{ ng l}^{-1}$  for Hg(II) and MeHg respectively. This total mercury species concentrations include thus both labile species in solution, non reactive species in solution (i.e. strong sulfide or organic complexes) and species associated to the cells (i.e. sorbed or uptaken).

## 2-5- Transformations and sequestration yields calculations

Yields of mercury transformations and sequestration were determined by adding trace quantities of isotopically enriched  $^{199}\text{Hg(II)}$  and  $\text{Me}^{201}\text{Hg}$  to the pure cultures selected. The amount of formed and recovered mercury species deriving from the enriched isotopes 199 and 201 (i.e.  $^{199}\text{Hg}^{\circ}$ ,  $^{201}\text{Hg}^{\circ}$ ,  $\text{Me}^{199}\text{Hg}$ ,  $\text{Me}^{201}\text{Hg}$ ,  $^{199}\text{Hg(II)}$ ,  $^{201}\text{Hg(II)}$ ) for each fraction (i.e. gaseous, labile, total) are calculated by using the equation described previously by Hintelmann et al. (1995). Further, the detection limits for the different concentrations calculated are evaluated using the equation given by Hintelmann et al. (1997).

Hg transformations yields, such as methylation, demethylation and reduction can be then calculated with their associated detection limit. The methylation yields are calculated by dividing the total amount of  $\text{Me}^{199}\text{Hg}$  formed by the amount of  $^{199}\text{Hg(II)}$  spiked. The lowest methylation yield detectable is 0.9% and corresponds to a formation of 2.7 ng of  $\text{Me}^{199}\text{Hg}$  per vial. Demethylation yields can also be calculated by two different ways: by determining



the percentage of  $\text{Me}^{201}\text{Hg}$  disappearing or the percentage of formed  $^{201}\text{Hg}(\text{II})$ . This last solution is not applicable in our case due to the high difference of concentrations ranges between the two mercury species. The demethylation yields are then obtained by dividing the amount of  $\text{Me}^{201}\text{Hg}$  disappearing with respect to the amount of  $\text{Me}^{201}\text{Hg}$  added. Under these conditions, a higher detection limit at 20% is obtained when compared to methylation. This low sensitivity for demethylation is due to the low spike of  $\text{Me}^{201}\text{Hg}$  in order to avoid any metabolic response to acute  $\text{MeHg}$  toxicity among the strains. Reduction reactions obtained were derived from the quantity of gaseous species formed from the  $^{199}\text{Hg}(\text{II})$  added. The reduction yield is then obtained by dividing the sum of gaseous elemental mercury amounts in the head space and the aqueous phase by the amount of spiked  $^{199}\text{Hg}(\text{II})$ . Under these conditions, the detection limit obtained is 0.05% for the reduction yield.

Finally, the levels of sequestration of mercury species were determined by comparing the amount of the labile fraction to the total fraction in order to calculate the sequestered fraction (i.e. total minus labile fraction). This sequestration yield can be obtained for recovered  $\text{Me}^{201}\text{Hg}$ , formed  $\text{Me}^{199}\text{Hg}$  and recovered  $^{199}\text{Hg}(\text{II})$ . These sequestration yields were assumed to represent the mercury species associated to refractory compounds, such as sulfide or organic compounds, and to the cells by sorption or assimilation.

### 3- RESULTS AND DISCUSSION

#### 3-1- Identification of Hg-resistant bacterial strains from estuarine sediments

The isolated bacterial strains were obtained from a sediment slurry from the Adour estuary which was spiked to a low level of Hg as Hg(II) of 0.2 mg l<sup>-1</sup>. The 15 different strains were isolated and identified on partial 16S rRNA sequence homology. Results are displayed in Table 1. The isolated strains presented very different metabolic functions such as strict or facultative fermenters (*Clostridium sp.*, *Georgenia muralis*), denitrifiers (*Pseudomonas stutzeri*), anoxygenic phototrophs (*Rhodopseudomonas palustris*, *Green sulfur bacterium*), sulfate-reducers (*Desulfovibrio sp.*, *Desulfomicrobium sp.*, *Desulfotomaculum sp.*) and *Bosea thiooxydans*. These bacteria belonged to different functional groups characteristic of anoxic environments. Most of the isolated strains were linked to sulphur cycling processes.

The isolation procedures were performed in the presence of culture media presenting concentration of Hg(II) ranging from 0.1 to 5 mg l<sup>-1</sup>. These levels are rather low when compared to resistance levels found for aerobic bacterial strains (Robinson et Tuovinen 1984). We believe that in our culture conditions, we did not induce strong pressure selection but have obtained a more realistic environmental selection of anaerobic strains insensitive to the presence of mercury in solution.

| Strains | Name                              | Homology (%) |
|---------|-----------------------------------|--------------|
| ADR 04  | <i>Pseudomonas stutzeri</i>       | 98           |
| ADR 09  | <i>Pseudomonas stutzeri</i>       | 99           |
| ADR 10  | <i>Clostridium sp.</i>            | 95           |
| ADR 15  | <i>Georgenia sp.</i>              | 96           |
| ADR 03  | <i>Bosea sp.</i>                  | 98           |
| ADR 08  | <i>Bosea sp.</i>                  | 97           |
| ADR 20  | <i>Clostridium sp.</i>            | 96           |
| ADR 25  | <i>Rhodopseudomonas palustris</i> | 95           |
| ADR 22  | <i>Desulfatomaculum sp.</i>       |              |
| ADR 13  | <i>Desulfovibrio africanus</i>    | 100          |
| ADR 14  | <i>Desulfovibrio africanus</i>    | 100          |
| ADR 19  | <i>Desulfomicrobium baculatum</i> | 99           |
| ADR 21  | <i>Desulfomicrobium baculatum</i> | 100          |
| ADR 26  | <i>Desulfomicrobium sp.</i>       | 95           |
| ADR 28  | <i>Desulfomicrobium sp.</i>       | 98           |

**Table 1:** Isolated bacteria strains from the Adour estuary sediment.

### 3-2- Specific toxicity of Hg species toward isolated strains

The sensitivity to heavy metals and MeHg of six different strains [belonging to the genera *Clostridium* (Gram+, fermentative), *Pseudomonas* (Gram-, denitrifier), *Rhodopseudomonas* (Gram-, phototroph), *Desulfovibrio* and *Desulfomicrobium* (Gram-, SRB)] was evaluated by determining the minimal inhibiting concentration (MIC) in the liquid medium. The results displayed in Table 2 showed that the four bacterial groups (fermentative, denitrifier, phototroph and sulfate reducer) presented variations with regards to the resistance towards various heavy and organo metals (i.e. Hg(II), Cu, Cd, MeHg). Our results demonstrate the occurrence of very high levels of resistance to both Hg(II) (64 and 128 mg l<sup>-1</sup>) and MeHg (>64 mg l<sup>-1</sup>) species for all the sulfate-reducers (ADR 14, 21 and 28). Bacterial growth still continued even for concentrations for 64 mg l<sup>-1</sup> of MeHg. The growth rates obtained are not significantly different from those found in the absence of MeHg. The resulting MIC for Hg(II) are higher than for the levels previously reported for Hg resistant bacteria (Robinson et Tuovinen 1984). The MIC for MeHg are also higher than those described by Baldi and al. (1993). These resistant strains appear to exhibit at least a similar resistance level for MeHg than compared to Hg(II). These SRB strains are also much more resistant to mercury species than to cadmium and copper. These results suggest the existence of specific resistance mechanisms of these strains to mercury species.

For the non-SRB strains, two important results can be pointed out. First, the Gram positive bacterium (*Clostridium* sp.) is more sensitive than the Gram negative bacteria. Such results have already been obtained by other authors (Baldi et al. 1993, Lascourrèges et al 1999). The structural and functional differences of the bacterial envelopes between the gram positive and gram negative bacteria probably result in the different levels of permeability to toxic substances. Second, the Hg(II) resistance obtained for the Gram negative bacteria were quite low (4-8 mg l<sup>-1</sup>) compared to the strains containing the detoxification system via *mer* genes expression (Robinson et Tuovinen 1984).

| Identification |                                   | MIC (mg L <sup>-1</sup> ) |     |        |      |
|----------------|-----------------------------------|---------------------------|-----|--------|------|
|                |                                   | Cu                        | Cd  | Hg(II) | MeHg |
| ADR 04         | <i>Pseudomonas stutzeri</i>       | 16                        | 32  | 8      | 0.5  |
| ADR 20         | <i>Clostridium</i> sp.            | 2                         | 2   | 2      | 0.5  |
| ADR 25         | <i>Rhodopseudomonas palustris</i> | 2                         | 8   | 4      | 0.5  |
| ADR 14         | <i>Desulfovibrio africanus</i>    | 16                        | 64  | 128    | > 64 |
| ADR 21         | <i>Desulfomicrobium baculatum</i> | 4                         | 64  | 64     | > 64 |
| ADR 28         | <i>Desulfomicrobium</i> sp.       | 16                        | 128 | 128    | > 64 |

**Table 2:** Minimal inhibited concentrations for Cu, Cd and mercury species.

### 3-3- Mercury methylation, demethylation and reduction potentials by the specific anaerobic isolated strains

Mercury methylation, demethylation and reduction potentials were determined for the 12 purified cultures of bacteria isolated from the Adour estuary sediment. The results of methylation, demethylation and reduction yields are calculated after incubation with isotopic tracers and are summarized in Table 3. Bacterial growth monitored by protein production shows that all strains developed well with concentrations of  $7 \mu\text{g l}^{-1}$  and  $0.07 \mu\text{g l}^{-1}$  of  $\text{Hg(II)}$  and  $\text{MeHg}$ , respectively.

| Strains | Protein production ( $\text{mg L}^{-1}$ ) | Methylation yield (%) | Demethylation yield (%) | Reduction yield (%) |
|---------|-------------------------------------------|-----------------------|-------------------------|---------------------|
| Medium  | 0                                         | <0.9                  | <20                     | 0.8                 |
| ADR 04  | 124.2                                     | <0.9                  | <20                     | 0.3                 |
| ADR 09  | 54.6                                      | <0.9                  | <20                     | 0.2                 |
| ADR 10  | 23.4                                      | <0.9                  | <20                     | 0.7                 |
| ADR 15  | 46.8                                      | <0.9                  | <20                     | 0.3                 |
| ADR 25  | 164.1                                     | <0.9                  | <20                     | 0.2                 |
| ADR 22  | 9.5                                       | <0.9                  | <20                     | 0.3                 |
| ADR 13  | 21.9                                      | 3.5                   | <20                     | 0.3                 |
| ADR 14  | 48.3                                      | 4.7                   | <20                     | 0.2                 |
| ADR 19  | 12.3                                      | <0.9                  | <20                     | 0.1                 |
| ADR 21  | 41.3                                      | 14.8                  | <20                     | 0.5                 |
| ADR 26  | 42.2                                      | 4.8                   | <20                     | 0.4                 |
| ADR 28  | 38.3                                      | 11.5                  | <20                     | 0.1                 |

**Table 3:** Methylation, demethylation and reduction yields of isolated strains from the Adour estuary.

*Methylation* - With regards to the methylation potentials, the five strains all belonging to SRB (ADR13, ADR14, ADR21, ADR26, ADR28) are able to methylate Hg compared to the uninoculated control medium. The final methylation yields obtained range from 3.5 to 14.8% under similar culture conditions and with no direct relationship to the proteins production (Table 3). The results also demonstrate that 2 of the SRB isolated are not able to methylate mercury (ADR22, ADR19) under our experimental conditions. Hg methylation potential is only present in SRB strains but both differs with respect to the bacterial group concerned (genus) and to the specific strain isolated.

To our knowledge, no study on Hg methylation by Gram + SRB strains has ever been attempted. In our results, the strain ADR 22 (*Desulfotomaculum sp.*) which is a Gram positive SRB does not methylate mercury.

Among the SRB methylating strains recovered from the Adour sediments, two different genera are identified: *Desulfovibrio* and *Desulfomicrobium*. Previous authors have shown that some SRB belonging to the genus *Desulfovibrio* could effectively methylate Hg (King et al. 2000, Jay et al. 2002, Pak et Bartha 1998, Ekstrom et al. 2003). Ekstrom et al. (2003) have demonstrated that *Desulfovibrio africanus* display a significant methylation yield of 2.1% after 48 h of incubation. This is comparable to the 3.5 et 4.7% found for our *Desulfovibrio africanus* strains isolated from the Adour estuary (ADR 13 and ADR 14). The *Desulfovibrio desulfuricans* strains have traditionally mostly been studied for their methylation potential (Pak et Bartha 1998, King et al. 2000, Jay et al. 2002, Ekstrom et al. 2003). The link between sulfate reduction and Hg methylation has always been demonstrated using either sediment experiments or pure SRB collection cultures, but no investigation has been directly performed on the methylation potential of isolated environmental bacteria.

In our study, among the four *Desulfomicrobium* strains isolated, the Hg methylation potential is found to be strain specific. The strain ADR 19 is not able to methylate Hg, Low levels of Hg methylation occur for ADR 26 strain. The highest Hg methylation yields are obtained in cultures of *Desulfomicrobium* strains ADR21 and ADR28 (Table 3). Such differences within the same genus and in between species have been also previously reported by Ekstrom et al. (2003) for *Desulfovibrio desulfuricans*. Mercury methylation appears to rely on specific strains to promote its formation in biochemical pathways. This strain specificity is more important than the general metabolism of SRB. Pure cultures studies (King et al., 2000) also revealed that SBR capable of utilizing acetate would methylate Hg more effectively than SRB strains that did not utilize acetate. Ekstrom et al. (2003) have also shown that some incomplete oxidizers are able to significantly methylate Hg through a pathway independent of the acetyl-CoA pathway.

*Demethylation* - Mercury demethylation potentials were evaluated for the 12 strains isolated under anaerobic conditions. No significant demethylation of MeHg is detectable (>20%) for any of these strains as well as for the uninoculated control (Table 3). In all cases, the Me<sup>201</sup>Hg spike is fully recovered independently of the strain studied (Table 4). These results suggest that these bacteria strains, MeHg resistant, and methylators are not able to demethylate MeHg under our anoxic conditions. Methylation/demethylation potentials studied from the same sediment (Rodriguez et al. 2004) have shown that abiotic conditions are more effective to promote MeHg demethylation. In our case, biotic demethylation appears to be minimal when compared to previous studies. In general, in anoxic sediments, the contribution of sulfidogens has been proposed to induce the demethylation of MeHg (Robinson and Tuovinen 1984, Oremland et al. 1991). Oremland et al. (1991) have isolated these strains but did not succeeded also in degrading MeHg with these strict anaerobes. MeHg demethylation has only been reported with a yield of 40% with a pure culture collection of *Desulfovibrio Desulfuricans*, (Compeau et Bartha 1984, Pak et Bartha 1998). It is also worth to mention that all these studies have used high spiked concentrations of MeHg (1 and 0.1 mg l<sup>-1</sup>) which are not representative of naturally occurring environmental levels. Under our

experimental conditions, the fact that low spiking levels of MeHg ( $0.07 \mu\text{g l}^{-1}$ ) may avoid acute metabolic response and explain the absence of high demethylation yield. The formation of gaseous species ( $\text{Hg}^0$  and  $\text{Me}_2\text{Hg}$ ) was monitored by injecting the head space of the culture vials in the CGC-ICPMS. No  $\text{Me}_2\text{Hg}$  could be detected ( $<0.1 \text{ ng l}^{-1}$ ) for all the different bacteria strains whereas [Baldi et al. \(1993\)](#) could report  $\text{Me}_2\text{Hg}$  formation by cultures of *Desulfovibrio desulfuricans* in the presence of MeHg.

**Reduction** - The reduction yields obtained from formed  $\text{Hg}^0$  amount (head space plus dissolved) with respect to spiked  $^{199}\text{Hg(II)}$ , range from 0.1 to 0.8 % (Table 3). For the uninoculated medium, the reduction yield is about 1%. The comparison of these results suggests that no biotic reduction by these strains is taking place. The  $\text{Hg(II)}$  resistant bacteria are generally able to reduce the  $\text{Hg(II)}$  via the Mer A protein (mercury reductase) ([Barkay et al. 2003](#)). The MeHg resistant bacteria will demethylate MeHg via the Mer B protein (MeHg lyase) to produce  $\text{Hg(II)}$  which is in turn reduced by the Mer A protein ([Barkay et al. 2003](#)). In both cases,  $\text{Hg}^0$  is produced and degassed. In our case, our methylating and MeHg resistant BSR strains not neither demethylate MeHg nor reduce  $\text{Hg(II)}$  to  $\text{Hg}^0$ . These results suggest that their resistance mechanisms are then not associated with *mer* operon mechanisms.

#### II-4- Sequestration of Hg species by the isolated anaerobic strains

Investigations on the behavior of mercury species with regards to its translocation and reactivity were followed in the pure cultures. The mercury species partition between the labile and total fractions was determined after the incubation and transformation of the different labeled species introduced. The results on the recoveries obtained and the partition for each species are presented in Table 4.

In the case of the  $^{199}\text{Hg(II)}$ , the spike can either be methylated or directly reduced to  $\text{Hg}^0$  during the incubation period. The global mass balance on all the  $^{199}\text{Hg}$  mercury species is established (sum of the formed  $\text{Me}^{199}\text{Hg}$ ,  $^{199}\text{Hg}^0$  and recovered  $^{199}\text{Hg(II)}$ ). The recoveries obtained for  $^{199}\text{Hg(II)}$  range between 63% and 101% for the different incubated strains and the culture medium. The lowest recoveries of  $^{199}\text{Hg(II)}$  are not due to losses via gaseous species formation but would mostly be due to incorporation in refractory compounds (organic or sulfidic) or direct adsorption to the flask walls. The formation of refractory species containing  $\text{Hg(II)}$  via microbial metabolism products may be an explanation for this limited recovery of  $^{199}\text{Hg(II)}$  in our experiments. However, in the case of the uninoculated control, the spike level is also not totally recovered (80%) despite of the fact that no organic or sulfidic compounds were added or produced. Such results would also highlight the possible contribution of direct adsorption on the wall of the culture flasks.

| Strains | Me <sup>201</sup> Hg recovered |               |               | total 201<br>recovery<br>(%) | <sup>199</sup> Hg<br>gaseous<br>formed<br>(ng) | Me <sup>199</sup> Hg formed |               |               | <sup>199</sup> Hg(II) recovered |               |               | total 199<br>recovery<br>(%) |
|---------|--------------------------------|---------------|---------------|------------------------------|------------------------------------------------|-----------------------------|---------------|---------------|---------------------------------|---------------|---------------|------------------------------|
|         | Labile<br>(ng)                 | Total<br>(ng) | Uptake<br>(%) |                              |                                                | Labile<br>(ng)              | Total<br>(ng) | Uptake<br>(%) | Labile<br>(ng)                  | Total<br>(ng) | Uptake<br>(%) |                              |
| Medium  | 1.03                           | 3.64          | 56            | 116                          | 2.64                                           | <0.16                       | <2.7          |               | 33.8                            | 249.9         | 76            | 80                           |
| ADR 04  | 0.69                           | 3.36          | 66            | 107                          | 0.99                                           | <0.16                       | <2.7          |               | 56.3                            | 319.2         | 70            | 102                          |
| ADR 09  | 0.82                           | 2.87          | 56            | 91                           | 0.50                                           | <0.16                       | <2.7          |               | 17.7                            | 293.4         | 89            | 93                           |
| ADR 10  | 0.79                           | 3.33          | 61            | 106                          | 2.15                                           | <0.16                       | <2.7          |               | 66.1                            | 197.5         | 50            | 63                           |
| ADR 15  | 0.87                           | 3.38          | 59            | 107                          | 0.99                                           | <0.16                       | <2.7          |               | 72.0                            | 316.2         | 63            | 101                          |
| ADR 25  | 0.52                           | 2.80          | 69            | 90                           | 0.66                                           | <0.16                       | <2.7          |               | 48.9                            | 271.3         | 69            | 86                           |
| ADR 22  | 0.53                           | 3.41          | 73            | 108                          | 0.99                                           | <0.16                       | <2.7          |               | <4.6                            | 241.3         | 77            | 77                           |
| ADR 13  | <0.11                          | 3.22          | 100           | 102                          | 0.83                                           | <0.16                       | 10.9          | 100           | <4.6                            | 273.2         | 100           | 90                           |
| ADR 14  | <0.11                          | 3.42          | 100           | 109                          | 0.66                                           | <0.16                       | 14.7          | 100           | 30.8                            | 275.3         | 100           | 92                           |
| ADR 19  | 0.87                           | 3.63          | 61            | 115                          | 0.17                                           | <0.16                       | <2.7          |               | <4.6                            | 234.8         | 80            | 75                           |
| ADR 21  | <0.11                          | 2.88          | 100           | 91                           | 1.49                                           | <0.16                       | 46.5          | 100           | <4.6                            | 186.5         | 100           | 74                           |
| ADR 26  | <0.11                          | 2.68          | 100           | 85                           | 1.32                                           | <0.16                       | 15.3          | 100           | <4.6                            | 207.7         | 100           | 71                           |
| ADR 28  | <0.11                          | 3.49          | 100           | 111                          | 0.33                                           | <0.16                       | 36.3          | 100           | 25.9                            | 218.3         | 100           | 81                           |

Table 4: Gaseous, dissolved and total mercury species formed and recovered in pure cultures.

On the contrary, most of the recoveries obtained after the Me<sup>201</sup>Hg spike and incubation vary between 85 to 116% with a maximum of results around 100 % suggesting that for the different strains tested and culture medium little to no loss or sorption of Me<sup>201</sup>Hg occur. This result could certainly suggest that most of the labeled MeHg is preferentially remaining in solution or absorbed by the cell cultures.

Results dealing with the distribution between the labile and the total fractions for the different species tagged with the isotope 199 clearly show that two different behaviors are observed (Table 4). For the non methylating SRB strains (i.e. ADR 22, 19) and other general strains (i.e. ADR 04, 09, 10, 15, 20, 08, 25) cultures, an important partitioning of <sup>199</sup>Hg(II) is observed between the labile and sequestered mercury fractions. The sequestered fraction can represent between 50 to 89% of the total fraction. A different behavior is observed for the methylating SRB strains (ADR 13, 14, 21, 26, 28). They display an important potential to sequester all Hg species. Both Me<sup>199</sup>Hg resulting from methylation and the <sup>199</sup>Hg(II) added are recovered in the total fraction whereas no detectable concentrations could be evidenced in the labile fraction. The same type of results is obtained with the spiked Me<sup>201</sup>Hg. The sequestered fractions range from 56 and 73% for the non methylating bacteria and are up to 100% for the methylating bacteria.

These results suggest that sequestration potentials of the Hg species appear to be dependent on the physiology and the ability to transform mercury. For the Hg methylating SRB strains, all Hg species are either co-precipitated or directly associated with the cells by sorption on the cell membranes or by assimilation in the intra-cellular media.

Taking into account the similarity of behavior of the Me<sup>201</sup>Hg added and of the Me<sup>199</sup>Hg formed, two important observations can be outlined. First, the sequestration mechanism is not directly associated to the formation of MeHg since both the MeHg formed and the MeHg added in the medium are similarly sequestered. Second, a specific sequestration potential of Hg would exist for the methylating strains studied. All methylating strain resulted in a sequestration of Hg species. Independently from the methylation location in or outside the cell, Hg is localized in the cell in a similar way to specific resistance mechanisms.

Little is still known of the specific mechanisms associated with uptake or sequestration mechanisms of SRB strain with regards to Hg species. Results obtained on *Escherichia Coli* strains demonstrated that the transport of Hg(II) across the cell membrane is a passive diffusion mechanism (Barkay et al. 1997). More recently, Kelly et al. (2003) have reported that for aquatic bacteria (*Vibrio anguillarum*) an active transport of Hg(II) across the cell membrane will differ according to the physiological state of the bacteria. The uptake mechanisms of MeHg and Hg(II) by Hg resistant *Pseudomonas* K62 has suggested to involve the *mer* genes in the Hg(II) transport but do not participate in the transport of MeHg (Kiyono et al. 1995). Our results would suggest that the uptake by the cells is strain specific with regards to their potential to methylate Hg and certainly involve a combination of Hg with proteins either intracellularly or in the cell walls.



### 3-5- Hg methylation and sequestration as a function of SRB metabolism

In order to further investigate the Hg methylation and sequestration processes by SRB, a series of experiments were performed on the two strains isolated and exhibiting the highest methylation potentials (ADR 21 and 28). These experiments are based on short time incubations with high biomass levels (resting cells). Four assays were realized with and without carbon source and with and without  $\text{MoO}_4^{2-}$ . The results are presented in Table 5.

| strain | Incubation condition                             | Me <sup>201</sup> Hg recovered |            |            | Me <sup>199</sup> Hg formed |            |            | <sup>199</sup> Hg(II) recovered |            |            | Methylation (%) |
|--------|--------------------------------------------------|--------------------------------|------------|------------|-----------------------------|------------|------------|---------------------------------|------------|------------|-----------------|
|        |                                                  | Labile (ng)                    | Total (ng) | Uptake (%) | Labile (ng)                 | Total (ng) | Uptake (%) | Labile (ng)                     | Total (ng) | Uptake (%) |                 |
| ADR 21 | Medium                                           | <0.11                          | 3.56       | 100        | <0.16                       | 45.0       | 100        | <4.6                            | 171        | 100        | 14.3            |
|        | Sterilization                                    | 3.02                           | 3.06       | 1          | <0.16                       | <2.7       |            | 261                             | 297        | 6          | <0.9            |
|        | Medium with $\text{MoO}_4^{2-}$                  | 2.79                           | 2.97       | 3          | <0.16                       | <2.7       |            | 270                             | 297        | 0          | <0.9            |
|        | Medium without C source                          | 2.61                           | 2.75       | 3          | 7.2                         | 8.1        | 6          | 306                             | 315        | 0          | 2.6             |
|        | Medium without C source with $\text{MoO}_4^{2-}$ | 2.57                           | 2.66       | 2          | <0.16                       | <2.7       |            | 234                             | 252        | 0          | <0.9            |
| ADR 28 | Medium                                           | <0.11                          | 2.97       | 100        | <0.16                       | 40.5       | 100        | <4.6                            | 153        | 100        | 12.9            |
|        | Sterilization                                    | 2.61                           | 2.75       | 3          | <0.16                       | <2.7       |            | 297                             | 306        | 0          | <0.9            |
|        | Medium with $\text{MoO}_4^{2-}$                  | 2.75                           | 2.97       | 4          | <0.16                       | <2.7       |            | 198                             | 243        | 10         | <0.9            |
|        | Medium without C source                          | 2.93                           | 3.02       | 2          | 10.3                        | 10.8       | 2          | 216                             | 252        | 8          | 3.4             |
|        | Medium without C source with $\text{MoO}_4^{2-}$ | 2.66                           | 3.06       | 7          | <0.16                       | <2.7       |            | 252                             | 261        | 2          | <0.9            |

**Table 5 :** Partition of Me<sup>201</sup>Hg recovered, Me<sup>199</sup>Hg formed and added <sup>199</sup>Hg(II) and methylation potentials for ADR 21 and ADR 28 for different incubation conditions.

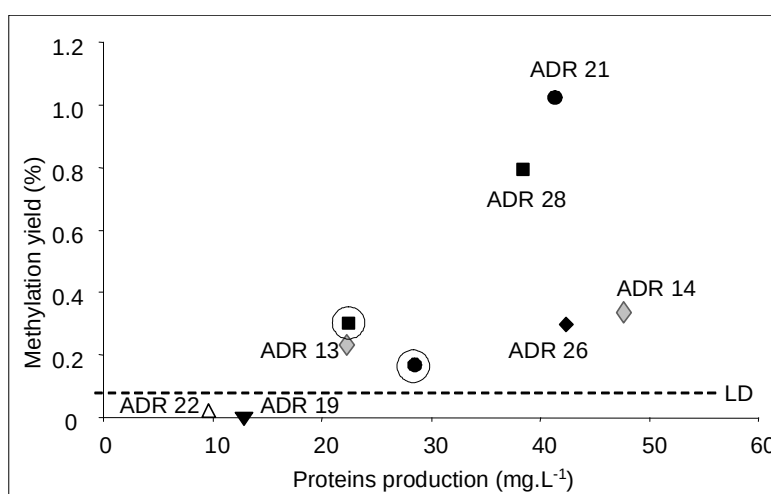
*Methylation* - To verify if the Hg methylation in SRB strains is linked to sulfate reduction activity, we have performed additional inhibition experiments with molybdate ( $\text{MoO}_4^{2-}$ ) which is a known inhibitor of the sulfate reducing activity. The results presented in Table 5 clearly show that no methylation of <sup>199</sup>Hg(II) in absence of sulfate-reduction activity both after addition of  $\text{MoO}_4^{2-}$  and after sterilization occur. The molybdate spike completely inhibits the methylation activity for the two bacterial strains. For the incubations with activity but no bacterial growth (no carbon source), the yield of MeHg formed is much lower than that obtained with the active cultures in the presence of a carbon source.

This indicates that  $\text{MoO}_4^{2-}$  addition plays a similar role than the sterilization with regards to Hg methylation in some SRB cultures. These results also confirm the close link between sulfate reduction and methylation mechanisms (Pak and Bartha 1998, Gilmour et al. 1992 and 1998). They also suggest that an active mechanism responsible for Hg methylation is directly dependant on the bacterial activity. Hg methylation requires energy and is not necessarily limited to sulfate-reducing activities. Benoit et al. (2001) have indeed demonstrated that Hg methylation can take place under fermentative conditions (no sulfate added) by *Desulfobulbus propionicus*.

*Sequestration mechanisms* - The partition between the labile and the total fraction either for the formed or recovered Hg species is also dependant on incubation conditions. When the sulfate reduction activity is inhibited by addition of  $\text{MoO}_4^{2-}$  or by sterilization, more than 90% of recovered Hg species are found in the labile fraction (Table 5). For the series of incubation with bacterial activity but no carbon source, MeHg is formed and more than 90% is recovered in the labile phase. On the contrary, with the cultures displaying high sulfate-reducing activity and in the presence of a carbon source stimulating the growth, MeHg is not found the labile fraction but is totally recovered after acid extraction which again suggests its sequestration by the bacteria.

With an important cellular density and low microbial activity, sequestration of MeHg by cells is less efficient than for fast growing bacteria. The sequestration of Hg species appears to be correlated with active metabolic processes of the bacteria strain such as the synthesis of proteins. These mechanisms would strongly block the MeHg species inside the cell probably associated to thiol containing compounds.

The different results obtained in this study can be summarized in figure 2. When plotting the Hg methylation yields against the amount of protein produced (ie. during bacterial growth), the different distribution patterns depending on the strains are clearly discriminated (Figure 2). For the ADR 21 and 28 (*Desulfomicrobium* strains), Hg methylation potential appears to be the highest when microbial production increases. However, for ADR 13, 14 (*Desulfovibrio* strains) and ADR 26 (*Desulfomicrobium* strain) strains, the production of MeHg appears to be regulated during the bacteria growth since the methylation yields reach a plateau. This suggests that Hg methylation is not directly related to a specific BSR group nor to a specific genus. These results highlight the fact that Hg methylation processes are specific metabolism reactions which expression level varies among the different bacterial strains and not only to the metabolism of the generic groups. These observations confirm the need of study for Hg methylation potentials of environmental isolated bacteria.



**Figure 2:** Hg methylation yield versus proteins production for isolated BSR. Black symbols represent *Desulfomicrobium* group, grey ones *Desulfovibrio* group and white one *Desulfotomaculum* group. Encircled points are relative to resting cells incubation (with associated protein content).

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## **Chapitre D**

### **Synthèse générale**





## Synthèse générale

Les objectifs principaux de ce travail étaient d'utiliser les potentiels des traceurs isotopiques stables à la fois pour la mise en œuvre de méthodes analytiques fiables pour les analyses de spéciation du mercure et des butylétains dans les différentes matrices des environnements aquatiques (eau, sédiment, tissus biologique) et pour l'étude des processus environnementaux régulant le cycle biogéochimique de ces polluants. Les questions initiales reposaient sur la compréhension du cycle biogéochimique et du devenir du mercure et des butylétains dans les environnements côtiers et plus particulièrement sur l'étude des processus de transformations et de transferts dans les différents compartiments. Les différentes parties de ce travail permettent de répondre à ces différents objectifs et d'apporter plusieurs éléments de réponse en proposant des techniques analytiques et expérimentales employant les traceurs isotopiques stables.

### Développement de techniques analytiques pour la spéciation du mercure et des butylétains

Afin de pouvoir appréhender les cycles biogéochimiques du mercure et des butylétains, il a été tout d'abord nécessaire de développer des techniques analytiques de spéciation performantes, justes et précises afin de déterminer les concentrations de ces composés dans les différentes matrices environnementales. Ce développement a été réalisé en utilisant la technique de dilution isotopique permettant la validation de chaque étape de la procédure analytique. Différentes procédures ont été développées dans ce travail démontrant les avantages de la quantification par dilution isotopique qui sont résumés ci-dessous :

- Elle améliore nettement les performances analytiques par rapport aux techniques de quantification traditionnelles.

En effet, la justesse sur les résultats est améliorée car cette technique permet de corriger les pertes éventuelles ou les rendements non quantitatifs dus à des effets de matrice. Elle offre aussi la possibilité de suivre les dégradations ou les transalkylations des différentes espèces chimiques qui sont susceptibles de se produire pendant les différentes étapes du protocole analytique. Par la détection et l'estimation du rendement de ces transformations, les résultats vont pouvoir être corrigés et donc être améliorés en terme de justesse.

Les différents résultats présentés dans ce travail montrent aussi l'important potentiel de cette technique pour améliorer la précision des résultats par rapport aux techniques conventionnelles. En effet, en comparant systématiquement les résultats obtenus par dilution isotopique à ceux obtenus par quantification classique (étalonnage externe, ajouts dosés), il apparaît que la dilution isotopique est nettement plus précise pour l'analyse des composés du mercure et des butylétains.

- Elle représente une technique unique pour l'évaluation des méthodes analytiques de spéciation.

En effet l'utilisation d'espèces enrichies isotopiquement a montré la possibilité de tester et de valider les techniques analytiques en permettant la détection des erreurs pour chaque étape de la procédure analytique. Ce point est essentiel pour le développement analytique des méthodes de spéciation qui souffrent souvent d'un manque de matériaux de référence certifiés pour la validation des résultats. Sa capacité à pouvoir évaluer et valider les méthodes analytiques est démontrée dans la première partie de ce travail. Ce potentiel pour le diagnostic analytique fait d'elle une méthode de référence pour les analyses de spéciation.

- Elle a permis le développement de nouvelles techniques analytiques pour la spéciation du mercure et des butylétains dans les matrices environnementales.

Le premier objectif de ce travail est de mettre à profit les avantages de la quantification par dilution isotopique afin de déterminer simultanément les concentrations en MMHg et TBT dans des tissus biologiques. Une méthode analytique rapide, juste et précise a été développée proposant des conditions d'extraction optimum pour extraire simultanément le MMHg et le TBT d'un tissu d'huître ainsi que des conditions analytiques pour le couplage GC-ICPMS. La procédure de mise à l'équilibre du traceur isotopique avec l'échantillon a aussi été améliorée permettant de réduire le temps de préparation de l'échantillon à cinq minutes.

Le développement d'une méthode pour la détermination simultanée des espèces du mercure et des butylétains dans les eaux naturelles a aussi été réalisé. Les difficultés analytiques généralement rencontrées pour ce type d'échantillon du fait des très faibles concentrations et des effets de matrice importants ont pu être contournées. Les résultats montrent que la méthode de propylation est la technique de dérivation la plus appropriée à l'analyse d'échantillons environnementaux d'eau de mer car son efficacité est peu affectée par les effets de matrice. La méthode a été appliquée à des échantillons réels d'eaux de mer et de neiges montrant son important potentiel pour l'analyse de ces composés à des concentrations environnementales seuils.

Enfin une technique analytique juste pour la détermination du MMHg dans les sédiments a été développée. Grâce à l'utilisation de traceurs isotopiques, il a été possible de suivre la formation accidentelle de MMHg pendant la préparation d'échantillon. A la vue des résultats, il apparaît que l'artéfact de méthylation ne se produit pas pendant les étapes de mise à l'équilibre de l'ajout et d'extraction. Les réactions de transalkylation semblent plutôt se produire dans la phase organique finale pendant l'éthylation. Une extraction supplémentaire dans un solvant organique a été insérée afin de réduire la quantité de IHg avant l'éthylation.

Malgré tous les avantages que présente la technique de dilution isotopique pour les analyses de spéciation, il est néanmoins nécessaire de citer ses limitations. Tout d'abord, à l'heure actuelle, il n'existe pas de standards commerciaux d'espèces organométalliques enrichies isotopiquement. Il est donc nécessaire de synthétiser les espèces recherchées ce qui peut représenter une étape critique. De plus, le prix de cette technique est encore relativement important du fait du coût élevé des métaux enrichis isotopiquement. Afin de

rendre cette technique accessible et surtout de pouvoir l'utiliser en routine, il est donc nécessaire de fournir des espèces organométalliques enrichies isotopiquement et d'en réduire leur coût.

### **Suivi des transferts et transformations des composés du mercure et des butylétains dans les environnements aquatiques**

Les techniques analytiques développées dans la première partie de ce travail, ont ensuite été utilisées pour l'analyse d'échantillons environnementaux afin d'étudier les cycles biogéochimiques du mercure et des butylétains. Les deux volets de cette étude consistaient à comprendre les processus de transferts et des transformations de ces polluants aux interfaces des environnements aquatiques.

➤ Tout d'abord, le transfert vers les organismes benthiques a été étudié. Une étude multidisciplinaire entre chimistes et biologistes a été conduite afin d'étudier la bioaccumulation des métaux et organométaux dans la macrofaune benthique de l'estuaire de l'Adour. Une méthode intégrée d'échantillonnage et d'analyse a été développée. L'échantillonnage a été réalisé de manière à classer les individus par groupes trophiques (suspensivores, dépositivores et prédateurs). Cette méthode a permis de déterminer la contamination et le transfert trophique des métaux et organométaux de la population benthique de l'estuaire de l'Adour. La biodiversité des organismes benthiques semble être liée à la contamination des sédiments. La bioaccumulation est dépendante du type de polluants métalliques et du groupe trophique. Les dépositivores sont généralement plus impactés par une contamination directe du fait de leur mode d'alimentation. La biomagnification parmi les groupes trophiques benthiques est démontrée uniquement pour le MMHg.

➤ Le principal objectif de ce travail était enfin de mettre au point des méthodes expérimentales utilisant les traceurs isotopiques stables afin de comprendre les processus biogéochimiques du cycle du mercure dans les différents compartiments des environnements aquatiques. Ce travail a permis la détermination des potentiels de méthylation, déméthylation et réduction du mercure dans les sédiments et la colonne d'eau ainsi que le transfert du MMHg vers le phytoplancton et les bactéries. La recherche systématique des facteurs prépondérants influençant ces réactions a aussi été mise en œuvre afin de déterminer l'influence des activités biologiques (bactériologique et phytoplanctonique) et des paramètres physicochimiques.

- Dans une première étude, les taux de méthylation et de déméthylation régulant la concentration en MMHg dans les sédiments et la colonne d'eau de l'étang de Thau où l'activité ostréicole représente un intérêt économique important. Des expérimentations in situ sont développées grâce à l'utilisation de traceurs isotopiques stables pour les différents compartiments. Les méthodes d'incubation ont été évaluées en terme de sensibilité et de justesse pour tracer les processus environnementaux. Les résultats ont montré que l'outil isotopique permet de suivre

avec justesse et précision les différentes transformations du mercure dans les sédiments et la colonne d'eau. Les activités biologiques (microbiologique, phytoplanctonique), qui jouent un rôle important dans ce type d'écosystème côtier, semblent être liées à la méthylation du mercure dans la colonne d'eau. Par comparaison des différents flux de transformations et de transferts à l'interface eau-sédiment, il semble que les processus de transformations sont plus importants que les échanges entre l'eau et les sédiments. Enfin, la déméthylation dans les sédiments apparaît comme un processus important régulant les concentrations en MMHg dans l'étang de Thau.

- Une deuxième étude a permis pour la première fois d'établir des taux de transformations des espèces de mercure dans les eaux de surface de la Méditerranée. L'utilisation de traceurs isotopiques a permis la détection de très faibles taux de transformations même à des niveaux de concentrations très bas. La méthylation du Hg est fortement corrélée à l'activité des micro-organismes (bactérie et phytoplancton). Les réactions photochimiques semblent majoritairement gouvernées la déméthylation et la réduction mais des processus biotiques secondaires existent. Ces résultats mettent en évidence la méthylation du IHg dans la zone euphotique ainsi qu'un transfert du MMHg vers le phytoplancton ce qui établit une nouvelle source potentielle de MMHg dans les environnements côtiers et marins.

- Enfin les potentiels de méthylation et de déméthylation ont été déterminés pour des souches bactériennes isolées d'un sédiment estuarien. Des études préliminaires sur ce sédiment avaient mis en évidence son potentiel de méthylation lié à l'activité de bactéries anaérobies. Le but de cette étude était d'isoler les différentes souches bactériennes présentes dans ce sédiment et de déterminer les potentiels de méthylation et de déméthylation pour chacune d'entre elles. L'utilisation d'espèces enrichies isotopiquement a permis de déterminer simultanément les taux de ces processus réversibles tout en utilisant des niveaux de concentrations naturels. Les résultats montrent que plusieurs souches bactériennes sont capables de méthyler le mercure, toutes appartenant au groupe des bactéries sulfato-réductrices. Ils suggèrent que les processus de méthylation du mercure sont régulés par des métabolismes spécifiques à certaines souches et pas nécessairement à des groupes phylogénétiques. Ces bactéries méthylantes sont non seulement très résistantes au MMHg mais elles sont aussi capables de séquestrer efficacement les espèces de mercure par assimilation intracellulaire ou sorption sur les membranes cellulaires.

## **Chapitre E – Annexe I**

**Revue sur les potentiels et limites de l'analyse de spéciation par dilution isotopique pour la métrologie et l'évaluation de la réactivité environnementale**



# Potential and limits of speciated isotope-dilution analysis for metrology and assessing environmental reactivity

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This paper describes the application of isotopically labelled elemental compounds for species-specific isotope-dilution (ID) analysis and for species transformation evaluation with the focus on organotin species and methylmercury in both analytical and environmental studies. Among others, the accurate measurement of organometallic compounds (speciation) in environmental samples can be hampered by processes such as incomplete extraction from solid samples, material loss, rearrangement reactions during extraction and/or derivatization, low recoveries, and signal drift. All these undesirable effects, with the exception of incomplete species extraction from the sample matrix, can be studied and/or corrected for using speciated ID analysis (IDA). The use of compounds enriched in stable isotopes with subsequent detection by inductively coupled plasma mass spectrometry (ICP-MS) is a powerful tracer technique to study dynamic environmental processes. We also present an overview of other work published in this area. We discuss limitations of the applicability of speciated IDMS.

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**Keywords:** Isotope dilution; Lead speciation; Mercury speciation; Metal speciation; Tin speciation

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## 1. Introduction

Though numerous questions about the biogeochemical cycle of metals in the environment have been answered, many still remain unresolved. Sensitive, specific and precise analytical methods are needed to perform studies at ambient levels. Accurate determinations of metal species are critical to understanding the biogeochemical cycling of the contaminant and estimating associated potential exposures of aquatic food webs. In particular, accurate determinations of the organometallic species monomethylmercury (MMHg) and tributyltin (TBT) pose a challenge to the analytical chemist.

Speciation analysis (i.e. the determination of specific chemical forms of an element) may be achieved in a number of ways [1]. Most commonly, the analyte species are extracted from the sample matrix, ensuring that there are no losses of, or alterations to, the different chemical forms. This is followed by separation of these chemical forms using a chromatographic technique, such as high-performance liquid chromatography (HPLC), gas chromatography (GC), supercritical fluid chromatography (SFE), or capillary zone electrophoresis (CZE). A number of detection methods are available, but its sensitivity, multi-elemental capability and the possibility of isotopic information make ICP-MS the detector of first choice.



Trends

Trends in Analytical Chemistry, Vol. 23, No. 3, 2004

Despite significant improvements in instrumentation, the quality of the results is mostly associated with sample pre-treatment stages [2] (e.g., GC-ICP-MS is only suitable for volatile species, and derivatization reactions are required prior to the detection of originally ionic compounds). Additionally, the analysis of solid samples, such as sediments or bio tissues, requires a leaching/digestion step prior to detection. However, there are traditional problems related to non-quantitative recoveries and, more recently, questions have arisen about the formation of artifacts and transformations of species during sample preparation.

To tackle the present problem of speciation data validation, the use of ID techniques offers great potential since quantitative recoveries are not necessary and rearrangement reactions are easily detected. Provided the enriched isotope species is present in an equilibrated and equivalent state to the natural isotope species, it can perform the role of the ideal internal standard. ID mass spectrometry (IDMS) has been considered to be a definitive method, offering the potential of small uncertainties [3].

Examples of species-specific ID (SSID) have been published for the speciation of iodine [4,5], selenium [6–8], chromium [9–11], lead [12,13], thallium [14], mercury [15–20], and tin [20–22]. Not only are they useful for routine determination, speeding analysis, but they also greatly assist in testing and diagnostics of new analytical methods and techniques. In particular, approaches using multi-labelled-multi-species spikes allow correct species concentrations to be obtained by calculating degradation factors from one species into another, in case rearrangement or decomposition reactions occur.

However, the application of stable isotopes in environmental studies is not limited to the classical IDA. Stable enriched tracers have an even greater potential in investigating dynamic environmental processes. With MS detection, it is possible to use enriched stable isotope tracers at sub-natural levels because of the high selectivity and sensitivity of the instrumentation. The technique is suitable for recording the transformations of different isotope-tracer species added to a system if they are produced from different enriched isotopes (e.g., information about methylation and de-methylation of mercury and methylmercury, respectively, can be extracted from a single experiment). Furthermore, it is possible to apply SSID for standardisation. In this way, the accuracy and precision of measurements are improved.

In this article, we discuss possible applications of isotopically enriched stable isotopes in speciation studies. We study the different steps in IDA, from the instrumental parameters that need to be evaluated to get precise isotope-ratio measurements to the synthesis and characterization of isotopically enriched species. We highlight the application of direct IDA as well as the

study of species transformations during both the analytical procedure and environmental studies. We also outline limitations of this technique.

## 2. Optimisation of instrumental parameters

For the application of speciated ID using online coupled techniques (HPLC-ICP-MS or GC-ICP-MS), instrumental separation and detection parameters must be carefully balanced with each other.

### 2.1. Separation conditions

Aspects to be considered relate on the one hand to the chromatographic species separation and on the other hand to ICP-MS acquisition constraints. When capillary GC (CGC) is employed as separation technique, very short transient signals are obtained. Here, ICP-MS data acquisition must be fast enough to realise good peak definition (i.e. a minimum of 15 data points per peak). Therefore, the number of isotopes measured must be balanced with dwell time per isotope and peak width. For example, there is a significant difference in the measurement of methylmercury, when changing from packed to capillary column: with a peak width of 60 s for packed column applications, isotope dwell times are not an important issue, but a peak width of 1.2 s with a capillary GC needs careful evaluation of the suitable number of isotopes and dwell times employed [15,16]. Clear advantages of capillary over packed column are superior separation power (i.e. peak resolution) and unequalled detection limits.

An example for CGC-ICP-MS operating conditions is given in Table 1.

Another important aspect is peak shape. Because isotope ratios are most commonly calculated on the basis of integrated peak areas, the chromatographic separation should deliver symmetrical peaks with no tailing in order to minimise peak integration errors.

### 2.2. ICP-MS detection conditions

Apart from spectral interferences and detector linearity, detector dead time, mass bias and data acquisition parameters need to be optimised.

**2.2.1. Detector dead time.** If the detection system is an electron multiplier operated in the pulse-counting mode, there is a finite time before and after each pulse during which the detector does not record (i.e. it is “dead”). If another ion impinges on the surface of the detector within this time, it will not be detected. This means that the actual count rate is higher than the observed rate. The dead time arises from the electronics of the detector system (amplifier, discriminator and counting system). The dead time of the ICP-MS instrument normally lies in the range of 15–100 ns.



**Table 1.** CGC-ICP-MS Operating conditions for MeHg determination by SSIDMS

|                                                          |                                                           |
|----------------------------------------------------------|-----------------------------------------------------------|
| Injector parameters                                      |                                                           |
| Mode                                                     | Splitless                                                 |
| Temperature                                              | 200°C                                                     |
| Injected volume                                          | 2 µL                                                      |
| GC parameters                                            |                                                           |
| Column                                                   | MXT Silcosteel, 30 m, i.d. 0.53 mm, d <sub>f</sub> 1.0 µm |
| Carrier gas                                              | Helium, 25 mL/min                                         |
| Make up gas                                              | Argon, 300 mL/min                                         |
| GC program                                               | 60°C/ ramp 50°C/min/180°C                                 |
| Transfer line                                            |                                                           |
| Length: 1 mInner: Silcosteel, i.d. 0.28 mm, o.d. 0.53 mm |                                                           |
| Outer: Silcosteel, i.d. 1.0 mm, o.d. 1/16"               |                                                           |
| ICP-MS parameters                                        |                                                           |
| Rf power                                                 | 1250 W                                                    |
| Gas flow:                                                |                                                           |
| Plasma                                                   | 15 L/min                                                  |
| Auxiliary                                                | 0.9 L/min                                                 |
| Nebuliser                                                | 0.9 L/min                                                 |
| Dwell time                                               |                                                           |
| 40 ms for <sup>201</sup> Hg; <sup>202</sup> Hg           |                                                           |
| 10 ms for <sup>203</sup> Tl; <sup>205</sup> Tl           |                                                           |

The effect of the detector dead-time,  $\tau$ , on the measured count rate can be expressed as

$$I_{\text{real}} = I_{\text{exp}} / (1 - \tau \times I_{\text{exp}}) \quad (1)$$

where  $I_{\text{real}}$ =real count rate, and  $I_{\text{exp}}$ =measured count rate.

The dead time may be calculated experimentally by different procedures (e.g., by applying the method proposed by Held and Taylor, using the following equation [23]:

$$\frac{R(^{202}\text{Hg}/^{201}\text{Hg})_{\text{theo}}}{R(^{202}\text{Hg}/^{201}\text{Hg})_{\text{exp}}} = 1 + [R(^{202}\text{Hg}/^{201}\text{Hg})_{\text{theo}} - 1] \times \tau \times I_{\text{exp}}(^{202}\text{Hg}) \quad (2)$$

where

$R(^{202}\text{Hg}/^{201}\text{Hg})_{\text{theo}}$ =theoretical isotope ratio,  
 $R(^{202}\text{Hg}/^{201}\text{Hg})_{\text{exp}}$ =measured isotope ratio,  
 $I_{\text{exp}}(^{202}\text{Hg})$ =measured count rate for isotope <sup>202</sup>Hg and  $\tau$ =detector dead time.

Plotting  $R(^{202}\text{Hg}/^{201}\text{Hg})_{\text{theo}}/R(^{202}\text{Hg}/^{201}\text{Hg})_{\text{exp}}$  over  $I_{\text{exp}}(^{202}\text{Hg})$  yields a straight line, from the slope of which ( $m=[R(^{202}\text{Hg}/^{201}\text{Hg})_{\text{theo}}-1] \times \tau$ ) the dead time can be calculated.

It should be noted that the detector dead time is not a fixed parameter and that it varies according to the age of the detector. It should therefore be re-evaluated at regular intervals.

**2.2.2. Mass bias.** Mass bias is another factor causing deviations of measured isotopic ratios from the expected ratios. Mass bias derives from the different transmission of ions of different mass from the point at which they enter the sampling device until they are finally detected by the electron multiplier. The true cause of these effects is not completely understood, but the overall result is an apparent increase in signal for the heavier isotopes, which distort the observed ratio.

The only way to overcome this problem is to analyse a certified reference material (CRM), such as NIST 981, 982 or 983, and compare the observed abundance ratios with the certified ones. The mass bias correction factor can then be calculated by using: Correction factor=true (certified value) ratio/experimental ratio. The correction factor may then be applied to all subsequent analyses. This approach describes the so-called "bracketing", where isotope-ratio standards are measured at regular intervals between the samples.

Another approach is the simultaneous mass bias correction using a suited isotope pair with known isotope ratio, which is mixed to the analyte (e.g., Tl for mass bias correction of lead isotopes). This approach was realized in the Rodríguez et al. wet plasma coupling, where thallium solution was introduced simultaneously during the GC measurements of methylmercury [16]. Mass bias for Hg isotopes was then corrected by using the <sup>205</sup>Tl/<sup>203</sup>Tl isotope ratio. The use of the Tl correction system has several benefits, mainly because, as the analyte introduced via GC and the solution used for mass bias correction are measured simultaneously, changes during a single measurement or drift occurring during the day are automatically detected and can be corrected for. Additionally, fewer measurements are required, and that allows instrument run time and costs to be reduced.

The following correction equation was applied [24]:

$$R(^{202}\text{Hg}/^{201}\text{Hg})_{\text{true}} = \frac{R(^{202}\text{Hg}/^{201}\text{Hg})_{\text{exp}}}{1 + an} \quad (3)$$

where  $a$  is the bias per mass unit and  $n$  the mass difference between isotopes <sup>202</sup>Hg and <sup>201</sup>Hg. Factor  $a$  was determined by comparison of  $(^{205}\text{Tl}/^{203}\text{Tl})_{\text{experimental}}$  with  $(^{205}\text{Tl}/^{203}\text{Tl})_{\text{theoretical}}$ .

Using this Tl correction, the accuracy was found to be 0.2%; without mass bias correction, the deviations were usually more than 1.5%. This demonstrates that, for SIDMS of mercury species, Tl is an adequate reference element for mass bias correction. This correction was also successfully used for lead [25]. For tin speciation, the same approach — using continuous nebulisation of an antimony solution (<sup>121</sup>Sb and <sup>123</sup>Sb)—was recently successfully employed [22].

### 2.3. Data-acquisition parameters

Parameters, such as the number of points per mass unit, the number of isotopes monitored and the integration time per isotope, need to be evaluated for the measurement of precise isotope ratios in fast chromatographic-ICP-MS peaks. Compromise conditions for data-acquisition parameters have to be selected, because of the opposite influence of integration time on sensitivity and peak profiles. The fast transient chromatographic peak is well defined at very short integration times. On the one hand, an increase in instrumental noise is then detected (as a result of worse counting statistics). On the other hand, by using very long integration times, peak definition worsens dramatically, spectral skew is observed and peak areas exhibit poor reproducibility. Eventually, IDA was performed using the tin isotopes 118, 119 and 120, and a 66-ms integration time per isotope [26].

### 3. IDA procedure

The general procedure for speciation analysis in a solid sample is illustrated in Fig. 1. ID is based on the addition of isotopically altered species to the sample matrix. Ideally, the amount of spike species added should result in an isotope ratio of spike to reference isotope close to 1. Equilibration of the spiked labelled species with the natural abundant species in the sample alters the isotope ratio that is measured and used for calculation.

For the results to be accurate, it is critical to achieve effective equilibrium of the enriched spike and the sample. If the sample is a liquid, gentle mixing will ensure that a homogeneous mixture is obtained. If the sample is a solid material, precautions should be taken to prevent preferential extraction of the spiked species over the analyte species. It is advisable to allow the spike to equilibrate ('soak in') for a minimum of 24 h before any extraction process is attempted. But, recently, a rapid method was developed (2 min) giving yield to good precision (uncertainty range 0.7–13.7%) using a simultaneous microwave extraction and spiking procedure [22]. However, this approach may be used only during the extraction step of quantitative recovery of species.

After spiking and stabilisation of the sample, extraction and chromatographic analysis are performed. Different extraction procedures have been used, such as distillation, microwave extraction or ultrasonication. For GC, a previous derivatization step is necessary, and, for LC, a pre-concentration is required. By ICP-MS detection, a chromatogram will be obtained for each isotope with peaks corresponding to individual analyte species. The area under each peak is determined for the spike and the reference isotope, and the isotope ratio is calculated. Recently, it has been shown that the preci-

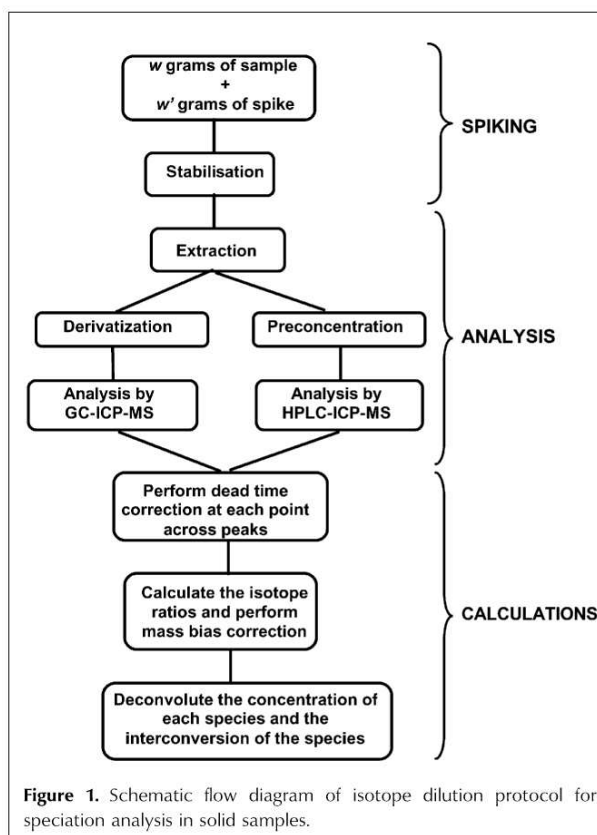


Figure 1. Schematic flow diagram of isotope dilution protocol for speciation analysis in solid samples.

sion obtained in isotope-ratio determinations can be improved by applying either the determination of ratios on single points in the peak in a "steady state" approach [27], or by defining a limited integration zone on the basis of the evolution of isotope ratio during peak elution [28].

The concentration of the species in the samples is calculated by applying the ID equation [3]:

$$c = \frac{c' w' A_r(RY' - X')}{w A_r'(X - RY)} \quad (4)$$

For the sample,  $c$ =concentration of sample solution;  $w$ =mass of sample solution;  $A_r$ =relative atomic mass of the element being determined;  $X$ =isotope abundance (atom-%) isotope 1; and,  $Y$ =isotope abundance (atom-%) isotope 2.

For the spike,  $c'$ =concentration of the spike solution;  $w'$ =mass of the spike solution;  $A_r'$ =relative atomic mass of the element in the spike;  $X'$ =isotope abundance (atom-%) isotope 1; and,  $Y'$ =isotope abundance (atom-%) isotope 2.

The final parameter that requires measurement is the isotope ratio,  $R$  ( $X/Y$ ). Apart from  $R$ , the other parameters are constants, masses and the concentration of the spike solution. Thus, the concentration in the unknown sample is ultimately based on the measurement of  $R$  and the specification of the spike material.



#### 4. Synthesis of isotopically enriched species

For the purpose of speciation analysis, it is essential that the added spike of enriched isotope and the indigenous element species for determination are present in the same chemical form. Thus, despite the substantial benefits on offer, IDA has remained an under-exploited method of analysis, primarily because of the lack of commercially available isotopically labelled species standards. In many cases, it is necessary to synthesise the relevant species from the isotopically enriched element.

The high cost of the enriched starting material for synthesis of isotopically enriched compounds necessitates the use of small quantities only. Synthetic methods for manufacture are readily available, but application of these methods on a small scale (<1 g) revealed several difficulties. For the same economic reasons, purification processes involving wasteful separations methods should be avoided.

Synthetic routes for the successful small-scale preparation of isotopically labelled organometallic compounds therefore require, whenever possible, the use of reasonably stable reagents, along with maintenance of high reaction yields, ease of product isolation and generic preparation apparatus. Such methods for the small-scale preparation of MMHg and organotin compounds have been developed.

Isotopically enriched MMHg has been synthesised from  $^{201}\text{HgO}$  using methylcobalamin [29]. Conditions were adapted to work on micro-scale (using only 150  $\mu\text{g}$   $^{201}\text{Hg}$ ) to get the maximum yield of MMHg and to try to avoid formation of dimethylmercury. The synthesised  $\text{MM}^{201}\text{Hg}$  was extracted with toluene. Recently, Clough et al. have synthesised, purified and characterised, by 1-H nuclear magnetic resonance spectro-

scopy (NMR), an Hg-199-enriched methylmercury chloride standard [30]. The stability of methylmercury during the analytical procedure was investigated. No evidence of decomposition was found.

Synthetic routes for the successful small-scale preparation of isotopically labelled butyl- and phenyl-substituted organotins have been established [31]. Organotin iodides were prepared from redistribution reactions between tin (IV) iodide and the corresponding tetraorganotin, which was prepared directly from the reaction between tin metal and iodobutane. These compounds were characterised by  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy.

Other procedures have been described for the synthesis of isotopically labelled DBT by reaction of  $^{118}\text{Sn}$ -enriched tin with butyl iodide in the presence of tetrabutylammonium iodide as catalyst and dimethylformamide as solvent [21]. A mixed spike containing  $^{119}\text{Sn}$ -enriched monobutyltin (MBT), dibutyltin (DBT) and TBT was prepared by direct butylation of  $^{119}\text{Sn}$ -enriched metal using a 1:3 molar excess of butylchloride with iodide and triethylamine as catalyst.

#### 5. Characterisation and analysis of synthesised product by reverse ID

Synthesised isotopically enriched species standards need to be characterized with respect to isotopic composition, purity and concentration. In Fig. 2, a chromatogram is shown for an isotopically enriched  $\text{MM}^{201}\text{Hg}$  standard. Natural isotope abundance is included for comparison. The MMHg determination was carried out by CGC-ICP-MS after ethylation. The isotopic composition was calculated from the measured peak areas [29]. The solution showed a  $^{202}\text{Hg}/^{201}\text{Hg}$  ratio of 0.0146,

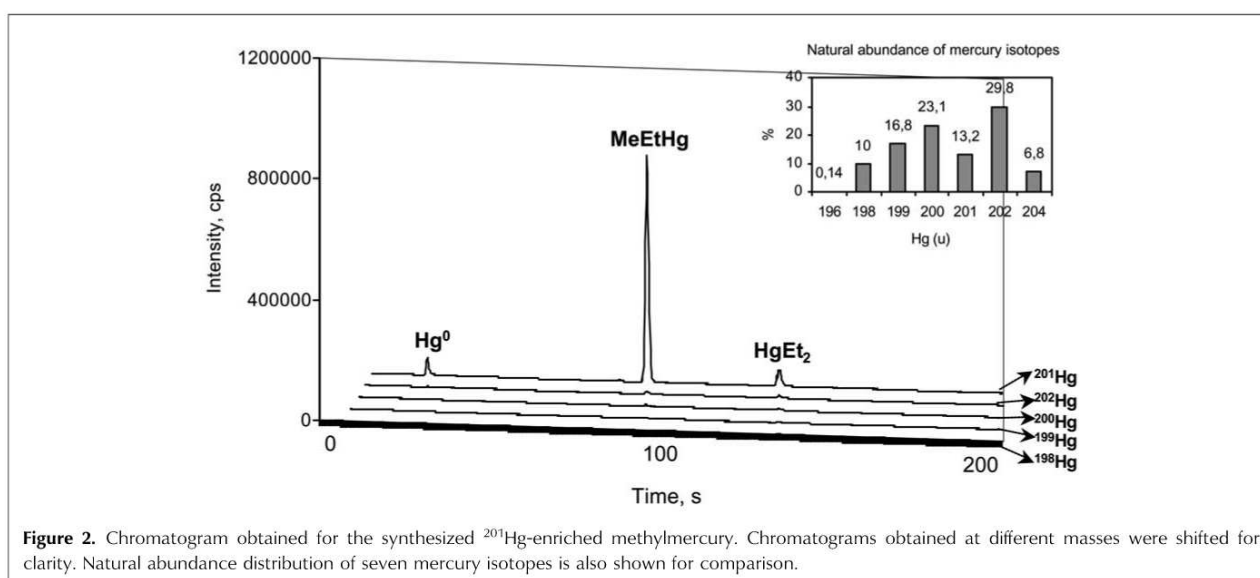


Figure 2. Chromatogram obtained for the synthesized  $^{201}\text{Hg}$ -enriched methylmercury. Chromatograms obtained at different masses were shifted for clarity. Natural abundance distribution of seven mercury isotopes is also shown for comparison.

greatly differing from the natural  $^{202}\text{Hg}/^{201}\text{Hg}$  ratio of 2.2655.

As an example for tin, the isotopic composition of the  $^{118}\text{Sn}$ -enriched DBT was calculated from the measured peak-area ratios after mass bias correction using

isotopically natural abundant Sn DBT species. This solution, with a  $^{120}\text{Sn}/^{118}\text{Sn}$  ratio of 0.0063, greatly different from the natural  $^{120}\text{Sn}/^{118}\text{Sn}$  ratio of 1.3452, will be used directly (spike solution) to perform IDA of DBT in real samples [21].

**Table 2.** Applications of speciated IDA

| Analyte  | Comments                                                                                                                                                                                                                                                                                                                                                                               | Ref. |
|----------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| Tin      | Preparation and characterization of isotopically enriched organotin compounds.                                                                                                                                                                                                                                                                                                         | [31] |
|          | Synthesis and analysis of $^{199}\text{Sn}$ -enriched MBT, DBT and TBT.                                                                                                                                                                                                                                                                                                                | [26] |
|          | An overview of HPLC-ID-ICP-MS for speciation studies.                                                                                                                                                                                                                                                                                                                                  | [33] |
|          | Different liquid-solid extraction techniques, including room-temperature leaching for IDA in sediments using GC-ICP-MS.                                                                                                                                                                                                                                                                | [21] |
|          | Simultaneous determination of MBT, DBT and TBT in sediments by IDA using GC-ICP-MS.                                                                                                                                                                                                                                                                                                    | [26] |
|          | Rapid and precise determination of TBT in sediments and biological samples by SSIDMS with microwave extraction-ethylation-CGC-ICP-MS. A new labelled TBT standard provided by LGC Limited was used. Different mass bias correction and spiking procedures are tested and compared.                                                                                                     | [22] |
|          | A method is described for accurate and precise determination of TBT by SSIDMS in sediments using SPME in combination with GC-ICP-MS.                                                                                                                                                                                                                                                   | [35] |
|          | A method for the simultaneous determination of MBT, DBT and TBT in coastal sea-water based on the application of ID methodologies using a $^{199}\text{Sn}$ -enriched spike.                                                                                                                                                                                                           | [34] |
|          | Different liquid-solid extraction techniques, including room-temperature leaching with mechanical shaking, ultrasonic and microwave-assisted extractions, have been evaluated for the quantitative speciation of tin for MBT, DBT and TBT in PACS-2 and BCR-646 CRMs.                                                                                                                  | [44] |
|          | Accelerated solvent extraction is evaluated for butyltin speciation in PACS-2 CRM. A double-enriched spike containing $^{199}\text{Sn}$ -enriched MBT and TBT and $^{118}\text{Sn}$ -enriched DBT allowed for the simultaneous determination of the three butyltin species and factors governing species interconversion.                                                              | [45] |
| Mercury  | Micro-scale preparation and characterization of isotopically enriched MMHg.                                                                                                                                                                                                                                                                                                            | [29] |
|          | Synthesis, purification and characterization by $^1\text{H}$ NMR spectroscopy of $^{199}\text{Hg}$ -enriched methylmercury chloride.                                                                                                                                                                                                                                                   | [30] |
|          | The stability of MMHg during the analytical procedure was investigated.                                                                                                                                                                                                                                                                                                                |      |
|          | Determination of MMHg in biological samples using GC-ICP-MS.                                                                                                                                                                                                                                                                                                                           | [16] |
|          | Various extraction techniques, such as distillation, acid and alkaline extraction, have been tested with regard to their potential to form a MMHg artifact from inorganic mercury, $\text{Hg}^{2+}$ is added to different reference materials in the form of enriched stable tracers and the formation of new methylmercury from that tracer is analysed by HPLC-ICP-MS and GC-ICP-MS. | [37] |
|          | Ultrasonic nebulisation was developed to couple HPLC separation and ICP-MS detection. The isotope-specific detection allows the application of the species-specific isotope addition method for the determination of methylmercury with a correction for artifact formation.                                                                                                           | [38] |
|          | SSID calibration for determination for $(\text{CH}_3)_2\text{Hg}$ , $\text{CH}_3\text{HgCl}$ and $\text{HgCl}_2$ in natural gas condensate. The determination was carried by both GC-ICP-MS and GC-furnace atomisation plasma ionisation mass spectrometry (FAPIMS).                                                                                                                   | [32] |
|          | Development of an ID-based GC-ICP-MS method, which can simultaneously determine incipient concentrations of methylmercury as well as the degree of methylation of inorganic mercury and de-methylation of methylmercury in brackish water sediment.                                                                                                                                    | [43] |
|          | Isotopically labelled MMHg was used to validate the derivatisation procedure by sodium tetraethylborate and to get a better knowledge of chloride interference mechanism in environmental water samples.                                                                                                                                                                               | [36] |
|          | Formation of artifact MMHg in IAEA 405 sediment reference material was studied. MMHg was extracted by both aqueous distillation and leaching with dilute solutions of nitric acid. MMHg was formed from both ambient and added inorganic mercury in extracts of IAEA 405 by either technique.                                                                                          | [42] |
|          | MMHg determination in fish tissues by SPME in combination with GC-ME and ICP-MS detection. Samples are digested with methanolic potassium hydroxide and the analytes were propylated.                                                                                                                                                                                                  | [18] |
| Lead     | Lead speciation in rain-water by HPLC-ID-ICP-MS. Reversed phase ion-pairing chromatography. Artificial rainwater analysed. Limits of detection = 3 ng/g trimethyllead and 14 ng/g triethyllead.                                                                                                                                                                                        | [12] |
| Chromium | Correction of species transformations in the analysis of Cr(VI) in solid environmental samples using speciated isotope dilution.                                                                                                                                                                                                                                                       | [11] |
|          | Accuracy in species analysis: SIDMS exemplified by the evaluation of chromium species.                                                                                                                                                                                                                                                                                                 | [9]  |
| Selenium | Selenite and selenate were determined at sub- $\mu\text{g/L}$ levels in natural waters by MIP-MS                                                                                                                                                                                                                                                                                       | [8]  |
| Thallium | Development of an IDMS method for dimethylthallium speciation and first evidence of its existence in the ocean.                                                                                                                                                                                                                                                                        | [14] |



The concentration of the enriched organometallic compounds in the spike solutions and the obtained reaction yield are calculated using reverse IDA. Experimentally, the spike solution is treated as a sample and is itself spiked with a standard (of known concentration and purity) that has a natural isotopic abundance. In Equation (4) above, the sample and the spike values are interchanged.

## 6. Application of ID for speciation analysis

Several applications of both GC and HPLC-ID-ICP-MS have been published. These include elements such as Hg, Sn, Pb, Cr, Mo and Tl. A short summary of these applications is given in Table 2. Not only are they useful for routine determination but they also greatly assist in testing and diagnostics of new analytical methods and techniques.

### 6.1. Species-specific ID analysis (SSIDA)

Accurate ID speciation methods have been proposed for several compounds and materials.

A method has been developed for MMHg determination in biological samples by CGC-ICP-MS [16]. Optimum conditions were established for the measurement of isotope ratios on the fast transient chromatographic peak. Mass bias was corrected by using the simultaneously measured thallium signals at  $^{205}\text{Tl}$  and  $^{203}\text{Tl}$ . MMHg was microwave extracted from the solid and then ethylated.

The procedure was applied to two fish-muscle CRMs (DORM-1 and CRM-463) with excellent results. A chromatogram obtained for CRM463 spiked with  $^{201}\text{Hg}$ -enriched monomethylmercury solution is shown in Fig. 3 (for masses 201 and 202). As can be seen, the isotopic composition of methylmercury has been altered drastically; a measured 202/201 isotope ratio of 1.21 instead of 2.26 was determined. The proposed procedure may serve as a reference method in control laboratories.

Isotopically enriched  $(\text{CH}_3)_2\text{Hg}$ ,  $\text{CH}_3\text{HgCl}$  and  $\text{HgCl}_2$  were prepared and then used for SSID calibration and method assurance tests for mercury species determination in natural gas condensate [32]. The method developed gives excellent detection limits and improved matrix tolerance compared with other speciation approaches for organic matrix samples.

Hill et al. applied HPLC-ID-ICP-MS for lead and tin speciation [33]. For organotin determination, a spiking experiment in which known amounts of analyte species were spiked into a sediment and left to equilibrate was applied. Then, the organotin species were extracted from the sediment with acetic acid using ultrasonication. Organolead species were determined in a similar way, but the sample was a simulated rainwater [33]. Certified materials NIST 981, 982 and 983 (natural lead ratio, equal atom lead and radiogenic lead) containing different (but very accurately known) ratios of  $^{206}\text{Pb}$ : $^{208}\text{Pb}$  were purchased. These starting materials underwent a Grignard derivatization to form the organolead species. These species were purified by recrystallisation and the purity determined using a number of techniques including NMR spectroscopy and flame atomic absorption spectrometry. Analysis of two simulated rainwater samples for trimethyllead yielded results close to the "expected" values (0.270 mg/L cf. 0.285 mg/L and 0.248 mg/L cf. 0.228 mg/L) with a precision of less than 2% RSD.

Ruiz Encinar et al. carried out the determination of DBT ions in sediments by IDA using a  $^{118}\text{Sn}$ -enriched DBT spike synthesised in the laboratory [21]. The different organotin compounds were separated by GC after aqueous ethylation using  $\text{NaBEt}_4$  and extraction into hexane. The spike was added directly to the solid sample and extracted with a methanol-acetic acid mixture. The method was applied to the determination of DBT in sediment reference materials with satisfactory results.

A mixed  $^{119}\text{Sn}$ -enriched spike was applied to the simultaneous analysis of MBT, DBT and TBT in certified

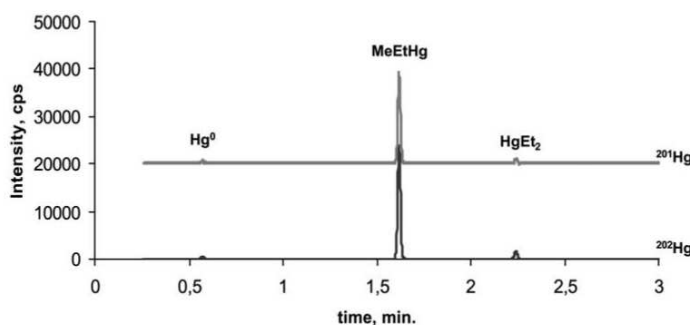


Figure 3. Chromatogram obtained for the CRM 463 extract spiked with  $^{201}\text{Hg}$ -enriched methylmercury solution. The chromatogram at mass 201 was shifted.

Trends

Trends in Analytical Chemistry, Vol. 23, No. 3, 2004

reference sediments, PACS-2 and CRM 646, with satisfactory results [26]. Sample pre-treatment consisted simply of spiking the dry sediment with the spike solution and extraction of the organotin species with methanol/acetic acid. After ethylation, the determination was carried out by CGC-ICP-MS.

A rapid, accurate, sensitive and simple method was developed for the simultaneous determination of MBT, DBT and TBT in coastal seawater samples [34]. The method was based on the application of ID methodologies using an  $^{119}\text{Sn}$ -enriched spike containing all three butyltin compounds.

Yang et al. described a method for the accurate determination of TBT using solid phase microextraction (SPME) in combination with GC separation and inductively coupled mass spectrometry (ICP-MS) detection [35]. Butyltin compounds were ethylated in aqueous solution with sodium tetraethylborate and the head space sampled with a polydimethylsiloxane-coated fused silica SPME fibre. An 18-fold improvement in the precision of TBT concentration measured using ID was observed, clearly demonstrating its superiority in providing more accurate results as compared to the method of standard additions.

Recently, a method was developed for simultaneous MMHg and TBT determination in biological samples [20]. Integrated simultaneous sample preparation for mercury and tin species included open focused microwave extraction and derivatization by ethylation. The determination was carried out by CGC-ICP-MS. The first biological reference material certified for multi-element speciation (CRM 710, IRMM, oyster tissue) was analysed with excellent accuracy (high precision and verified trueness). Fig. 4 shows a chromatogram obtained for the CRM 710 spiked with  $^{201}\text{MMHg}$  and  $^{117}\text{TBT}$ .

More applications can be found in Table 2.

## 6.2. Species transformations studies

ID calibration provides an important diagnostic tool for the development of new analytical methods in terms of isotopes used as tracers. If rearrangement reactions between the different species are taking place during extraction from the solid sample, clean-up and/or derivatization, they can be revealed by measuring the isotope ratios in the non-spiked species.

The capabilities of the CGC-ICP-MS in measuring isotope ratios were used to optimise sample derivatization by aqueous ethylation with  $\text{NaEt}_4$  with respect to MMHg degradation pathways and quantitative recovery [16]. Using the coupled CGC-ICP-MS technique and isotope-ratio measurements, the potential occurrence of chemical transformation during sample treatment was evaluated by analysing a mercury solution with an altered isotope abundance. Thus, it was possible to confirm the provenance of elemental mercury ( $\text{Hg}^0$ ) as a breakdown product of  $\text{Et}_2\text{Hg}$  and  $\text{MeEtHg}$  during the derivatization step in biological samples. The mechanism could not be described completely, but it seems to be significantly affected by the combined effects of several variables, such as pH, buffer and  $\text{NaEt}_4$  concentration.

Isotopically labelled MMHg was applied in connection with CGC-ICP-MS to validate the derivatization procedure by sodium tetraethylborate for methylmercury determination in water samples [36]. It was found that, in most cases, transformations of MMHg into elemental mercury ( $\text{Hg}^0$ ) took place. From investigations of synthetic solutions, it was stated that halide ions are responsible for this transformation process. Chloride and bromide converted MMHg into  $\text{Hg}^0$ . Iodide hinders the ethylation of methylmercury by  $\text{NaEt}_4$ . These differences in the transformation of methylmercury by the different halides must be caused by various mechanisms during the transformation process. It was also

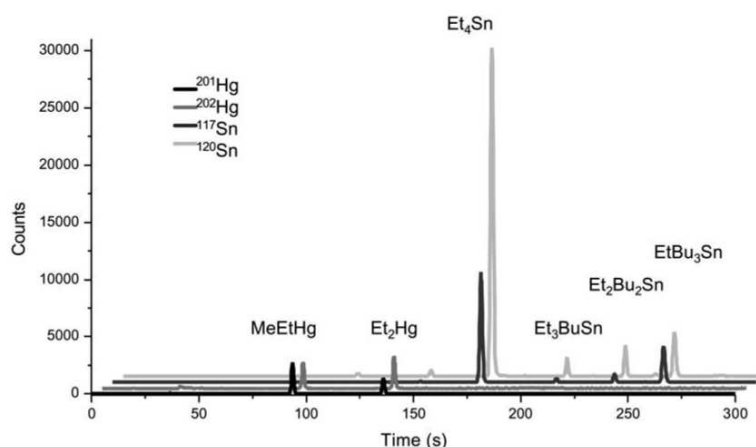


Figure 4. Chromatogram obtained for standard mussel tissue CRM 710 spiked with  $^{201}\text{MMHg}$  and  $^{117}\text{TBT}$ .



shown that transformation of MMHg took place only during the derivatization step.

In contrast to ethylation, propylation with NaBPr<sub>4</sub> did not cause any transformation. However, accurate results of MMHg determination could be obtained by propylation as well as by ethylation when the ID procedure was applied. Equilibration between sample MMHg and spike isotopically enriched MMHg took place prior to the derivatization procedure so that partial loss of the isotope-diluted methylmercury by transformation and by subsequent steps of analysis has no effect on the analytical results. This demonstrates one of the great advantages of IDMS, in which loss of substance after the isotope spike has taken place does not influence the accuracy of the analytical results.

Doubts about the accuracy of methylmercury determinations in sediments when using the water vapour distillation method came up when Hintelmann et al. added an isotopically enriched Hg<sup>2+</sup> spike to the sample [37]. By determining the distilled MMHg after ethylation and subsequent analysis with GC-ICP-MS, they found that substantial amounts of methylmercury can be formed during the distillation process from inorganic mercury ions. In sediment samples, methylmercury levels typically represent only a few percent or less of the total inorganic mercury and even minor artificial methylmercury formation during distillation will result in significant systematic errors. A correction can be carried out with a single measurement when stable Hg isotopes and detection by ICP-MS are used. By measuring different isotopes, it is possible to determine the apparent concentration (Isotope 1), the artifact formation factor (Isotope 2) and the procedural recovery (Isotope 3). Wilken and Falter also used the determination of MM<sup>200</sup>Hg generated from spiked <sup>200</sup>Hg<sup>2+</sup> to correct for artifact formation during sample preparation [38].

Based on these experiments, critical comments concerning certified MMHg values in reference materials were made [39]. The controversy led to a workshop, of which the conclusions were summarized in a special issue of *Chemosphere*, published in 1999. The conclusions were that existing CRMs fulfil the purposes of verifying the accuracy of current methods and achieving data comparability but further studies on artifact MMHg formation should be carried out [40,41]. The use of isotopically enriched species was found to be a very useful tool for these studies.

Hammerschmidt and Fitzgerald investigated the potential formation of artifact MMHg in both IAEA405 reference material and sediment from Long Island Sound (LIS) [42]. The experiments were designed to examine MMHg synthesis from either ambient (i.e. naturally present) inorganic mercury, or Hg<sup>2+</sup> that was added to samples before extraction with either acid leaching or aqueous distillation. MMHg was formed from both ambient and added inorganic mercury in

extracts of IAEA-405 by either technique. Mercury methylation occurred in solution and was a function of both the availability of ambient reactive mercury (Hg<sub>R</sub>) and a methylation potential. Formation of artifact MMHg in LIS sediments was not conclusive, but addition of inorganic mercury demonstrated that a potential exists.

Lambertsson et al. developed an ID-based GC-ICP-MS method that could simultaneously determine incipient concentrations of methylmercury as well as the degree of methylation of inorganic mercury and de-methylation of methylmercury in brackish water sediments [43]. Methyl and inorganic mercury were extracted from the sediments using a solution containing KBr-H<sub>2</sub>SO<sub>4</sub>-CuSO<sub>4</sub>. MM<sup>202</sup>Hg was added as a species-specific internal standard and <sup>199</sup>Hg<sup>2+</sup> to monitor artifact formation of methylmercury during sample work-up. For investigations of mercury methylation and demethylation during sample incubation, <sup>201</sup>Hg<sup>2+</sup> and MM<sup>198</sup>Hg<sup>+</sup> were added. When using bromide for extraction, degradation of methylmercury into elemental mercury and the probable formation of methylmercury bromide during sample pre-treatment and/or analysis were observed. The extraction procedure based on KBr, H<sub>2</sub>SO<sub>4</sub> and CuSO<sub>4</sub> shows a relatively low (i.e. below 0.05%) artificial transformation of inorganic mercury to methylmercury.

Systematic experiments have been carried out to localize the sources of unintentional abiotic methylmercury formation during analysis by CGC-ICP-MS after open-focused microwave assisted acid extraction [17]. Different spiking and derivatization (either ethylation, propylation and Grignard) procedures were tested. Artifact formation does not take place during the spiking or the extraction step, but during the final derivatization/extraction, with the amount of inorganic mercury in this last step being the determining factor. Solvent extraction with CH<sub>2</sub>Cl<sub>2</sub> prior to ethylation allows adequate control of artifact formation. A critical analysis of the results obtained has shown that artifact MMHg formation depends on not only inorganic mercury content, but also organic carbon and sulphur levels in the sediments investigated.

During TBT analysis of sediment extracts by HPLC-ID-ICP-MS, Hill et al. noticed potential species transformation [33]. The sediments were spiked with the <sup>116</sup>Sn-enriched TBT and the presence of several (as yet unidentified) peaks with greatly enriched <sup>116</sup>Sn signal was detected. The butyltin peak has the expected natural isotope ratio. Thus, the species transformation is probably related to the presence of phenylated tin compounds and it is possible that the unidentified peaks may be mixed butyltin and phenyltin compounds.

The use of double-spike methodologies has been also evaluated for the investigations of extraction procedures in relation to the degradation of butyltin



compounds during extraction. Different liquid-solid extraction techniques, including room-temperature leaching with mechanical shaking, ultrasonic and microwave-assisted extractions have been evaluated for the quantitative speciation of MBT, DBT and TBT in PACS-2 and BCR-646 CRMs. A methanol-acetic acid mixture was used as the extractant in all cases [44]. A mixed spike containing  $^{119}\text{Sn}$ -enriched MBT,  $^{118}\text{Sn}$ -enriched DBT and  $^{119}\text{Sn}$ -enriched TBT was synthesized, characterized and used for IDA. In the analysis of the certified sediments, the sample was spiked with the mixture, extracted under different conditions, derivatised with sodium tetraethylborate and extracted into hexane. Isotope ratios  $^{120}\text{Sn}/^{118}\text{Sn}$  and  $^{120}\text{Sn}/^{119}\text{Sn}$  were determined as peak area ratios for all butyltin species after GC-ICP-MS analysis. Sequential degradation reactions during extraction (from TBT to DBT, from DBT to MBT and from MBT to inorganic tin) were assumed, and mathematical equations were developed that allowed the determination of the correct species concentrations and the decomposition factor for each of the transformation reactions. For ultrasonic extraction and mechanical shaking, negligible degradation reaction was observed. However, for microwave assisted extractions, degradation factors up to 7% (TBT to DBT) and 16% (DBT to MBT) were obtained for both reference materials when high-MW energy was applied in the extraction step. MBT seems to be strongly bound to the solid matrix, and, for quantitative recovery, a harsher extraction technique and longer extraction time are required. No evidence of transbutylation was found on the basis of the results obtained for TBT  $^{120}\text{Sn}/^{119}\text{Sn}$  and  $^{118}\text{Sn}/^{119}\text{Sn}$  isotope ratios under all extraction conditions tested.

The use of accelerated solvent extraction (ASE) for the extraction of butyltin compounds from PACS-2 was investigated and the corresponding degradation factors under different extraction conditions were calculated. A double-enriched spike containing  $^{119}\text{Sn}$ -enriched MBT and TBT and  $^{118}\text{Sn}$ -enriched DBT allowed the simultaneous determination of the three butyltin species and the factors governing species interconversion [45].

## 7. Application in environmental tracer studies

Traditionally, radiochemical procedures have been used to study processes such as partitioning between dissolved and particulate phase, uptake and accumulation into aquatic organisms and transformation of inorganic into organic species (i.e. inorganic mercury into the highly toxic MMHg). However, the results obtained are questionable because, since commercially available radioactive material have low specific activity, high concentrations of metals must be added to the system.

Hence, the experiments might not represent natural processes, since structural effects in the case of partitioning or toxic effects in uptake experiments are likely or cannot be ruled out. Additionally, only net effects are detectable, but most environmental processes comprise at least two sub-processes (e.g. adsorption and desorption, oxidation and reduction, uptake and depuration, methylation and demethylation) [15].

Stable enriched isotope tracers offer great potential for investigating dynamic environmental processes. The use of stable isotopes in conjunction with ICP-MS offers several benefits. The response is not calculated from the total metal concentration, but rather from changes in the isotope ratio. Isotope ratios can be determined very precisely, allowing the use of very low spike concentrations that do not overwhelm natural levels. Typically, an increase in ambient concentration of 1-10% creates sufficient isotopic difference for effects to be easily measured. In all these experiments, not only the fate of the added tracer, but also the behaviour of the ambient metal present in the system can be monitored, simply by measuring another isotope of that metal. Additionally, coupled and often overlying processes can be measured together by using double labelling tracers.

For the investigation of mercury methylation and demethylation during sample incubation,  $^{201}\text{Hg}^{2+}$  and  $\text{MM}^{198}\text{Hg}$  were added to sediment layers at 20% of the ambient concentrations of the two species [43]. Mercury methylation and de-methylation activities differed by a factor of four between depths and were highest in the top 3 cm of the sediment. In this region, 9.5% of the added  $^{201}\text{Hg}^{2+}$  tracer was methylated and 79% of the  $\text{MM}^{198}\text{Hg}$  tracer was de-methylated. The methylmercury concentration changed about five times over the depth profile. The added species may initially be more available for transformations compared to incipient species; also the chemical form in which the spike is added (e.g., as  $\text{Hg}(\text{NO}_3)_2$  or  $\text{HgCl}_2$ ) can influence the transformation rate.

Hintelmann et al. used GC-ICP-MS and stable enriched isotope tracers for simultaneous determination of methylation and de-methylation rates of mercury species in anaerobic fresh water sediment slurry [46]. Solutions containing stable isotope tracers of  $^{199}\text{Hg}^{2+}$  and  $\text{MM}^{202}\text{Hg}$  were spiked into lake sediments at sub-ambient concentrations. The formation of  $\text{MM}^{199}\text{Hg}^+$  and the decrease in  $\text{MM}^{202}\text{Hg}$  were measured simultaneously in time series experiments. Specific rate constants for the two processes were calculated and compared to rate constants obtained by monitoring changes in concentration of the ambient methylmercury in the same sample. The inorganic mercury tracer  $^{199}\text{Hg}^{2+}$  was methylated faster than the ambient inorganic  $\text{Hg}^{2+}$ , which indicates that the added tracer  $\text{Hg}^{2+}$  is more available for transformation



reaction than the ambient  $\text{Hg}^{2+}$ . The degradation of tracer and ambient methylmercury proceeded at a similar rate, showing no significant differences between added tracer and ambient methylmercury. The calculated half-life for methylmercury in sediments was 1.7 d, suggesting a rapid turnover and low persistence of methylmercury in lake sediments. Different Hg species were investigated regarding their availability for methylation reactions.

Further work using simultaneous assays is necessary to decide which environmental factors regulate the two opposing processes, thereby determining the methylmercury levels in sediments. Recently, mercury methylation and demethylation studies using isotopically enriched species have been carried out in our laboratory to correlate these activities with prevailing environmental parameters, like redox potential and microbial activity [47]. Sediments (sterilized and non-sterilized) were spiked with both isotopically enriched inorganic mercury ( $^{199}\text{Hg}^{2+}$ ) and monomethylmercury ( $\text{MM}^{201}\text{Hg}$ ) at real environmental levels to avoid perturbing the system and incubated under both aerobic and anaerobic conditions. Results clearly showed that methylmercury levels in sediments were controlled by competing and simultaneous methylation and demethylation reactions. In estuarine sediments, the synthesis and stability of monomethylmercury were enhanced under anaerobic non-sterile conditions. Consequently, the aerobic sterile and non-sterile and the anaerobic non-sterile are either less favourable for MMHg synthesis or are more conducive to its destruction. An attempt was made to identify the reaction product of the demethylation process by measuring the volatile mercury fraction produced during the experiments. The production of total gaseous mercury was found to be minimal, although it could be demonstrated to be related to the demethylation process.

To investigate the dynamics of mercury newly deposited onto a terrestrial ecosystem, an enriched stable isotope of mercury ( $^{202}\text{Hg}$ ) was sprayed onto a Boreal forest sub-catchment in an experiment that allowed, for the first time, the fate of "new" mercury deposition to be monitored and to be distinguished from native mercury historically deposited in the ecosystem [48]. During one season, only 8% of the added  $^{202}\text{Hg}$  volatilised to the atmosphere and less than 1% appeared in run-off.

Within a few months, approximately 66% of the applied  $^{202}\text{Hg}$  remained associated with above ground vegetation, the rest being incorporated into the soil. The fraction of  $^{202}\text{Hg}$  bound to vegetation was much higher than was seen for native Hg (< 5% vegetation), suggesting that atmospherically derived mercury enters the soil pool with a time delay, after plants senesce and decompose. A small but significant fraction volatilised, while most of the new mercury attached tightly to vegetation and a lesser amount to underlying

soils. This suggests that a large fraction of newly deposited mercury is not immediately mobile.

## 8. Limitations of isotopically enriched stable isotopes

It should be noted that ID does not provide a panacea for all problems encountered in speciation analysis. In all these methods, it is assumed that the added spike of enriched isotope and the indigenous element species behave in a similar way. However, care should be taken about this point. If the spike is extracted from the solid more efficiently (e.g., because it was not allowed to equilibrate) than the analyte present in the sample, incorrect data will be obtained. If the spike is more available than the natural element, the conclusions drawn from environmental studies may not be representative, although they would at least assist in detecting this problem.

Species-specific spike ID operated in the typical way (a mixture of species with a single enriched isotope) cannot compensate for non-quantitative extraction from the solid or for species degradation reactions in the initial liquid-solid extraction step.

The high accuracy of IDA in trace metal speciation is based on the fact that, once isotope equilibration has taken place, decomposition of the species will not affect the final result; however, if the decomposition product of one species is another compound that also has to be determined, errors will occur in the concentration of the second species (i.e. in the case of MMHg artifact).

For example, changes in speciation during the extraction process cannot be overcome, although there is compelling evidence that breakdown or alteration of such species is occurring. Mathematical equations can be developed to allow computation of both the correct species concentrations by IDA and the possible degradation factors from one species to another.

Additionally, ID cannot overcome the problem of contamination.

## 9. Conclusions

IDMS has been demonstrated to be very valuable for studying the transformations of organotin and mercury species in environmental samples and for improving precision and accuracy of results. It therefore seems that multi-isotope labelled IDA techniques will become a most powerful tool for optimising and validating procedures for trace-metal speciation, going from species extraction from the solid to derivatization, separation and detection of those species for accurate final determination. However, care should be taken to ensure that

Trends

Trends in Analytical Chemistry, Vol. 23, No. 3, 2004

the isotopically enriched stable isotope behaviour is representative of the natural element species, quantitative solid extraction is achieved and contamination is prohibited, as these are the main limitations.

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## **Chapitre E – Annexe II**

**Détermination du TBT dans des sédiments et tissus biologiques par  
l'analyse de spéciation en dilution isotopique, extraction micro-  
ondes et chromatographie en phase gazeuse-ICPMS**



## Rapid, accurate and precise determination of tributyltin in sediments and biological samples by species specific isotope dilution-microwave extraction-gas chromatography-ICP mass spectrometry

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The rapid and precise determination of tributyltin (TBT) in sediments and biological tissues has been performed by species specific isotope dilution mass spectrometric analysis (IDMS) using an inductively coupled plasma mass spectrometer as detector after capillary gas chromatography (CGC-ICP-MS). A new labelled TBT standard (<sup>117</sup>TBTCl in methanolic solution) commercial isotope provided by LGC Limited (Teddington, UK) was used for these determinations. Parameters affecting the ICP-MS performances, such as correction for detector dead time and mass bias correction, were carefully studied. The mass bias was corrected using two different methods: the bracketing mode and an on-line mode based on a continuous nebulisation of an antimony solution (<sup>121</sup>Sb and <sup>123</sup>Sb). The on-line mode has been successfully applied for mass bias corrections and allows simultaneous GC-ICP-MS analysis of organotin. Three spiking procedures were compared using isotopically enriched TBT (<sup>117</sup>TBT) to compare the efficiency of the extraction procedures for the different samples studied. A rapid method was developed (2 min) giving yield to good precision (uncertainty range between 0.7 and 13.7%) using a simultaneous microwave extraction and spiking procedure. The accuracy and precision of the different protocols has been validated on certified reference materials, such as PACS 2 (980 ng Sn g<sup>-1</sup>) and CRM 462 (70 ng Sn g<sup>-1</sup>) for the sediments, CRM 477 (2200 ng TBT g<sup>-1</sup>) and CRM 710 (135.1 ng TBT g<sup>-1</sup>) for the biological tissues. The results obtained were in all cases in good agreement with the certified reference values.

### Introduction

Tributyltin (TBT) is well known as a major toxic compound widely dispersed in the environment. TBT has been extensively used as a biocide in antifouling paints since the 1970s<sup>1,2</sup> and released in the marine environment, resulting in extensive damages to non-target organisms such as oyster or mussels for concentrations as low as 1 ng l<sup>-1</sup>.<sup>3,4</sup> With the evidence of increasing endocrine disrupting effects on marine life, and the imposex at the bottom of the sea correlated with the tracks of shipping routes,<sup>5</sup> the Marine Environmental Protection Committee (MEPC) has proclaimed a world-wide recommendation of banning TBT in 2003.<sup>6</sup> The magnitude of the environmental impacts and the establishment of important regulations calls for improvements in current analytical requirements to obtain an accurate and sensitive analytical speciation method.

The speciation analysis of organotin compounds may be achieved in a number of different ways. Currently, most analytical techniques developed for the speciation of organometallic compounds usually combine separation techniques such as HPLC<sup>7,8</sup> or GC<sup>9-11</sup> with a specific detection mode such as PFPD,<sup>12,13</sup> AA,<sup>14,15</sup> AE<sup>16,17</sup> or ICP-MS.<sup>9,18-22</sup> The introduction of ICP-MS as a detector allows us to reach a wide choice of capabilities, including simultaneous detection on several isotopes quasi-simultaneously. The coupling of GC to ICP-MS appears to be one of the more promising techniques for organotin analysis because of its multi-element capability, the possibility of isotopic information and its high sensitivity.<sup>9,19,22</sup>

In addition, the quality of the results of speciation determination is closely associated with the quality and efficiency of the sample pre-treatment stages. Routinely, for analysis using GC-ICP-MS, the analytes are extracted from the sample matrix and derivatised to yield volatile compounds. It is most important that no losses or alterations to the different chemical forms occur. Most sample preparation techniques for tributyltin speciation in a solid matrix combine different complex preparation steps using agitation,<sup>23,24</sup> ultrasonic bath<sup>24-26</sup> or supercritical fluid extraction.<sup>27</sup> Open microwave assisted digestion has also gained wide acceptance as a rapid and efficient method for sample decomposition in inorganic analysis.<sup>11-13</sup> Investigations into the behaviour of organotin compounds under a microwave field were carried out and the procedures were optimised to improve extraction recoveries and reduce the time for sample preparation.<sup>11,26</sup> Once the extraction step is performed, the organometallic species are derivatised in order to obtain volatile species. This step is generally performed using a Grignard reagent, hydride generation or ethylation.<sup>28</sup>

To avoid problems of losses, or alteration of species during the sample preparation steps, the use of isotope dilution techniques is a good alternative method.<sup>29</sup> Quantitative recoveries are not necessary and rearrangement reactions are easily detected. Isotope dilution mass spectrometry (IDMS) has been used in organic analysis<sup>30</sup> for some years but has recently been transferred to inorganic speciation analysis. Isotope dilution is based on the addition of a precise amount of an isotopically labelled form of the analyte to the sample. With



the known isotopic abundance of both spike and sample, the amount of the spike added to the sample and the altered isotope ratio, the concentration of the species can be calculated. The analyte of interest must have more than one stable isotope, which is the case with tin, which has 10 different isotopes, and the isotopes used for IDMS analysis should be interference free. IDMS relies purely on isotopic ratio measurement. The problem of incomplete extraction is negated but the spike sample must be extracted with the same efficiency as the analyte already present in the sample. With IDMS it is particularly important that full equilibrium between the species tested and the enriched species is achieved so that their behaviour will be similar during the whole analytical procedure. Very few applications of isotope dilution for speciation analysis have been published,<sup>10,18,31–35</sup> but most of them advocate that the spike must be added at the earliest point of the analysis and the time for equilibration must be sufficiently long. For tin, Ruiz-Encinar *et al.*<sup>10</sup> developed a GC-ICP-MS technique for the separation and determination of DBT in sediments with a precision of 1% after an extraction procedure of 12 h with mechanical agitation. The same authors applied the same method to the determination of MBT, DBT and TBT in sediments with an average precision of 0.8%.<sup>22</sup>

In this work, GC-ICP-MS was applied to the measurement of isotope ratios for tributyltin in an attempt to demonstrate the usefulness of species specific isotope dilution analysis in trace metal speciation in different types of environmental matrices. Special attention was paid to the spiking and the extraction procedures, as well as to the operating conditions (dead time analysis and mass bias correction) of the GC-ICP-MS for the isotope ratio measurements. The analytical procedures developed were applied to the analysis of reference sediments PACS-2 (NRCC, Canada), CRM 462 (IRMM, Belgium) and biological tissues CRM 477 (IRMM, Belgium) and CRM 710 (candidate reference material, IRMM, Belgium).

## Experimental section

### Reagents and materials

**Reagents.** Natural tributyltin chloride (TBTCl) and enriched TBTCl (<sup>117</sup>TBTCl, 90.5 µg ml<sup>-1</sup>) were obtained from LGC Limited (Teddington, UK). The purification and synthesis of these standards is described in Sutton *et al.*<sup>36</sup> A 1000 µg (Sn) g<sup>-1</sup> dilution of TBTCl was prepared in methanol. This solution was kept in dark refrigeration. A 1000 ng (Sn) ml<sup>-1</sup> dilution of the previous solution was finally diluted to a 10 ng (Sn) ml<sup>-1</sup>. This last solution was daily prepared and used for the calibration. A dilution of the <sup>117</sup>TBTCl spiking solution was prepared by weighing ~0.1 g of a 90.5 mg g<sup>-1</sup> enriched solution and diluting in ~10 g of water. The concentration of the enriched TBT solution obtained was calculated by reverse isotope dilution mass spectrometry (RIDMS). Three independent isotope dilution experiments were performed and each solution was injected five times. A 0.980 ± 0.001 µg (<sup>117</sup>TBTCl) g<sup>-1</sup> (or 0.354 ± 0.004 µg (<sup>117</sup>Sn) g<sup>-1</sup>) solution was obtained with an abundance for the isotope 117 of 99.97%.

Antimony (1004 ± 3 mg Sb l<sup>-1</sup>) and tin (998 ± 3 mg Sn l<sup>-1</sup>) were obtained from Spex Certiprep (Metuchen, NJ, USA). Inorganic antimony and tin were used for the mass bias and dead time correction, respectively. Acetic acid, sodium acetate and HPLC grade isooctane were purchased from Sigma Aldrich (Poole, Dorset, UK) and sodium tetraethylborate (purity higher than 99%) from Strem Chemicals (Newburyport, MA, USA). 1% (w/w) sodium tetraethylborate (NaBEt<sub>4</sub>) solutions were prepared daily and kept in the dark. Ammonium hydroxide was obtained from Fluka (Buchs, Switzerland) and 65% ultrapure nitric acid and methanol from Merck (Darmstadt, Germany).

**Materials.** An analytical balance, Sartorius Model BP211D, with a precision of 10<sup>-5</sup> g for a mass lower than 80 g and 10<sup>-4</sup> g for a mass higher than 80 g (Göttingen, Germany), was used for all the weighing. An open focused vessel microwave oven Prolabo A301 (Fontenay-sous-Bois, France) was used for the extractions. The analyses were performed in a HP 5290 Series II gas chromatograph (Agilent, Palo Alto, CA, USA) coupled to a HP7500 Series inductively coupled plasma mass spectrometer (Agilent, Yokogawa, Japan). Briefly, a home-made heated steel transfer line was used, connected to the ICP torch by a glass T-piece.<sup>20,21</sup> The transfer line is made of an inner capillary (MXT, "guard column", id 0.28 mm) inserted into a second capillary (Silcosteel treated stainless steel, od 1/16", id 1 mm). The make-up argon flows through the outer capillary. The length of the whole system is approximately one meter. The GC and ICP-MS operating conditions are given in Table 1. <sup>126</sup>Xe is introduced as a gaseous species to check the sensitivity of the instrument during the day.

**Reference materials.** Two sediments with certified contents of tributyltin were used: PACS-2 (marine sediment with a certified value for TBT of 980 ng Sn g<sup>-1</sup>) from the National Research Council of Canada (NRCC, Canada) and CRM 462 (harbor sediment with a certified value for TBT of 70 ng Sn g<sup>-1</sup>) from the Institute for Reference Materials and Measurements (IRMM, Belgium). Two biological tissues were obtained from the IRMM: the reference material CRM 477 (mussel tissue) and a candidate reference material for tributyltin (CRM 710) which is an oyster tissue certified for 2200 and 135.1 ng TBT g<sup>-1</sup>, respectively.

### Procedures

**Spiking procedures for isotope dilution.** Isotope dilution is based on the addition of a precise amount of an isotopically labeled form of the analyte to the sample. When the natural abundance of the analyte in the sample and in the spike are known, the concentration of the analyte in the sample can be calculated if a known amount of the spike is added and equilibrated with the analyte in the sample. Provided that the isotope ratio of the mixture is measured accurately, the spiking procedure is a critical stage to ensure full equilibrium and the same behavior of the analyte and the analogue during the

**Table 1** GC and ICP-MS conditions for the separation of MBT, DBT, TBT and TPrT

| GC parameters              |                                             |       |
|----------------------------|---------------------------------------------|-------|
| Column                     | MXT Silcosteel 30 m,<br>id 0.53 mm, df 1 μm |       |
| Injection port             | Splitless                                   |       |
| Injection port temperature | 250 °C                                      |       |
| Injection volume           | 1 μl                                        |       |
| Carrier gas flow           | He 30 ml min <sup>-1</sup>                  |       |
| Make up gas flow           | Ar 300 ml min <sup>-1</sup>                 |       |
| Oven programme:            |                                             |       |
| Initial temperature        | 80 °C                                       |       |
| Time                       | 0 min                                       |       |
| Ramp rate                  | 50 °C min <sup>-1</sup>                     |       |
| Final temperature          | 250 °C                                      |       |
| Final time                 | 2 min                                       |       |
| ICP-MS parameters          |                                             |       |
| Rf power                   | 1250 W                                      |       |
| Gas flow                   |                                             |       |
| Plasma                     | 15 L min <sup>-1</sup>                      |       |
| Auxiliary                  | 0.9 L min <sup>-1</sup>                     |       |
| Nebuliser                  | 0.9 L min <sup>-1</sup>                     |       |
| Isotope dwell times        | Sn: 117, 118, 120                           | 30 ms |
|                            | Sb: 121, 123                                | 5 ms  |
|                            | Xe: 126                                     | 5 ms  |

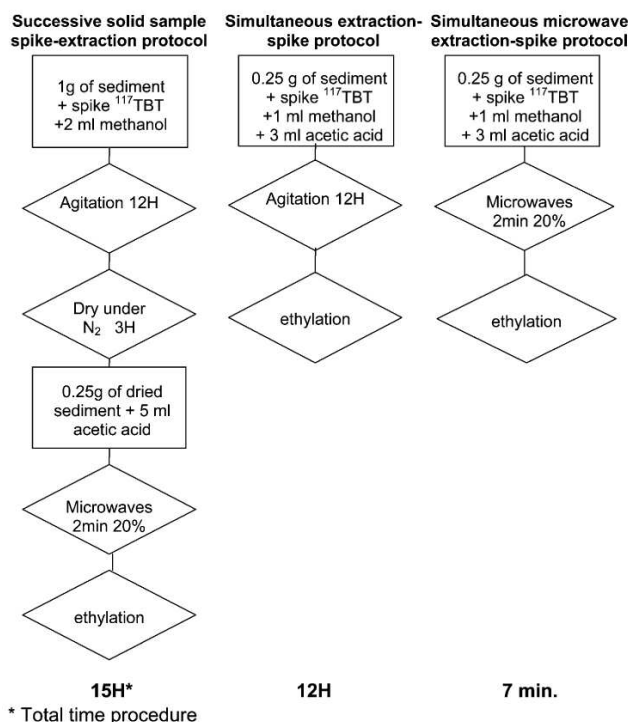


Fig. 1 Schematic flow chart of the isotope dilution spiking-extraction protocols.

analytical procedure. In accordance with the above remarks, three different spiking procedures were tested to evaluate the influence of the equilibration between the spike and the analyte on their behavior during the sample preparation. A flow chart of the simplified isotope dilution spike and extraction procedures is presented in Fig. 1.

**Successive solid sample spike-extraction protocol (Method 1).** An aliquot of 1 g of dry sample was accurately weighed in a glass tube and spiked with a known amount of the enriched  $^{117}\text{TbTCI}$  solution. 2 ml of methanol were then added, the tube was capped and mechanically shaken overnight in the dark. After shaking, the methanol was evaporated using a gentle stream of nitrogen ( $2\text{ ml min}^{-1}$ ). The dried subsample of the sediment was then stirred for homogenization before microwave extraction.

**Simultaneous mechanical extraction-spike protocol<sup>10,22</sup> (Method 2).** A sample 0.25 g in size was accurately weighed in a glass tube and spiked with a known amount of enriched  $^{117}\text{TbTCI}$  solution. 1 ml of methanol and 3 ml of acetic acid were added. The tube was then immediately capped and mechanically shaken overnight in the dark. Once the agitation step was over, the sample was centrifuged for 5 min at 2500 rpm and the supernatant was quantitatively transferred to a glass tube with a Teflon cap for derivatization by ethylation. The extraction using mechanical agitation was validated by Ruiz-Encinar *et al.*<sup>10,22</sup> and provided good data in agreement with the certified value for the PACS-2.

**Simultaneous microwave extraction-spike protocol (Method 3).** An amount of 0.25 g of sample was accurately weighed in a microwave extraction vessel and spiked with a known amount of the enriched  $^{117}\text{TbTCI}$  solution. 1 ml of methanol and 3 ml of acetic acid were added and an air-cooled condenser was placed on top of the extraction vessel and then submitted to microwave extraction.

In all spiking procedures, minimisation of errors on the isotope ratio in the final determination was obtained by addition of the amount of enriched standard to the sample, so that the isotope ratio of the spiked isotope to that of the isotopes of the analyte were close to unity. Each experiment was performed six times for sediments and in triplicate for the biological materials. Blanks for each spiking method were also determined to check for any contamination.

**Microwave extraction procedure.** The microwave extraction for organotin compounds has been optimized by Szpunar *et al.*<sup>11</sup> An amount of 0.25 g of sample was accurately weighed in the extraction vessel. 5 ml of acetic acid were added and an air-cooled condenser was placed on top of the extraction vessel to minimize the possible lost of any volatile species. The mixture was gently stirred, placed in the open microwave cavity and extracted for 2 min at 20% of irradiation power (40 W). After the extraction period, the sample was allowed to cool at room temperature. The extract was quantitatively transferred into a glass tube with a Teflon cap, 2.5 ml ( $\times 2$ ) of Milli-Q water were added to rinse the extraction vessel and then transferred to a glass tube. The extract was centrifuged for 5 min at 2500 rpm. The supernatant was then transferred to a clean tube and submitted to ethylation.

**Derivatization of the extract.** A volume of 5 ml of a  $1.77\text{ mol l}^{-1}$  acetic acid-sodium acetate buffer and 1–3.8 ml of ammonium hydroxide were added to the supernatant to set the pH at 5.0. Then, 2 ml of iso-octane and 1 ml of 1% (w/w) sodium tetraethylborate were added. The tube was immediately capped and shaken by hand for 5 min. The organic phase was finally transferred to an injection vial and stored at  $-18^\circ\text{C}$  until measurement.

**GC-ICP-MS analysis.** The operating conditions used for the gas chromatographic separation and the ICP-MS detection are illustrated in Table 1. For isotope dilution analysis, each



sample was injected five times and the chromatographic peak integration was performed using the Chromatography Data Analysis software from the HP Chemstation (Agilent, Yokogawa, Japan). Isotope ratios were always measured as peak area ratios and corrected by a mass bias factor. In addition, the concentration of TBT for each sample was analyzed by external calibration using TPrT as an internal standard and by standard addition using the isotope 120 which is not affected by the enriched  $^{117}\text{TBT}$  spike.

## Results and discussion

### Optimisation of the ICP-MS conditions for isotope ratio determination

**Detector dead time analysis.** To evaluate the detector dead time, tin standard solutions which ranged from  $30 \text{ ng Sn g}^{-1}$  to  $100 \text{ ng Sn g}^{-1}$  were prepared in 1% nitric acid and injected by continuous aspiration. The measurements were performed by using the isotope ratio acquisition mode of the HP7500 with an integration time of 5 s per isotope. The detector dead time was then calculated by applying a method proposed by Held and Taylor.<sup>37</sup> This method gave a detector dead time of 45 ns, which was chosen for the rest of measurements.

**Mass bias correction.** The mass bias is the difference between the measured isotope ratio and the true value. This difference can be caused by different sensitivity response displayed by the instrument with respect to the mass.

To calculate the mass bias correction factor two methods were tested. In the first case, a standard solution of antimony was nebulised continuously during acquisitions or a standard solution of TBT was injected between each acquisition. This latter mode is often known as the *bracketing mode*.

For this purpose, the following solutions were prepared. Four solutions of 5 ml containing 0, 1, 10 and  $100 \mu\text{g (Sn) kg}^{-1}$  of tributyltin (TBT) were prepared. The samples were ethylated following the ethylation procedure mentioned above. A  $100 \text{ ng (Sb) g}^{-1}$  standard solution in 1% nitric acid was prepared from a  $10\,000 \text{ ng (Sb) g}^{-1}$  solution, which had been prepared in advance from a  $1000 \mu\text{g (Sb) g}^{-1}$  solution. This solution was added as a continuous standard for the mass bias correction.

The TBT standard solutions were injected five times each in the following order:  $10 \text{ ng Sn g}^{-1}$ , blank,  $1 \text{ ng Sn g}^{-1}$ ,  $10 \text{ ng Sn g}^{-1}$ ,  $100 \text{ ng Sn g}^{-1}$  and  $10 \text{ ng Sn g}^{-1}$ . The  $10 \text{ ng Sn g}^{-1}$  standard solution of TBT was injected at the beginning, in the middle and at the end of the whole process to see whether it could be used as a mass bias standard in the bracketing mode.

The correction of mass bias ( $a$ ) was calculated using the following linear eqn. (1):

$$R_t = \frac{R_m}{(1+an)} \quad (1)$$

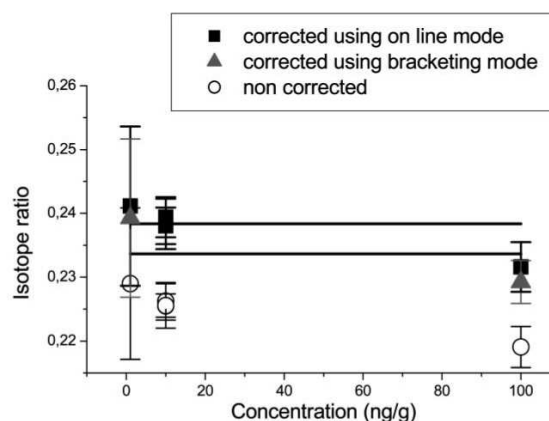
where  $R_t$  is the theoretical isotope ratio,  $R_m$  represents the experimentally measured isotope ratio,  $n$  is the mass difference between isotopes and  $a$  is the mass bias per mass unit. In this case,  $a$  was determined from the continuous measurement of antimony isotopes 121 and 123 (eqn. (2)):

$$a = \frac{(R_m - R_t)}{nR_t} \quad (2)$$

The average signal from minute 3 to minute 5 for  $^{121}\text{Sb}$  and  $^{123}\text{Sb}$  was obtained for each sample injected. This range covered the retention time period of chromatographic peaks of organotin compounds.

For the bracketing mode,  $R_m$  is the measured isotope ratio ( $^{117}\text{Sn}/^{120}\text{Sn}$ ) for the standard solution of 10 ppb of tributyltin.

Fig. 2 displays how the corrected isotope ratios ( $^{117}\text{Sn}/^{120}\text{Sn}$ ) change with concentration for correction using either the continuous nebulization of antimony standards or with the



**Fig. 2** Corrected (■,▲) and non-corrected (○)  $^{117}\text{Sn}/^{120}\text{Sn}$  isotope ratios at different concentrations of tin (in  $\text{ng Sn g}^{-1}$ ). Correction was carried out using the on line method with antimony standard and the bracketing mode with TBT standard. Straight lines indicate the theoretical upper and lower limit of the  $^{117}\text{Sn}/^{120}\text{Sn}$  isotope ratio.

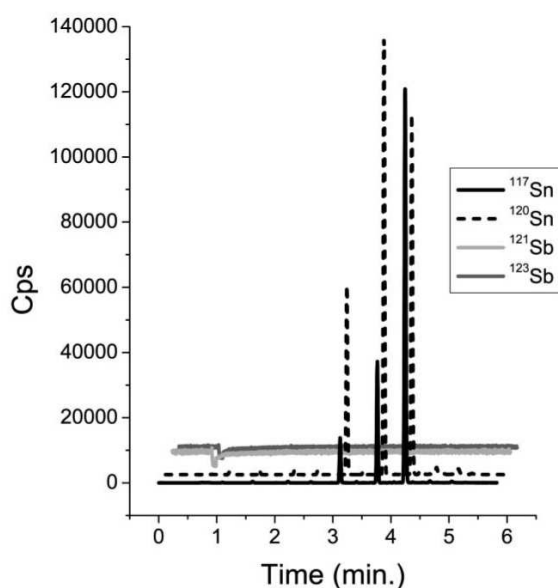
bracketing approach with the TBT standards. The black lines represent the theoretical upper and lower limits of the isotope ratios and were estimated considering the theoretical value plus/minus one percent of error. Better isotope ratios were always obtained after mass bias correction with both correcting methods. The corrected isotope ratios for different concentrations were within the limits of the theoretical isotope ratios for the on-line mode. Using the bracketing mode, the correction did not provide an accurate isotope ratio for the  $100 \mu\text{l l}^{-1}$  standard solution. At the end of the procedure the corrected isotope ratios for the two methods are not significantly different even if the average mass bias per unit for this experiment was  $-0.0175 \pm 0.0007$  for the correction with antimony and  $-0.0145 \pm 0.0004$  for the bracketing mode.

The mass bias correction method using continuously nebulised antimony was selected since it allows a simultaneous correction, calculating the mass bias for each acquisition. A chromatogram (Fig. 3) shows the signal of the two isotopes of antimony during the acquisition for organotin compounds. This method also allows the discrimination of a direct variation in the sensitivity of the apparatus. Finally, this method is easier and less time consuming since it was not necessary to inject a standard after each measurement.

**Data acquisition parameters.** For isotope dilution analysis, data acquisition parameters settings are very important in obtaining well defined chromatographic peaks and hence generating good isotopic ratios. Dwell times of 30 ms for tin isotopes (117, 118, 120), 5 ms for antimony isotopes (121, 123) and 5 ms for  $^{126}\text{Xe}$  were chosen under our operating conditions. The total integration time was 105 ms. Since the shape of a normal chromatographic peak is approximately 3 s, the peaks under these conditions were defined by about 28 point measurements. Isotope ratios have always been calculated using peak area ratios and corrected for mass bias.

**Analytical performances.** The repeatability of the isotope dilution method was evaluated at high ( $100 \text{ ng (Sn) g}^{-1}$ ) and low ( $10 \text{ ng (Sn) g}^{-1}$ ) concentration levels. Each prepared solution was injected ten times. A relative standard deviation (RSD%) of 0.7% and 0.5% was obtained for the high and the low level of concentration, respectively.

The absolute detection limits for the GC-ICP-MS was estimated by using three times the standard deviation of 6 blank measurements. The detection limits were better than 40 fg for  $^{117}\text{Sn}$  and 30 fg for  $^{120}\text{Sn}$ .



**Fig. 3** Chromatogram obtained for the PACS-2 spiked with the enriched  $^{117}\text{TBTCl}$  solution.

However, it is important to note that these are the instrumental detection limits for CGC-ICP-MS. The detection limits for isotope dilution analysis depend on the precision of isotope ratios, the mass of spike and the enrichment of the spike. Yu *et al.*<sup>38</sup> have developed a novel complete formulation for the determination of IDMS detection limits which is a function of the enrichment of the isotopic spike (eqn. 3)

$$LD = \frac{\sqrt{L_{Da}^2 + R_p^2 L_{Db}^2}}{|A_x - R_p B_x|} \quad (3)$$

where  $L_{Da}$  and  $L_{Db}$  are the linear calibration detection limits for isotopes A and B, respectively,  $R_p$  is the ratio of isotopes A and B for the spike, and  $A_x$  and  $B_x$  are the atom fraction of isotopes A and B in the sample. IDMS detection limit, calculated according to this equation, was 93 fg as tin for TBT.

This excellent analytical performance allows good operating conditions for routine isotope dilution measurements on various types of environmental samples.

#### Application of speciated isotope dilution analysis of TBT in sediments and biological tissues

A chromatogram obtained for the PACS-2 spiked with  $^{117}\text{Sn}$ -enriched tributyltin solution is shown in Fig. 3 for mass 117 and 120. The isotopic composition of tributyltin has been altered drastically. A measured 117/120 ratio of 1.215 is obtained, instead of the natural value of 0.236. The results

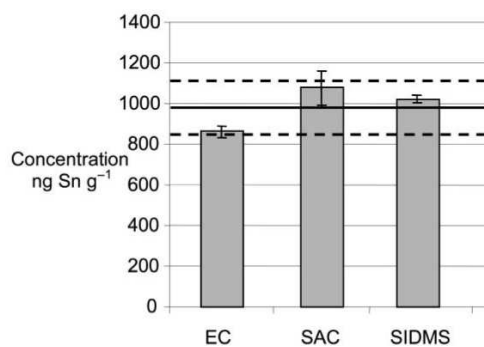
obtained for the certified reference sediments and biological tissues are summarized in Table 2.

**Validation of the method with CRMs.** In parallel with the SIDMS analysis, the concentrations of TBT with each spiking procedure were evaluated using external calibration and standard addition calibration. The results for the PACS-2 by method 3 are presented in Fig. 4. The three calibration methods give concentrations within the confidence interval of the certified value. Similar results were found for the other reference materials. These results confirm the fact that extractions with acetic acid are quantitative for TBT in sediments and biological tissues. In addition no degradation of TBT is observed.

**TBT in sediments.** The species specific isotope dilution analysis of TBT was applied to sediments PACS-2 and CRM 462. Six independent spiking experiments were carried out for each spiking procedure. TBT was extracted following the procedures indicated above and each sample was injected five times in the GC-ICP-MS. The results are given in Table 2.

The results for the PACS-2 show both good accuracy and good precision for the three different spiking procedures. The recoveries range between 97 and 105%. For CRM 462, the results obtained are satisfactory, with recoveries of 103%, 101% and 106% for methods 1, 2 and 3, respectively.

**TBT in biological tissues.** The same procedures were applied to the mussel and oyster tissues. Three independent replicates were performed for each procedure and injected five times into the GC-ICP-MS. The results are reported in Table 2. The recovery range is between 100% and 104% for the different spiking procedures. The results for the reference material, CRM 477, are in good agreement with the certified value and show good precision. The results obtained for the candidate



**Fig. 4** Concentrations of TBT (in  $\text{ng Sn g}^{-1}$ ) obtained for PACS-2 using external calibration (EC), standard addition calibration (SAC) and speciated isotope dilution mass spectrometry (SIDMS) with the simultaneous spiking microwave extraction procedure. Black lines indicate the certified value with the confidence interval.

**Table 2** Concentration means in  $\text{ng Sn g}^{-1}$  and RSD values obtained for CRM 462 and PACS-2 sediments and in  $\text{ng TBT g}^{-1}$  and RSD values obtained for CRM 477 and CRM 710

|                                                                       | CRM 462             | PACS-2            | CRM 477            | CRM 710                           |
|-----------------------------------------------------------------------|---------------------|-------------------|--------------------|-----------------------------------|
| Certified value                                                       | 70.0 ± 14 (20%)     | 980 ± 130 (13.3%) | 2200 ± 190 (8.6%)  | 135.1 ± 27.4 <sup>b</sup> (20.3%) |
| Successive spike-extraction protocol (15 h) <sup>a</sup>              | 72.1 ± 2.4 (3.3%)   | 949 ± 34 (3.6%)   | 2283 ± 20 (0.9%)   | 121.4 ± 4.0 (3.3%)                |
| Simultaneous mechanical extraction-spike protocol (12 h) <sup>a</sup> | 70.7 ± 3.6 (5.1%)   | 966 ± 42 (4.3%)   | 2296 ± 6 (0.3%)    | 144.0 ± 7.8 (5.4%)                |
| Simultaneous microwave extraction spike protocol (7 min) <sup>a</sup> | 74.1 ± 10.2 (13.8%) | 1031 ± 12 (1.2%)  | 2199 ± 15.2 (0.7%) | 138.0 ± 12.0 (8.7%)               |

<sup>a</sup>Total time procedure. <sup>b</sup>Recommended value.

reference material, CRM 710, for all the spiking-extraction procedures are in good agreement with the recommended value.

**Analytical performance of the different spiking protocols.** The spiking procedure is a critical stage in IDMS to ensure the same behavior of the analyte and its analog during the analytical procedure. The three different spiking protocols for isotope dilution analysis of TBT were applied to sediment and biological tissue reference materials. From the results obtained, we could conclude that all the spiking procedures gave similar results for both sediments and biological materials. However the three methods present significant differences in time and matrix conditions for equilibration of the spike and extraction and in analytical time of the procedures.

Full equilibration between the spike and the analyte in the sample is a prerequisite for achieving accurate results using isotope dilution analysis. The results could be wrongly estimated if a good equilibration between the spike and its analog was not achieved and if the spike was recovered with higher efficiency. To avoid non-quantitative sample preparation it is essential that the added spike mimics the analyte. To elucidate this process, three spiking extraction methods have been tested. Method 1 is considered as the reference method because it makes use of a long equilibration time, maximizing the equilibrium of the spike, and because due to its drying stage before extraction it mimics the initial matrix the closest.

In the successive spike extraction method, the spike is added to the sample and methanol is added for homogenization. An equilibration time of 12 h is used to ensure full equilibrium of the spike and the natural TBT. This lengthy spiking procedure allows the compound spiked to behave similarly to the natural species. This result is obtained by the direct introduction of the analytes within the matrix. Agitation in a solvent slurry followed by a drying step allows us to recompose the initial matrix. The extraction step using microwave heating is fast (3 min). The results are in good agreement with the certified values with a low relative standard deviation ranging between 0.9 and 3.6%.

For the simultaneous mechanical extraction spike protocol, the equilibration time for the spiked specie is also 12 h. The spike is added in the extractant solution (acetic acid and methanol). The spiking step and the extraction step take place at the same time. Therefore, here also, full equilibration between the spike and the analog compound is occurring in solution and not really within the natural matrix. Naturally occurring TBT is extracted from the solid and kept in the solution containing the enriched TBT. The results obtained on the reference materials display both good accuracy and precision (RSD from 0.3 to 5.1%). We could not observe any difference with the use of the successive spiking extraction procedure. The extraction with acetic acid is therefore quantitative for TBT in sediments and biological tissues since full equilibration of the spiked and the natural specie could be achieved in the extractant solution. This statement is confirmed by the results based on external calibration and standard additions. Acetic acid is an efficient extractant for TBT and no intermediate degradation products such as DBT and MBT could be observed during the extraction step.

With the simultaneous microwave extraction spike method the equilibrium between the spike and the natural analyte occurs in the extractant solution but with a shorter equilibration time. The spike is added to the sample with acetic acid. The slurry is directly submitted to microwave extraction for 3 min. The sample is then directly extracted after the spike without any significant equilibration period. We can also observe here that the results still agree with the certified values for all the reference materials. It further confirms that extraction with acetic acid is quantitative for TBT. No degradation of TBT was observed during the microwave heating and no equilibration

time was found necessary for the enriched TBT. The equilibration between the spike and the analyte was apparently rapidly achieved when using the microwave extraction. This result could possibly be attributed to the processes taking place during microwaves irradiation. Indeed, during microwave irradiation chemical reactivity occurs simultaneously at a molecular level due to the rapid rotation of polar molecules in the samples. This mode of action certainly ensures rapid homogenization between the natural analyte and the additional spike.

Comparing the performances of each protocol, the relative standard deviations for methods 1 and 2 are similar for each sediment and each biological tissue achieving similar precision. The standard deviations of methods 1 and 2 depend on TBT concentration. For method 3, the standard deviation seems independent of the TBT concentration. A systematic error could indeed be generated during the sample preparation. During the simultaneous microwave extraction spiked method, the mass of the sample and the mass of the spike have been weighed directly in the microwave bombs. In this case the precision of the mass balance is  $10^{-4}$  g (total mass > 80 g) instead of  $10^{-5}$  g for the methods 1 and 2 (total mass < 80 g). The low precision on the weight of the spike leads to a systematic error in the final result, estimated to be  $12 \text{ ng g}^{-1}$ . However, the relative standard deviation is in the same range as those of the two other methods. This approach should be improved using a more efficient balance (with high precision for total mass > 80 g) or smaller microwaves bombs.

In summary, the different spiking procedures do not apparently have any effect on the quality of the results. Even when the equilibration time was the shortest, the spike and its natural analogue behave in a similar manner during the whole of the preparation stages. Acetic acid is an efficient extractant for TBT in sediments and biological tissues. Microwave heating also did not degrade TBT and allows rapid and efficient extraction. According to these results, two main conclusions can be drawn. First, the microwave extraction procedure used in this work gave efficient extractions since, in the simultaneous microwave extraction spike method, no equilibration time was necessary. This procedure is thus a very fast and accurate method and can be set on a routine basis. Second, no degradation of the spike occurred for procedures using a long equilibration time with the matrix and the reagents or using microwave heating. Both types of results may originate from the rapid agitation and rotation of the dipolar compounds present in the sample and submitted to the microwave field.

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## **Chapitre E – Annexe III**

**Détermination du TBT dans un sédiment marin par dilution  
isotopique Exercice d'intercomparaison du Comité Consultatif pour  
la Quantité de Matière (CCQM)**



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## PAPER IN FOREFRONT

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## Determination of tributyltin in marine sediment: Comité Consultatif pour la Quantité de Matière (CCQM) pilot study P-18 international intercomparison

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**Abstract** The capabilities of National Metrology Institutes (NMIs – those which are members of the Comité Consultatif pour la Quantité de Matière (CCQM) of the CIPM) and selected outside “expert” laboratories to quantitate  $(\text{C}_4\text{H}_9)_3\text{Sn}^+$  (TBT) in a prepared marine sediment were assessed. This exercise was sanctioned by the 7th

CCQM meeting, April 4–6, 2001, as an activity of the Inorganic Analysis Working Group and was jointly piloted by the Institute for National Measurement Standards of the National Research Council of Canada (NRC) and the Laboratory of the Government Chemist (LGC), UK. A total of 11 laboratories submitted results (7 NMIs, and 4 external labs). Two external laboratories utilized a standard calibration approach based on a natural abundance TBT standard, whereas all NMIs relied upon isotope dilution mass spectrometry for quantitation. For this purpose, a species specific  $^{117}\text{Sn}$ -enriched TBT standard was supplied by the LGC. No sample preparation methodology was prescribed by the piloting laboratories and, by consequence, a variety of approaches was adopted by the participants, including mechanical shaking, sonication, accelerated solvent extraction, microwave assisted extraction and heating in combination with Grignard derivatization, ethylation and direct sampling. Detection techniques included ICP–MS (with GC and HPLC sample introduction), GC–MS, GC–AED and GC–FPD. Recovery of TBT from a control standard (NRCC CRM PACS-2 marine sediment) averaged  $93.5 \pm 2.4\%$  ( $n=14$ ). Results for the pilot material averaged  $0.680 \pm 0.015 \mu\text{mol kg}^{-1}$  ( $n=14$ ;  $80.7 \pm 1.8 \mu\text{g kg}^{-1}$ ) with a median value of  $0.676 \mu\text{mol kg}^{-1}$ . Overall, performance was substantially better than state-of-the-art expectations and the satisfactory agreement amongst participants permitted scheduling of a follow-up Key comparison for TBT (K-28), a Pilot intercomparison for DBT (P-43), and certification of the test sediment for TBT content and its release as a new Certified Reference Material (HIPA-1) with a TBT content of  $0.679 \pm 0.089 \mu\text{mol kg}^{-1}$  (expanded uncertainty,  $k=2$ , as Sn) ( $80.5 \pm 10.6 \mu\text{g kg}^{-1}$ ).

**Electronic Supplementary Material** Supplementary material is available in the online version of this article at <http://dx.doi.org/10.1007/s00216-003-2016-9>.

**Keywords** Tributyltin · Marine sediment · International intercomparison

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## Introduction

Organotin compounds are ubiquitous contaminants in the environment, several of which exhibit deleterious effects in aquatic ecosystems. In particular, the use of tributyltin as a marine antifouling agent has led to its near global dispersal and it is now prominent in the coastal waters of all developed nations [1]. Tributyltin (TBT) persists in sediments, despite international efforts to curtail its usage in TBT-based paints since 1982 in France and subsequently in other countries. TBT levels in water and in marine sediment samples remain relatively high and continue to pose an ecotoxicological threat in the aquatic environment.

Quantification of organotin species is a difficult task due to their instability, low concentration levels and questions concerning extraction efficiency. Pellegrino et al. [2] and Quevauviller et al. [3] recently summarized methodologies and performance characteristics for numerous current approaches, only to conclude that no single methodology can be recommended for consistent use.

This problem was deemed to be of sufficient interest to the analytical and environmental communities that pilot study P-18 of the Comité Consultatif pour la Quantité de Matière (CCQM) was sanctioned at the 7th meeting, April, 2000, to task the Inorganic Analysis Working Group to conduct an inter-comparison on the determination of TBT in sediment. This determination broadens the scope and degree of difficulty of measurements heretofore addressed by this Group and is the first to raise the issue of organometallic speciation by the CCQM. The exercise was jointly piloted by the Institute for National Measurement Standards of the National Research Council of Canada (NRC), Ottawa, and the Laboratory of the Government Chemist (LGC), Teddington, UK. It was agreed that the NRC would make available and distribute both a control standard (PACS-2 Marine Sediment CRM certified for TBT content) as well as a test sediment material, and that the LGC would make available a species specific calibration standard based on a synthesized  $^{117}\text{Sn}$ -enriched TBT to be used by participants wishing to conduct isotope dilution mass spectrometry (ID-MS). The advantages of application of such calibration methodology to the enhancement

of data quality have recently been demonstrated by Encinar et al. [4] who also noted significant degradation of tributyltin to dibutyltin (and monobutyltin), which was easily detected with use of enriched isotopic species.

A call for participation was sent out to all NMIs in October, 2000, at which time several external laboratories identified as expert in the technique were also invited to contribute to the effort. By the end of April, 2001, all respondents were sent one bottle of CRM PACS-2 control sediment and two bottles of the pilot material from the NRC along with calibration standards from the LGC comprising either a 100  $\mu\text{L}$  aliquot of >99% purity TBTCI standard and/or a vial containing a nominal 90.5  $\text{mg kg}^{-1}$  methanolic solution of the  $^{117}\text{Sn}$  enriched TBTCI to be used for ID-MS calibration. Instructions for handling and storage of all samples and calibration standards were provided to each participant. Results were requested to be submitted to NRC no later than October 1, 2001.

## Experimental

A summary of participating laboratories and their affiliations is presented in Table 1.

### Samples and standards

The P-18 pilot sample sediment was prepared in-house at NRC following careful 9:1 (*m/m*) gravimetric blending of two NRC CRM marine sediments, PACS-2 and HISS-1, the latter containing negligible concentrations of TBT and thus serving as a solid, inert diluent. The mixture was tumbled for 12 days at room temperature in a large glass container and sample homogeneity ascertained through measurement of several certified minor elements. ICP-OES was used for this purpose following a complete decomposition of sub-samples using pressurized microwave digestion with a mixed acid. Samples were withdrawn over a period of several days in an effort to track the progress of the blending process. Final variability within the blend was <3% RSD and <1% from target values of TBT based on the dilution factor. The sample was then partitioned into 336 pre-cleaned glass bottles, each containing nominal 25 g amounts of sample. All bottles were gamma-irradiated (minimum 2.5 Mrad) to ensure long-term stability and then labelled and packaged in trilaminar foil pouches. Analysis of randomly selected bottles of P-18 samples before and after gamma-irradiation revealed that this process altered the TBT content, as ex-

**Table 1** CCQM P-18 initial study participants

| Institution/organization |                                                              | Country             | Contact            |
|--------------------------|--------------------------------------------------------------|---------------------|--------------------|
| BAM                      | Federal Institute for Materials Research and Testing, Berlin | Germany             | T. Win             |
| Crompton                 | Crompton GmbH Research Analytic                              | Germany             | P. Schultze        |
| IRMM                     | Institute for Reference Materials and Measurements           | European Commission | H. Schimmel        |
| LGC                      | Laboratory of the Government Chemist                         | United Kingdom      | R. Wahlen          |
| LNE                      | Laboratoire National d'Essais                                | France              | B. Lalere          |
| Limnol.                  | Limnologisches Institut Dr Nowak                             | Germany             | T. Brandsch        |
| NARL                     | National Analytical Reference Laboratory                     | Australia           | L. Mackay          |
| NMIJ                     | National Metrology Institute of Japan                        | Japan               | K. Inagaki         |
| NRCC                     | National Research Council of Canada                          | Canada              | R. Sturgeon        |
| Oviedo                   | University of Oviedo, Dept. of Chemistry                     | Spain               | J.I. Garcia Alonso |
| Pau                      | Université de Pau et des Pays de l'Adour, Lab. de Chimie     | France              | O. Donard          |

**Table 2** Analytical methods and instrumental techniques used by CCQM P-18 participants

| Participant | Calibration <sup>a</sup>                              | Method                                                                  | Instrumentation              |
|-------------|-------------------------------------------------------|-------------------------------------------------------------------------|------------------------------|
| BAM         | ID-MS                                                 | (a) HOAc/MeOH/sonicate/ethylation<br>(b) HOAc/MeOH/microwave/ethylation | (1) GC-MS<br>(2) GC-AED      |
| Crompton    | SA-IS                                                 | KOH/MeOH/heat/ethylation                                                | GC-FPD                       |
| IRMM        | ID-MS                                                 | HCl/tropolone/hexane; ASE extraction; Grignard pentylation              | GC-MS                        |
| LGC         | ID-MS                                                 | HOAc/MeOH; ASE extraction                                               | HPLC-ICP-MS                  |
| LNE         | ID-MS                                                 | 12 h HOAc/shaking; ethylation                                           | GC-MS                        |
| Limnol.     | EC-IS                                                 | KOH/MeOH/heat/ethylation                                                | GC-FPD                       |
| NARL        | ID-MS                                                 | (a) HOAc/microwave<br>(b) HOAc/microwave/ethylation                     | (1) HPLC-ICP-MS<br>(2) GC-MS |
| NMIJ        | ID-MS                                                 | HOAc/MeOH/tropolone; microwave; ethylation                              | GC-ICP-MS                    |
| NRCC        | ID-MS                                                 | HOAc/microwave                                                          | HPLC-ICP-MS                  |
| Oviedo      | ID-MS: (1) <sup>117</sup> Sn<br>(2) <sup>119</sup> Sn | HOAc/MeOH/sonicate/ethylation                                           | GC-ICP-MS                    |
| Pau         | (1) ID-MS<br>(2) SA-IS<br>(3) EC                      | HOAc/microwave/ethylation                                               | GC-ICP-MS                    |

<sup>a</sup>ID-MS, isotope dilution mass spectrometry; SA, standard additions; IS, internal standard; EC, external calibration

pected [5]. The concentration of TBT in irradiated samples was determined (in-house) to be 13% lower than in non-irradiated samples. A homogeneous distribution of TBT between bottles was also verified. No significant difference was noted for the within bottle (8 determinations) versus between bottle homogeneity, as initially assessed by the precision of the NRC in-house SPME-GC-MS results (12.4% RSD, (1s, n=14) for 7 bottles). The decision to proceed with sample distribution was based on an examination of available literature relating to TBT determinations in sediment, and the state-of-the-art characterizing CRMs certified for TBT, suggesting that this level of homogeneity would be fit for purpose (it was typical of that characterizing available water, sediment and tissue CRMs [6, 7]). Subsequent, more detailed studies, using a more precise species specific isotope dilution MS technique, revealed that the distributed pilot sediment was quite homogeneous with respect to TBT, having less than 5% variation between bottles (1s, n=12), based on 1 g test samples.

Calibration standards were provided by LGC and were distributed direct to participants as requested, i.e., either a >99% purity natural abundance TBTCI standard (100 µL in a sealed vial) or both this standard as well as a 4 mL methanolic solution containing nominally 90.5 mg kg<sup>-1</sup> TBTCI in the form of <sup>117</sup>Sn-enriched material for those laboratories undertaking isotope dilution mass spectrometry. Safety data sheets accompanied the standards, as did instructions for their use and storage. The material was deemed stable for at least 5–6 months at room temperature in their amber vials.

### Methodologies

As noted earlier, the choice of protocol presented in the instructions document was left to the discretion of the participant. Apart from a decision to employ ID-MS or calibration with a natural abundance standard in combination with selected internal organotin standards (standard additions calibration with an accompanying internal standard (SA-IS) and external calibration (EC) with and without the internal standard), there are a number of extraction techniques currently in use for determination of TBT [2], including mechanical shaking, sonication, microwave assisted extraction, heating and accelerated solvent extraction coupled with solvents such as KOH, HCl, acetic acid and methanol. Further, extracts may be analysed directly or immediately derivatised with alkylating (ethylation) or Grignard reagents (pentylation) prior to detection. Detection is achieved following separation with either gas chromatography (GC) in tandem with mass spectrometry (MS), flame

photometry (FPD), atomic emission (microwave induced plasma) and inductively coupled plasma mass spectrometry (ICP-MS) or by high performance liquid chromatography (HPLC) coupled to ICP-MS. Table 2 summarizes the methods used by the individual participants. The Department of Physical and Analytical Chemistry of the University of Oviedo, Oviedo, Spain utilized ID-MS based on the LGC supplied <sup>117</sup>Sn enriched calibration material and also based on an in-house synthesized <sup>119</sup>Sn enriched TBTCI spike. For analysis of the P-18 material, the majority of participants selected 0.5–1 g sub-samples for work-up, the exceptions were 0.25–0.35 g (LGC, Univ. of Pau, Univ. of Oviedo), and 2.0 g (NRC). No target value for uncertainty was specified at the commencement of this study.

The majority of the participants applied ID-MS, except for Crompton GmbH Research Analytic and Limnologisches Institut Dr Nowak. A standard additions (SA) approach was taken by Crompton GmbH Research Analytic, wherein a natural abundance TBTCI and several organotin reference standards, i.e. tetrapropyltin, tripropyltin, diheptyltin, and monoheptyltin chlorides, were used to normalize for response. The Limnologisches Institut relied on an external calibration (EC) but also incorporated tripropyltin chloride as the internal standard during the derivatization step.

Details of the extraction methodologies and instrumentation used by the individual participants are summarized in the supplement to this study.

### Results

Several laboratories submitted more than one independent result, based on an alternative detection methodology, calibration strategy or extraction procedure. In such case, the resultant mean was treated as an independent value and is delineated as the appropriate laboratory acronym accompanied by a numerical suffix (e.g., NARL-1).

In only few cases was the functional relationship for ID-MS (i.e., equation or other approach) and a list of quantities defined along with associated quantified uncertainty components made available by the participants in their submitted reports. As a consequence, there are cases where it was not possible for the piloting laboratories to re-evaluate the results based on the information provided.

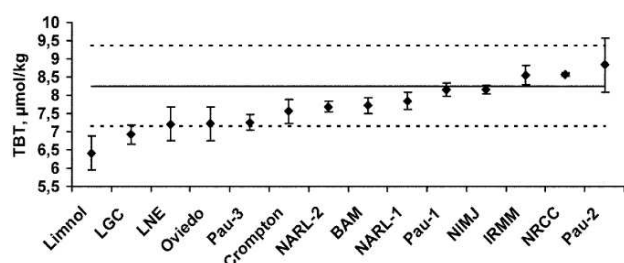
**Table 3** Results for determination of TBT in PACS-2 CRM<sup>a</sup>

| Participant | Total TBT cation, $\mu\text{mol kg}^{-1}$ (mean $\pm$ 1s, [n])                                                    | Recovery of certified value (%)                     | Report Date        |
|-------------|-------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------|--------------------|
| BAM         | (2) 7.72 $\pm$ 0.21 [4]                                                                                           | 93.5 $\pm$ 2.5                                      | October 1, 2001    |
| Crompton    | 7.56 $\pm$ 0.33 [4] (SA) <sup>b</sup>                                                                             | 91.5 $\pm$ 4.4                                      | November 18, 2001  |
| IRMM        | 8.55 $\pm$ 0.26 [4]                                                                                               | 103.5 $\pm$ 3.1                                     | September 27, 2001 |
| LGC         | 6.93 $\pm$ 0.26 [3]                                                                                               | 83.9 $\pm$ 3.8                                      | October 1, 2001    |
| LNE         | 7.21 $\pm$ 0.47 [3]                                                                                               | 87.3 $\pm$ 6.5                                      | October 10, 2001   |
| Limnol.     | 6.42 $\pm$ 0.17 [3](EC) <sup>c</sup>                                                                              | 77.7 $\pm$ 2.6                                      | September 27, 2001 |
| NARL        | (1) 7.85 $\pm$ 0.24 [3]<br>(2) 7.69 $\pm$ 0.14 [3]                                                                | (1) 95.0 $\pm$ 2.9<br>(2) 93.1 $\pm$ 1.7            | September 29, 2001 |
| NMIJ        | 8.16 $\pm$ 0.12 [3]                                                                                               | 98.5 $\pm$ 1.4                                      | October 9, 2001    |
| NRCC        | 8.57 $\pm$ 0.05 [3]                                                                                               | 103.8 $\pm$ 0.6                                     | September 14, 2001 |
| Oviedo      | 7.22 $\pm$ 0.46 [3] ( <sup>119</sup> Sn)                                                                          | 87.8 $\pm$ 5.6                                      | September 18, 2001 |
| Pau         | (1) 8.15 $\pm$ 0.18 [6]<br>(2) 8.83 $\pm$ 0.73 [3] (SA) <sup>b</sup><br>(3) 7.26 $\pm$ 0.22 [3] (EC) <sup>c</sup> | 98.7 $\pm$ 1.7<br>106.9 $\pm$ 8.8<br>87.9 $\pm$ 2.7 | September 21, 2001 |

<sup>a</sup>Reference value: 8.26 $\pm$ 1.10  $\mu\text{mol kg}^{-1}$  (95% CI)

<sup>b</sup>Standard additions (with tripropyltin and other organotin species serving as internal standards)

<sup>c</sup>External calibration (with tripropyltin chloride serving as internal standard)

**Fig. 1** Results for TBT in PACS-2 CRM sediment. Note that error bars represent $\pm$ 1s. Horizontal lines represent CRM PACS-2 certified value and associated 95% confidence interval

Participants' results for the control CRM marine sediment reference material PACS-2 are presented in Table 3, from which recovery data were calculated, based on the certified value of 0.98 $\pm$ 0.13 mg kg<sup>-1</sup> (as tin) or 8.26 $\pm$ 1.10  $\mu\text{mol kg}^{-1}$  (as TBT cation). Results are displayed graphically in Fig. 1. The mean result, calculated from these data, is

7.72 $\pm$ 0.18 ( $n=14$ )  $\mu\text{mol kg}^{-1}$  TBT cation with a median of 7.71, corresponding to an average recovery of the target analyte of 93.5 $\pm$ 8.2% (1s). With the exceptions of Univ. of Pau and NMIJ, no uncertainty budgets were developed for the determination of TBT in PACS-2, hence only data for the sample standard deviation (1s) reported by each participant is presented in Table 3 and illustrated in Fig. 1.

Explicit experiments aimed at elucidating recovery of TBT were not reported by any participant; values summarized in Table 3 were thus calculated from the ratio of determined TBT : certified value in PACS-2. For the purposes of this calculation, it was assumed that the efficiency of extraction of the endogenous TBT from the sediment was identical to that of the added spike. As application of ID-MS (or analyte standard addition) is based on the premise that complete equilibration of the added spike occurs in both the chemical and physical domains, if the resulting data are to be accepted, then it must also be accepted that the recovery data are also accurate. A standard additions approach was used by both Univ. Pau (Pau-2)

**Table 4** Results for the determination of TBT in P-18 pilot sediment

| Participant | $\mu\text{mol kg}^{-1}$ (total TBT cation), mean $\pm$ 1s [n] <sup>a</sup>                           | $\mu\text{mol kg}^{-1}$ (total TBT cation), mean $\pm$ U <sub>c</sub>                                 | k               |
|-------------|------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------|-----------------|
| BAM         | (1 & 2) 0.669 $\pm$ 0.033 [7]                                                                        | 0.669 $\pm$ 0.030                                                                                     | 2.2             |
| Crompton    | 0.623 $\pm$ 0.032 [4]                                                                                | 0.623 $\pm$ 0.101 <sup>b</sup>                                                                        | CI <sup>b</sup> |
| IRMM        | 0.800 $\pm$ 0.051 [12]                                                                               | 0.800 $\pm$ 0.054                                                                                     | 2               |
| LGC         | 0.657 $\pm$ 0.033 [11]                                                                               | 0.657 $\pm$ 0.082                                                                                     | 2               |
| LNE         | 0.586 $\pm$ 0.008 [6]                                                                                | 0.586 $\pm$ 0.031                                                                                     | 2               |
| Limnol.     | 0.551 $\pm$ 0.036 [6]                                                                                | 0.551 $\pm$ 0.029 (0.709 $\pm$ 0.037) <sup>c</sup>                                                    |                 |
| NARL        | (1) 0.686 $\pm$ 0.015 [6]<br>(2) 0.678 $\pm$ 0.016 [6]                                               | (1) 0.686 $\pm$ 0.044<br>(2) 0.678 $\pm$ 0.034                                                        | 2               |
| NMIJ        | 0.674 $\pm$ 0.015 [6]                                                                                | 0.674 $\pm$ 0.025                                                                                     | 2               |
| NRCC        | 0.748 $\pm$ 0.015 [6]                                                                                | 0.748 $\pm$ 0.052                                                                                     | 2               |
| Oviedo      | (1) 0.637 $\pm$ 0.035 [10, ( <sup>119</sup> Sn)]<br>(2) 0.611 $\pm$ 0.044 [11, ( <sup>117</sup> Sn)] | (1) 0.637 $\pm$ 0.056 ( <sup>119</sup> Sn)<br>(2) 0.611 $\pm$ 0.044 <sup>d</sup> ( <sup>117</sup> Sn) | 2(?)            |
| Pau         | (1) 0.722 $\pm$ 0.022 [6]<br>(2) 0.722 $\pm$ 0.017 [3]                                               | (1) 0.722 $\pm$ 0.043 (ID)<br>(2) 0.722 $\pm$ 0.020 (SA)                                              | 2               |

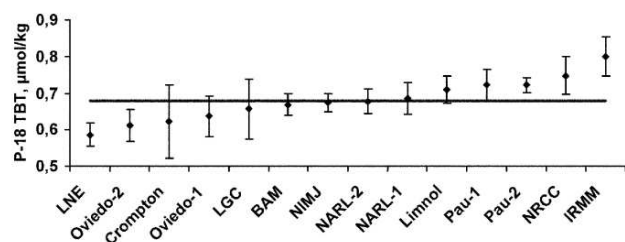
<sup>a</sup>One standard deviation of "[n]" replicate results

<sup>b</sup>SA-IS results, uncertainty is a simple 95% CI ( $n=3$ ), it does not include other error sources

<sup>c</sup>EC-IS results, value corrected for recovery (Table 3) given in parentheses

<sup>d</sup>Uncertainty reported as standard deviation of mean ( $n=11$ )

784



**Fig. 2** Results for TBT in P-18 Pilot sediment. Note: error bars depict combined expanded uncertainties, except where indicated otherwise. Horizontal line represents simple mean of all data. Error bars for Oviedo-2 represent sample standard deviation ( $\pm 1s$ ,  $n=11$ ), those for Crompton GmbH Research Analytic are their 95% *CI* ( $n=3$ ), and do not include other error sources

and Crompton GmbH Research Analytic; it was assumed, as in the case of ID-MS, that these added spikes were fully equilibrated with the sample prior to extraction. The Limnologisches Institut Dr Nowak utilized an external calibration approach, as did Univ. of Pau (Pau-3). It is not possible to draw any firm conclusions from these data regarding an optimum methodology for the extraction of TBT from the PACS-2 sediment; a variety of approaches (Table 2) appear appropriate, encompassing several solvent mixtures (HOAc, HCl, MeOH, KOH, toluene, hexane). Further, there is no relationship between subsample size and final results. It is noteworthy that a simple standard additions approach (Pau-2 and Crompton GmbH, Research Analytic) also yields acceptable data within the uncertainty of the certified value when either microwave assisted extraction or simple methanolic KOH with heating is applied, indicating that spike equilibration is readily achieved with this matrix and discordant results generated using ID-MS likely cannot be attributed to this factor.

Results for the determination of total TBT cation (all isomers, dry mass basis) in the pilot sediment are summarized in Table 4 and graphically presented in Fig. 2. A recovery factor, based on the analysis of PACS-2 (Table 3) was used to correct data submitted for the pilot sediment from the Limnologisches Institut; no other participant's data were similarly treated (it was assumed that the two matrices behaved identically with respect to extraction efficiency). Data provided by BAM were pooled results obtained from using both ID GC-MS and GC-AED detection. The uncertainties associated with all data are, unless noted otherwise, combined expanded uncertainties, having a nominal coverage factor of  $k=2$ . As there was no consistent treatment of uncertainties presented by the participants, particularly from the 4 external laboratories, a simple mean and associated standard deviation of the mean of all results along with the median value for the TBT concentration have been calculated [8], i.e., mean =  $0.680 \pm 0.015$  ( $n=14$ ); median =  $0.676 \mu\text{mol kg}^{-1}$  TBT.

Detection limits, estimated by all participants, are summarized in Table 5. There was no common method used to estimate this figure of merit, some participants choosing to define the LOD as the concentration giving a response

**Table 5** Estimated detection limits for TBT in sediment

| Participant | Estimated detection limit, $\text{nmol kg}^{-1}$ TBT cation | Detection technique |
|-------------|-------------------------------------------------------------|---------------------|
| BAM         | 6.3                                                         | GC-AED              |
| Crompton    | 170                                                         | GC-FPD              |
| IRMM        | 13                                                          | GC-MS               |
| LGC         | 70                                                          | HPLC-ICP-MS         |
| LNE         | 60                                                          | GC-MS               |
| Limnol.     | 72                                                          | GC-FPD              |
| NARL        | 20                                                          | HPLC-ICP-MS         |
| NMIJ        | 9.3                                                         | GC-ICP-MS           |
| NRCC        | 49                                                          | HPLC-ICP-MS         |
| Oviedo      | 14                                                          | GC-ICP-MS           |
| Pau         | 7 ( $^{117}\text{Sn}$ ); 5 ( $^{120}\text{Sn}$ )            | GC-ICP-MS           |

equivalent to three times the standard deviation of the blank, others deriving this figure of merit from a regression analysis of their calibration curve, noting that the value will change depending on the concentration range (and hence sample mass processed) in question.

## Discussion

Results for determination of TBT in the PACS-2 control sample were acceptable for the majority of the participants. This is illustrated by the data in Table 3 and Fig. 1. Results submitted by LGC and the Limnologisches Institut were biased low with respect to the accepted measurement window defined by the certified value and associated 95% *CI*, and data for LNE and the Univ. of Oviedo are just inside this "window". This is also reflected in the recovery data, summarized in Table 3, wherein these laboratories attained TBT recoveries of less than 88%.

The majority of laboratories subjected the sediments to a vigorous and efficient extraction procedure relying on microwave assisted or accelerated solvent extraction techniques. Although the data base is small in this study, it is suggestive that low recoveries of TBT from PACS-2 are systematically obtained by those laboratories utilizing the potentially less efficient extraction techniques, such as sonication (Oviedo), mechanical shaking (LNE) or heat (Crompton GmbH Research Analytic and Limnologisches Institut Dr Nowak). Nevertheless, results from LGC are inconsistent with this general conclusion, as an accelerated solvent extraction approach was used for sample extraction in this laboratory. Further, despite use of only heat, Crompton GmbH Research Analytic achieved acceptable results, and data obtained by NARL (not presented in the tables) following application of a sonication extraction process were in good agreement with their microwave assisted extraction results (i.e.  $<0.7\%$  divergent), suggesting that the source of the problem with the Limnologisches Institut Dr Nowak was simply the use of an EC protocol (also evident in Pau-3 data).

The TBT target value for the P-18 sediment (based on a 10-fold gravimetric dilution of PACS-2) was  $0.83 \mu\text{mol kg}^{-1}$



TBT cation. The resultant calculated mean of  $0.680 \pm 0.015$  ( $n=14$ ) obtained in this study reveals a significant disparity between the target and obtained TBT concentration. This appears to be a consequence of the gamma-irradiation sterilization process, which is known to degrade TBT (amongst other species) [5]. As a consequence, the approximate target value of  $100\text{--}150 \mu\text{g kg}^{-1}$  (as Sn) originally suggested in the covering letter to participants was considerably lower than anticipated and resulted in a re-wading scrutiny of data by all participants. As noted earlier, in-house measurements indicated that the irradiated samples were 13% lower in TBT content than non-irradiated samples. An examination of the participants' data confirmed this; the ratio of reported TBT in the PACS-2 compared to the P-18 material should be 10.0 (based on the gravimetric dilution factor) whereas an average of 8.61 was achieved with a range of values from 8.13 to 9.48 (based on uncorrected data for Limnologisches Institut Dr Nowak).

For the Limnologisches Institut laboratory, for which an external calibration approach was used, a correction factor for TBT recovery, based on their reported PACS-2 data, was subsequently applied to their P-18 data. This was based on the assumption that a matrix effect occurred and that the added internal standard (i.e., tripropyltin) served to only correct/compensate for volumetric factors. No other participants' P-18 sample data were likewise corrected. It is acknowledged that this is not a completely suitable approach as the uncertainty in the correction factor (assumed to be zero) will, in fact, be large because of the uncertainty in the certification range for the PACS-2. It may thus be prudent to exclude data for the pilot material from this laboratory.

The mean for Oviedo-2 appears biased low and it is to be noted that the measure of uncertainty used in this figure for this laboratory is a sample standard deviation ( $n=11$ ). These results were obtained on samples subjected to ultrasonic extraction in vessels that were later deemed (by the participant) to be of inappropriate geometry and hence the extraction efficiency was questionable.

Dry mass correction for sample humidity is relatively insignificant when compared to other variables likely responsible for the spread in the reported values. Moisture correction in the P-18 material ranged from a high of 0.98% (Pau) to a low of 0.14% (LNE) with a mean of  $0.64 \pm 0.24\%$  (1s).

Major sources contributing to overall uncertainty identified by the participants are summarized in Table 6 and were frequently related to the measurement of the reference-to-spike ratio in the blends. Only NARL noted the need to consider a correction factor (1.000) associated with possible degradation of the TBT to DBT during the extraction procedure and included a relative uncertainty component (0.97%) associated with this term. The LGC, IRMM, NARL and NMIJ provided explicit estimates of the contribution to overall uncertainty arising from the extraction efficiency. NMIJ equated it to the relative standard error of the mean of independent sample measurements (0.0092) and included this factor ( $E=1.000$ ) in their

**Table 6** Major sources of uncertainty

| Participant | Dominant source                                                           |
|-------------|---------------------------------------------------------------------------|
| BAM         | Method precision<br>Int. standard/analyte ratio                           |
| Crompton    | Calibration function                                                      |
| IRMM        | LOD (extraction efficiency)                                               |
| LGC         | Ref/spike ratio measurement<br>(extraction component, 65%)                |
| LNE         | Ref/spike ratio measurement                                               |
| Limnol.     | Sample work-up                                                            |
| NARL        | (1) Blank and degradation of TBT to DBT<br>(2) Method precision and blank |
| NMIJ        | Primary std<br>Extraction efficiency                                      |
| NRCC        | Ref/spike ratio measurement                                               |
| Oviedo      | Primary std assay<br>Ratio measurement                                    |
| Pau         | Unclear                                                                   |

equation for the ID-MS expression for the result. NARL did likewise, assessing the extraction uncertainty (0.0017) from measurement of successive extractions of the P-18 material. IRMM equated the uncertainty of the extraction process with the relative uncertainty of the limit of detection and propagated this component into their overall expression for expanded uncertainty. LGC equated the uncertainty associated with the extraction process to the blend-to-blend variation of the results over 11 determinations. The question of extraction efficiency is one that needs to be resolved; it impacts on results of speciation measurements substantially more than on previous inter-comparisons of total metal recoveries from various matrices [9, 10]. This issue has been discussed in some detail in subsequent publications from this laboratory [11, 12].

Several participants provided information arising from the reverse spike ID-MS determination of the concentration of TBT in the LGC isotopically enriched calibration standard. As supplied, this standard was stated to contain  $90.5 \text{ mg kg}^{-1}$  TBTCI (as Sn). Pau reported this standard to be  $98.1 \pm 1.1 \text{ mg kg}^{-1}$ ; Oviedo, 100.1; NRC,  $105.5 \pm 1.4$  and NMIJ, 101.5. Clearly, there may have been a need to precede this Pilot exercise with a determination of TBT in a simple calibration solution, as practised in some of the earlier CCQM inter-comparisons and it would have been useful to ask those participants using ID-MS to provide an independent measure of the concentration of the calibration standard by reverse spike techniques. Perhaps in this manner some trend in the data may arise which will relate scatter to calibration and not extraction efficiency.

Despite a 10-fold decrease in the concentration of TBT in the P-18 test sediment compared to the PACS-2 control, the precision of determination was not degraded for this material (8.4% RSD vs 8.2% for PACS-2, 1s data). It is likely that this arises because there is no detectable blank reported by most participants and the concentration of TBT in the sample was, in most cases, typically 20–100-fold above reported detection limits (cf. Table 5). In this connection, no single detection system can be recom-

**Table 7** Summary statistics amongst participants for P-18 sample

| TBT                              | All participants |
|----------------------------------|------------------|
| In $\mu\text{mol kg}^{-1}$       |                  |
| Mean                             | 0.680            |
| Median                           | 0.676            |
| Sample standard deviation, 1s    | 0.057            |
| Standard deviation of mean       | 0.015            |
| $ts / \sqrt{n}$ (95% confidence) | 0.033 ( $n=14$ ) |
| In percent                       |                  |
| 1s                               | 8.4              |
| RSD of mean                      | 2.3              |
| $ts / \sqrt{n}$ (95% confidence) | 4.9              |
| Range <sup>a</sup>               | 31               |

<sup>a</sup> (highest value – lowest value) / mean

mended for application at this concentration and it is likely that the skill and experience of the operator is more important than the hardware available. It is also significant that the performance of the external laboratories is not at all inferior to that of the NMIs as, in many cases, they have historically had more experience with the determination of this analyte than most NMI laboratories.

Only one of the participants (NARL) discussed the issue of isomer resolution when using GC techniques. This issue was also originally raised by NARL, which prompted issue of a statement by the pilot laboratories that if isomer resolution was achieved, the integrated response from all was to be taken as a measure for quantitation.

Summary statistics for this study are given in Table 7. In view of the disparate treatments used to arrive at the associated expanded measurement uncertainties, a median is the preferred statistic [8] characterizing these data.

### Challenges in CCQM P-18

This the first time the CCQM has challenged participants with the quantitation of an organometallic analyte. Determination of tributyltin chloride requires significant sample preparation to ensure reproducible extraction of the TBT without decomposition, as well as derivatization of the analyte prior to detection (in most cases). Estimated limits of detection suggest that the determination is being attempted by some participants at a level only 4-fold above their LOD. Precision and accuracy can be enhanced through application of ID-MS techniques but equilibration must be assured if results are to be acceptable. More attention should be given to the impact of extraction efficiency on the overall results and associated uncertainty.

It should be emphasized that the extremely good results obtained in this Pilot study are probably limited entirely to the determination of TBT, and likely DBT, in a sediment matrix. Application to tissue matrices or MBT or, indeed, to any other organometallic species, cannot be confidently extrapolated.

### Certification of HIPA-1

Participants' data for the test sediment contributed to the calculation of a certified value ( $0.679 \pm 0.089 \mu\text{mol kg}^{-1}$  or  $80.5 \pm 10.6 \mu\text{g kg}^{-1}$ , as Sn,  $k=2$ ) and characterization uncertainty ( $u_{\text{char}}=2.40 \mu\text{g kg}^{-1}$ ) for TBT in the Pilot material, which was given the acronym HIPA-1. For this purpose, the duplicate sets of data provided by NARL, Oviedo and Pau (cf. Table 4) were averaged to a single mean value for each laboratory and the unweighted means of all laboratories, excepting that from the Limnologisches Institut Dr Nowak, were then averaged to derive the certified value. Data from the Limnologisches Institut Dr Nowak were excluded as this participant used an external calibration curve for determination of TBT that would have required a recovery factor to account for reasonable results.

A detailed assessment of the homogeneity of the test sediment was undertaken in the NRC laboratories to derive "within" and "between" bottle components using ID-GC-ICP-MS following derivatizations/extractions. An uncertainty component due to homogeneity was estimated to be  $u_{\text{hom}}=4.45 \mu\text{g kg}^{-1}$ . A long-term stability study, based on a 38 month storage experiment for a similar sediment (PACS-2) for TBT [13] provided for an uncertainty component,  $u_{\text{stab}}$ , of  $1.61 \mu\text{g kg}^{-1}$  for a specified shelf lifetime (expiry date) of 5 years (September, 2006) under the conditions that the material is stored in the dark at  $4^\circ\text{C}$  or lower ( $-20^\circ\text{C}$  recommended). An uncertainty component associated with the short-term stability (transport) was calculated based on an isochronous study wherein measurements were conducted on samples stored for one month at  $-20^\circ\text{C}$ ,  $+4^\circ\text{C}$  and  $22^\circ\text{C}$ . No significant contribution associated with short-term stability was applied. The total expanded uncertainty ( $U_{\text{CRM}}$ ,  $k=2$ ) is thus  $10.6 \mu\text{g kg}^{-1}$ .

### Conclusion

The success of this Pilot inter-comparison has validated the widespread state-of-the-art capability for the accurate determination of TBT in sediment matrices. The international participation of experts and access to species specific isotope dilution mass spectrometry has permitted the certification of a new reference material for TBT in marine sediment (HIPA-1).

The CCQM Inorganic Analysis Working Group has recommended the organization of a Key Comparison (K-28) for the determination of TBT in a sediment matrix as well as a Pilot inter-comparison (P-43) for the estimation of dibutyltin in a sediment matrix. Based on the results of this Pilot P-18 study, an expanded uncertainty ( $k=2$ ) of  $<5\%$  can be realistically targeted. It will be of interest to acquire information values and their associated statistics for MBT using the same methodologies.

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## **Chapitre E – Annexe IV**

**Flux benthiques de métaux (Cu, Cd, Pb, Mn, U et iHg) et  
d'organométaux (MMHg, TBT, DBT et MBT) dans l'étang de Thau  
Rôle de l'activité biologique**





## Benthic fluxes of metal (Cu, Cd, Pb, Mn, U AND iHg) and organometal (MMHg, TBT, DBT and MBT) in the eutrophicated Thau Lagoon (Mediterranean Coast, France): Role of biological activity

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### ABSTRACT

In situ benthic chamber experiments were conducted at four stations in the Thau lagoon in order to directly and simultaneously determine exchanges of trace metals (Cu, Cd, Pb, Mn and U), mercury species (iHg, MMHg) and organotin compounds (MBT, DBT, TBT) at the sediment-water interface. Results indicate that trace metal benthic fluxes range from -130 to 286 nmol.m<sup>-2</sup>.day<sup>-1</sup> for Cd, -0.5 to 4.2  $\mu$ mol.m<sup>-2</sup>.day<sup>-1</sup> for Pb, -0.35 to 4.76  $\mu$ mol.m<sup>-2</sup>.day<sup>-1</sup> for Co, -7.5 to 20.4  $\mu$ mol.m<sup>-2</sup>.day<sup>-1</sup> for U, -11.4 to 30.8  $\mu$ mol.m<sup>-2</sup>.day<sup>-1</sup> for Cu and -11 to 246  $\mu$ mol.m<sup>-2</sup>.day<sup>-1</sup> for Mn. For mercury species, iHg and MMHg fluxes range from -6.8 to 2.6 nmol.m<sup>-2</sup>.day<sup>-1</sup> and -147 to 334 pmol.m<sup>-2</sup>.day<sup>-1</sup>, respectively. For organotin species, TBT, DBT and MBT display fluxes in the range -0.5 to 11.6 nmol.m<sup>-2</sup>.day<sup>-1</sup>, 25.4 to 111.8 nmol.m<sup>-2</sup>.day<sup>-1</sup>, and 3.7 to 27.3 nmol.m<sup>-2</sup>.day<sup>-1</sup>, respectively. The variability of fluxes within the chambers at the different stations is significantly correlated to the flux of Mn for most trace metals and mercury species, suggesting Mn as an important element to identify microscale redox gradient changes. Advective pathways, such as biorrigation and bioturbation processes are overriding diffusive fluxes for the exchanges of metals and organometals between the sediment and the water column. Because of the eutrophication processes, large amount of macrophytic algae (*Ulva spp.*, *Gracilaria spp.*) at the sediment surface changes significantly the direction and the levels of metals, mercury species and organotin compounds benthic fluxes. This study demonstrates that benthic organisms, both macrofauna and macrophytes, are responsible for significant seasonal, diurnal and spatial variability for trace metals benthic fluxes in the lagoon. Estimation of benthic flux contribution and cycling in such coastal environment remains thus extremely problematic. In this paper we propose to consider the biofilm as an active pump for O<sub>2</sub> renewal/consumption, promoting the diagenetic sequence. Tentative budgets integrating all the contributive pathways demonstrate with evidence that most of the metals and organometals fluxes are mediated by the biological activity of the different macrofaunal trophic groups, leading the contribution of diffusive flux to be finally negligible.

**KEYWORDS:** trace metals, mercury, organotin, speciation, benthic flux, biological activity

## 1- INTRODUCTION

The coastal ocean is an important domain in the global biogeochemical cycles of carbon, nutrient elements and trace elements in the ocean (Mantoura et al., 1991; Rabouille et al., 2001). In the coastal zone (10% of the global ocean surface) approximately 25% of global oceanic primary production) and 80% of global organic carbon burial take place (Smith and Hollibaugh, 1993). During the last century, the coastal ocean has been exposed to large perturbation, mainly consequences of N and P- enrichments. But in the modern scheme proposed by Cloern (2001), the conceptual model of the coastal eutrophication, responses of coastal ecosystem appears highly variable. In this context, we have now to consider not only nutrient enrichment, but many interacting stressors (Breitburg, 1999) such as trace anthropogenic contaminants and to focus on the complex linkages of these multiple stressors to evaluate ecosystem response (Grall & Chauvaud, 2002).

Compared to nutrients and carbon cycle that has been extensively documented (Walsh et al.1981; Christensen et al., 1989, Mantoura et al., 1991; Rabouille et al., 2001), the global cycle of trace elements in coastal environments represents actually an important lack of knowledge first because of the complexity of the pathways involved and second because of the analytical difficulties to measure trace elements concentrations in these diluted ecosystems. In coastal environment, the main sink of trace contaminants corresponds to their settling in the nearest shallow sedimentation zones. This sedimentary stock represents an enormous potential repository for contaminants, with the capability of remobilization, leading to a possible transfer to the water column and to the pelagic food web (Mucha et al., 2004). A few studies have considered and quantified this pathway in the global cycling of trace elements in coastal environments. It has been recently suggested to be a possible route to explain the non-conservative behaviour of elements in estuaries (Chiffoleau et al., 1994; Warnken et al., 2001). Further all, in Galveston Bay (Warnken et al., 2001) and San-Francisco Bay (Rivera-Duarte and Flegal, 1994) it has been considered to represent one of the major input of trace metals to the water column.

The benthic fluxes correspond to the exchange of metallic contaminants between the water column and the sediment. In these exchanges, two additional and different natures of fluxes must be considered. First, the diffusive flux corresponds to the natural diffusion of trace elements between the water column and the porewater with a direction and a range depending on the depleted (or enriched) compartment (Zhang et al., 1995). Second, the advective flux corresponds to an exchange of metals between the sediment and water driven by an external factor. The latter is assumed to depend on the water currents, but it results also from the biological activity in terms of sediment bioirrigation and bioturbation (Gill et al., 1999; Warnken et al., 2001; Zheng et al. 2002).

The reactivity and cycling of trace elements at the sediment surface has been for long considered to be influenced by the O<sub>2</sub> balance representing a redox barrier at the sediment surface (Swarzenski et al., 1999; Chaillou et al., 2002; Robert et al., 2004). This redox gradient depends on two main factors. First, the carbon

cycle (Hammond et al., 1999) leading to successive diagenetic transformations starting from the microbial degradation of organic matter (Froelich et al., 1979; Postma and Jacobsen, 1996). The influence of the diagenetic sequence on trace metal mobility has been demonstrated using sensitive tracers of redox conditions such as Mn or Fe (Chiffoleau et al., 1994; Zwolsman et al., 1997; Warnken et al., 2001). The second factor influencing the redox gradient at the sediment surface is the biological activity that affects also the near-surface O<sub>2</sub> balance (Boucher et al., 1998; Grenz et al., 2003;). Its direct impact on nutrient budgets has been documented (Chauvaud et al., 2000; Ragueneau et al., 2002;) whereas it is also strongly suspected to influence trace metals (Gill et al., 1999; Warnken et al., 2001; Zheng et al., 2002).

The different techniques employed for trace metal benthic flux measurements are essentially based on the nature of the measured flux. For diffusive flux, the technique is based on the local/small scale determination of the concentration gradient between bottom waters and porewaters using in situ gel probes (Davison et al., 1991; Davison et al., 1994; Zhang et al., 1995). For benthic flux measurements, the deployment of benthic chambers has been documented. This technique integrates both the diffusive flux and the advective flux (Boucher et al., 1998). This last technique results in the integration of all the different factors influencing trace metals mobility at the sediment-water interface (diffusive flux, bioirrigation flux, bioturbation flux, biofilm influence and reactions occurring at the sediment surface). The use of benthic chambers has been originally developed to measure benthic respiration rates (Boucher et al., 1998; Grenz et al., 2003), nutrients and scarcely devoted for trace metals and mercury species (iHg, MMHg) except a few publications (Ciceri et al., 1992; Covelli et al. 1999; Gill et al., 1999; Zago et al., 2000; Warnken et al., 2001; Choe et al., 2002; Warnken et al., 2003) and never applied for organotin compounds.

The comparison of fluxes determined by porewaters gradient and by benthic chambers leads most of the time to significant differences because of measuring different types of fluxes (Warnken et al., 2001; Choe et al., 2002; Berelson et al., 2003). These differences have been recently proposed to be directly linked to the biological activity (Hammond and Fuller, 1979; Berelson et al., 2003). These authors demonstrated that the two techniques can give complementary informations about the flux characteristics. However, the single consideration of diffusive flux is not sufficient to estimate the whole exchanges of trace elements between the sediment and the water column particularly during intense biological activity periods. In addition, the microscale consideration of gel probes and their respective time of deployment is not adapted to the environments presenting important spatial variability, heterogeneity and fast temporal changes.

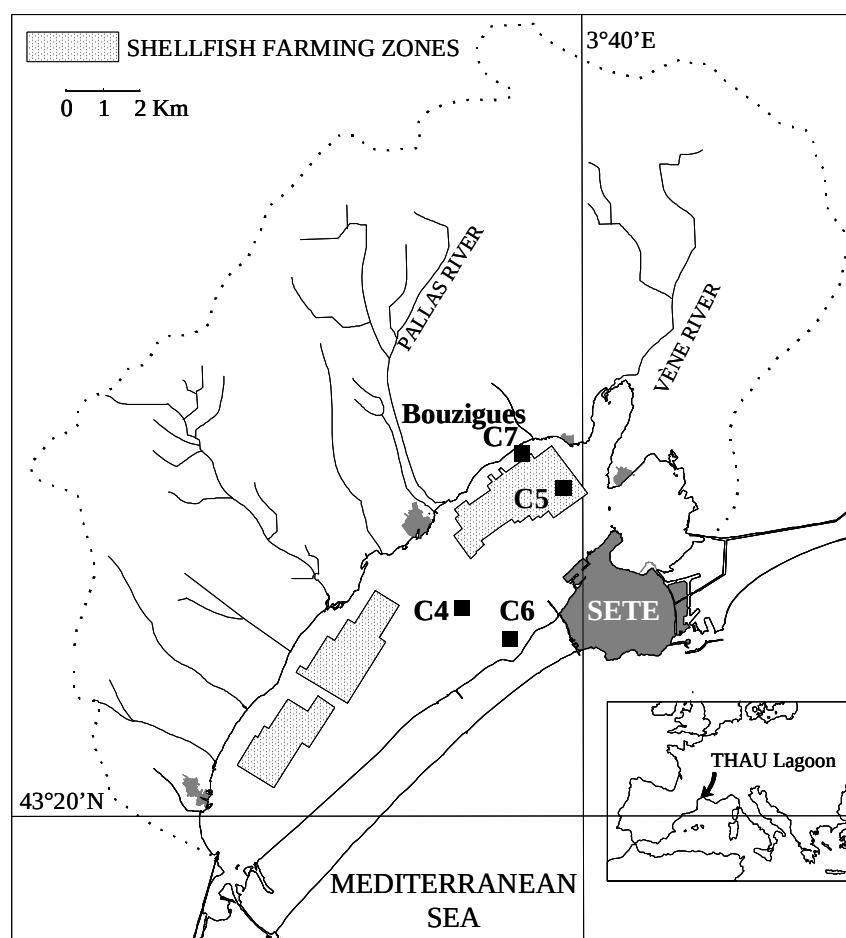
In this work, we propose to investigate fluxes at the water sediment interface in Thau lagoon (Mediterranean Sea, France) and the processes affecting trace metals cycling with particular attention to the diagenetic processes and the concomitant impact of 'biofilms', ie. biological presences and activities (feeding and respiration) at the sediment/water interface. A companion paper (Thouzeau et al. this issue) presents a

complete description of this eutrophicated driven biofilm considering macrophytic and/or macrofaunal distributions. This study was performed in the Thau lagoon, a shallow microtidal enclosed bay exhibiting shellfish farming zones (Southeastern, France). To achieve the accurate determination of trace metal (Cu, Cd, Pb, Mn and U), mercury species (iHg and MMHg) and organotin compound (MBT, DBT, TBT) fluxes, a robust integrated procedure employing benthic chambers is proposed. This study was part of a larger multidisciplinary research project (MICROBENT/PNEC program), which goal was to characterize and describe the nature and variability of biogeochemical fluxes occurring at the sediment-water interface.

## 2- MATERIAL AND METHODS

### 2.1- Site Description

The Thau Lagoon is located in the South East of France (Figure 1). This Lagoon covers an area of 75 km<sup>2</sup>. The connection of the lagoon to the Mediterranean Sea makes the water to present high levels of salinity ranging from 35 to 38 g.l<sup>-1</sup>. The depth in the lagoon varies from 4 to 10 m with a mean depth of 5 m. One particular characteristic of this lagoon is the presence of shellfish farming areas which cover 20 % of the total surface (Figure 1). The present study was carried out during two campaigns realized in April 2002 and May 2003, respectively. In this lagoon, the surface sediments consist of very fine particles (silt and clay) displaying a mean grain size ranging between 10 and 20µm (Schmidt et al., this issue). A part of the lagoon is also influenced by dense macrophytic covers (Gerbai and Verlaque, 1995).



**Figure 1:** Sampling sites and location of the four stations (C4, C5, C6 and C7).

The parameters measured and the four sampling sites for the two campaigns are summarized in Table 1:

- Station C4: located in the middle of the lagoon and outside the shellfish farming zone. The macrofaunal trophic groups are mainly composed of suspension feeders and deposit feeders (Thouzeau et al., this issue).
- Station C5: located inside the shellfish farming zone. No macrofaunal high size organisms (>1mm) were determined for the two campaigns (Thouzeau et al., this issue).
- Station C6: was a seagrass meadow located close to the city of Sète. it was only investigated in April 2002. The suspension feeders were presenting the highest proportion of total biomass (Thouzeau et al., this issue).
- Station (C7) was located close to the city of Bouzigues, in a corridor between oyster ropes. In this station, high densities of macroalgae (*Ulva spp.*, *Gracilaria spp.*) covering the sediment surface were observed in May 2003 (Figure 1).

Note that C4 and C5 are the two reference stations followed by the pluridisciplinary approach proposed within the MICROBENT project (Prevot et al., this issue), leading to a complete set of parameters available.

## April 2002

| Parameter                                                                        |                     | C4-1     | C4-2     | C5       | C6-1      | C6-2     | C7      |
|----------------------------------------------------------------------------------|---------------------|----------|----------|----------|-----------|----------|---------|
| Date                                                                             |                     | 9/4/02   | 10/4/02  | 13/4/02  | 12/4/02   | 12/4/02  | 14/4/02 |
| Incubation time (h)                                                              |                     | 2.3±0.2  | 3.6±0.4  | 2.6±0.1  | 3.14±0.07 | 2.1±0.3  | 2.4±0.2 |
| Depth (m)                                                                        |                     | 7.5      | 7.5      | 7.4      | 3.3       | 3.3      | 2.6     |
| Temperature (°C)                                                                 |                     | 14.2     | 14.2     | 13.9     | 14.3      | 14.2     | 13.5    |
| Salinity                                                                         |                     | 36.2     | 36.2     | 36.5     | 36.3      | 36.3     | 36.5    |
| Light ( $\mu\text{mol quanta.m}^{-2}.\text{s}^{-1}$ )                            |                     | 23±1     | 33±2     | 33±13    | 300±37    | 65±1     | 333±14  |
| Sulfate reduction rate ( $\text{mmol SO}_4^{2-}.\text{m}^{-2}.\text{day}^{-1}$ ) |                     | 9.9±1.0  | -        | 19±1.2   | -         | -        | -       |
| O <sub>2</sub> Flux ( $\text{mmol.m}^{-2}.\text{h}^{-1}$ )                       |                     | -0.8±0.7 | -1.6±0.3 | -1.8±0.3 | -1.9±1.1  | -3.4±0.5 | 0.7±1.2 |
| Dissolved Contaminants                                                           | Trace Metals        | x        | x        | x        | x         | x        | x       |
|                                                                                  | Mercury species     | -        | -        | -        | -         | -        | -       |
|                                                                                  | Organotin Compounds | -        | -        | -        | -         | -        | -       |
| Sediment Collection                                                              |                     | x        | -        | x        | -         | -        | x       |
| Benthic Fauna                                                                    |                     | x        | -        | x        | x         | -        | x       |

## May 2003

| Parameter                                                                        |                     | C4        | C5        | C7-1     | C7-2      |
|----------------------------------------------------------------------------------|---------------------|-----------|-----------|----------|-----------|
| Date                                                                             |                     | 12/5/03   | 15/5/03   | 14/5/03  | 14/5/03   |
| Incubation time (h)                                                              |                     | 6.63±0.03 | 5.18±0.02 | 2.9±0.2  | 2.8±0.2   |
| Depth (m)                                                                        |                     | 7.5       | 7.4       | 2.6      | 2.6       |
| Temperature (°C)                                                                 |                     | 17.1      | 18.6      | 19.8     | 19.8      |
| Salinity                                                                         |                     | 33.7      | 33.0      | 32.9     | 32.9      |
| Light ( $\mu\text{mol quanta.m}^{-2}.\text{s}^{-1}$ )                            |                     | 30±1      | 39±2      | 591±4    | 0.00      |
| Sulfate reduction rate ( $\text{mmol SO}_4^{2-}.\text{m}^{-2}.\text{day}^{-1}$ ) |                     | 16.9±2.0  | 31±5      | -        | -         |
| O <sub>2</sub> Flux ( $\text{mmol.m}^{-2}.\text{h}^{-1}$ )                       |                     | -0.7±0.3  | -3.9±0.2  | 23.9±2.6 | -11.1±0.7 |
| Dissolved Contaminants                                                           | Trace Metals        | x         | x         | x        | x         |
|                                                                                  | Mercury Species     | x         | x         | x        | x         |
|                                                                                  | Organotin Compounds | x         | x         | x        | x         |
| Sediment Collection                                                              |                     | -         | -         | -        | -         |
| Benthic Fauna                                                                    |                     | x         | x         | x        | x         |

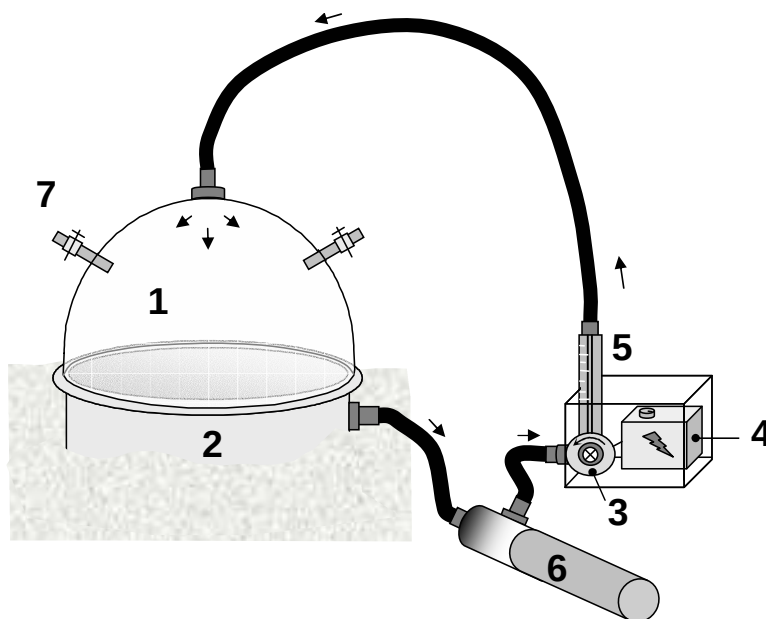
Table 1: List of the parameters measured at the different stations.



## 2.2- Benthic Chamber Operating Conditions

### 2.2.1- Benthic chamber characteristics

In situ incubations were carried out using diver-operated benthic chambers derived and adapted from previous work (Boucher and Boucher-Rodoni, 1988; Boucher et al., 1994; see Thouzeau et al., this issue). These chambers were composed of two separated parts: a clear acrylic hemisphere (dome) fastened to a base (Figure 2). The base employed in April 2002 was made of Polyvinylchloride (PVC) whereas a base in acrylic was employed in May 2003. The internal surface area of a chamber is 0.2 m<sup>2</sup> and the internal volume of incubated water varied from 48 to 60 l depending on the depth to which the bases were driven into the sediments. A submersible pump connected to a waterproof battery provided water circulation (Tricocclair Polyethylene tubing) in each chamber (Figure 2) allowing also continuous pH, temperature and oxygen concentration measurements using YSI 6920 probes refitted in a waterproof container and connected to a data logger as documented in detail elsewhere (Thouzeau et al., this issue). Incident irradiance was measured continuously in one of the chambers using a LI-192SA quantum sensor; the data were averaged and logged every minute in a LI-1000 data logger.

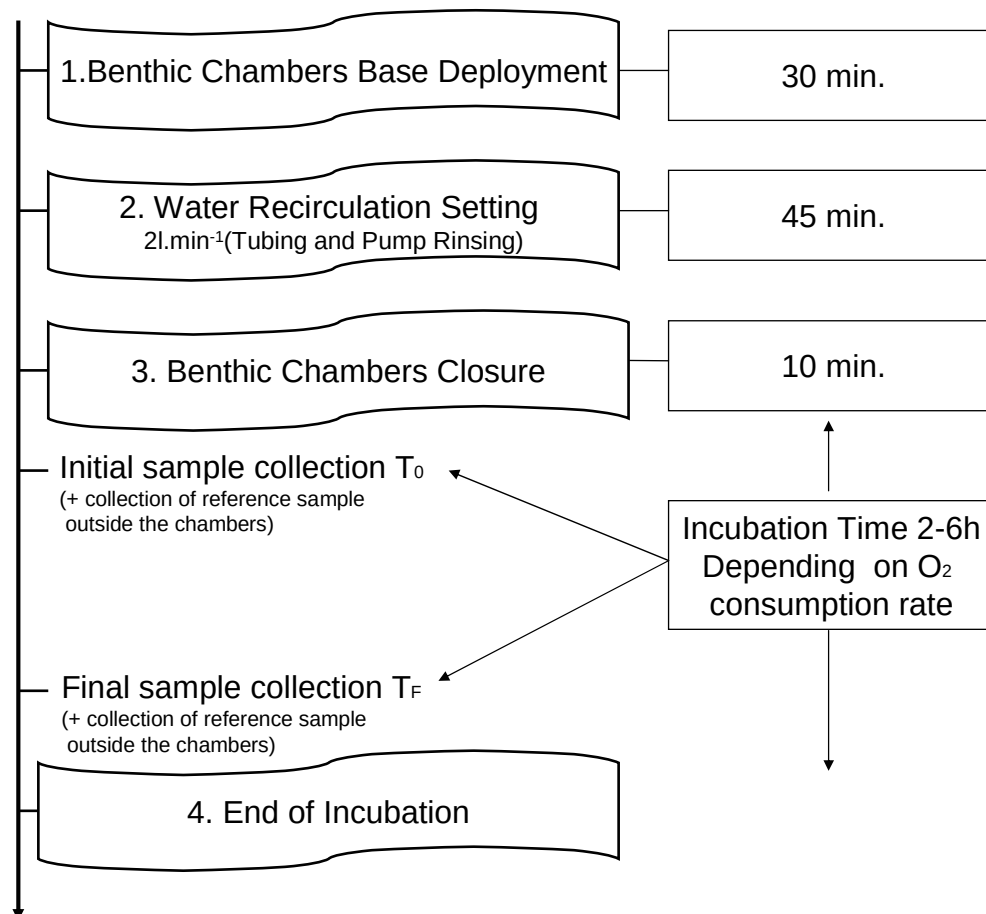


**Figure 2:** Benthic chamber characteristics. The experimental apparatus is composed of a transparent dome (1) fitted to a PVC or acrylic basis (2). The water is homogenised by a pump (3) supplied by a battery in a watertight housing (4). The water flow is checked with a flowmeter (5). Depth and oxygen concentration, temperature and salinity inside the enclosure, are recorded by a YSI 6920 probe (6). Water samples are collected through taps on the dome (7).

### 2.2.2 Benthic chamber operating procedure

The complete incubation procedure required three individual steps (Figure 3), all realized by divers. Three benthic chambers separated from each other by a distance of 2 m were deployed simultaneously at each station and for each incubation series to determine fluxes variability and representativity.

First, the chamber bases were carefully and slowly deployed at the sediment surface, followed by 30 minutes of delay allowing the mobilized particles to settle. Second, the water recirculation system was enabled for 45 minutes at a flow rate of  $2 \text{ l.min}^{-1}$  for both tubing and oxygen probe rinsing. Third, the chamber dome was installed on top of the base and water circulation was established. The initial samples ( $T_0$ ) were collected after 10 minutes using polypropylene syringes (500 ml) connected to the chamber by a polyethylene tap. Outside water was admitted through a second tap during sampling to avoid porewater release from the sediment (Figure 2). The number of syringes employed ranged from 5 to 9 depending on the number of parameters to be determined. After an incubation time ranging from 2 to 6h and optimized with respect to oxygen consumption rate, the final samples ( $T_F$ ) were collected with the same polypropylene syringes. This short-time incubation was particularly chosen to avoid intense  $\text{O}_2$  breakthrough during the experiments. The variation of  $\text{O}_2$  concentration during the experiments was lower than 20% of deviation in most cases. These incubating conditions are assumed to be suitable to preserve the original redox gradient occurring at the sediment-water interface, to keep unchanged the biological activity and to maintain as much as possible representative in situ conditions and equilibrium within the enclosures. The aspects devoted to trace metal and organometals measurements are presented in this study, whereas nutrients, metabolic fluxes ( $\text{O}_2$  and  $\text{CO}_2$ ) and benthic ecology considerations are presented in detail in Thouzeau et al. (this issue).



**Figure 3 :** Benthic chamber operation and procedures.

### 2.2.3 Benthic chamber cleaning and trace metal contamination control

Before deployment, all internal surfaces (dome and base) of the benthic chambers were cleaned first with a biocide detergent (RBS50, STC, France), before final decontamination with diluted Hydrochloric acid (1% w/v). Final rinsing was achieved using abundant volumes of commercial spring water. Then, all chamber parts were stored in polyethylene bag before their transport and deployment on the sampling sites.

A blank procedure was performed at the laboratory for each chamber to evaluate its specific background. The same cleaning protocol than previously cited was first employed, before filling the three chambers with commercial spring water. The blank incubation with continuous recirculation of 2 l.min<sup>-1</sup> was run for 5h and repeated twice. For all the studied elements except DBT and MBT, the first blank values were significantly low compared to the measured levels in the lagoon waters. The replacement of PVC bases (April 2002) by acrylic ones (May 2003) significantly decreased the DBT and MBT levels, allowing their determination in May 2003.

To check the contaminations during field experiments, we have compared the concentrations of all metals and organometals in the initial samples collected in the chambers ( $T_0$ ) with the respective values of the overlaying waters collected simultaneously. Identical syringes in both cases were used and the overlaying waters were collected at 30 cm over the sediment surface at 1m of distance of the chambers. No significant differences of concentrations between the initial sample of the incubation ( $T_0$ ) and the reference overlaying waters were measured suggesting no evidence of contamination except for DBT and MBT when using PVC bases (April 2002). The same procedure was applied to the final samples ( $T_F$ ). The comparison of the metal and organometal concentrations in the two reference overlaying water samples demonstrates the homogeneity of the water column during the incubation. We have also employed the external reference overlaying water concentrations to calculate the dilution effect of the incoming waters in the chambers when collecting large volumes of final samples ( $T_F$ ) (Eq1)

$$C_c = \frac{(C_f * (V_c - V_s) + C_{ref} * V_s)}{V_c} \quad (\text{Eq.1})$$

With  $C_c$ : the concentration corrected from the dilution effect

$C_f$ : The concentration in the sample collected

$C_{ref}$ : The external reference overlaying water concentrations collected simultaneously

$V_c$ : The volume of water in the chamber

$V_s$ : The total volume of samples collected

It is worthwhile mentioning that the total volume of samples collected never exceed more than 6% of the total volume of the chambers, leading most of the time this correction to be limited.

### 2.3- Sediment and benthic fauna sampling

At the end of the incubation, sediment cores were carefully taken by divers in order to maintain the integrity of the sediment-water interface. Cores were collected in 30 mm × 30 cm Plexiglas tubes and sealed underwater with rubber caps. Three individual cores in each chamber were collected. Other set of 5 cores were also collected by the divers in the benthic chambers for benthic fauna identification in terms of species and abundance. The whole procedure is described in detail in Thouzeau et al. (this issue).

### 2.4- Sample treatments and analysis

#### 2.4.1- Sample treatments

Samples for trace metals and organometals determination were treated according to clean procedures. All plasticware was cleaned using a 3 steps procedure. The plasticware was first decontaminated with a biocide detergent for 24h (RBS50 STC, France). After rinsing, 2 successive procedures were realized consisting in soaking the materials in a first 10% Nitric acid (Analytical grade, Carlo Erba) and a second 10% Hydrochloric acid (Analytical grade, J.T. Baker) solution for 1h in an ultrasonic bath.

All water samples were first filtered within one hour after collection under class 100 laminar flow hood (ADS Laminaire, France) using 500mL Polysulfone filtering units (Sartorius) fitted with 0.45µm acid pre-cleaned Durapore PVDF filters (47 mm d., Millipore). For trace metals, the dissolved fractions were collected in 125 ml (LDPE) Nalgene bottles and stabilized with 1% sub-boiling HNO<sub>3</sub> (J.T Baker, Ultrex). In April 2002, the analysis and processing of trace metals sample was performed in the laboratory, whereas in May 2003 it was done directly in the field. For Hg and Sn speciation, the dissolved fractions were pored in 500 ml PFA Nalgene bottles and stabilized with 1% sub-boiling HCl (J.T Baker, Ultrex) before final storage in double Ziplock plastic bags at 4°C. Blanks were regularly performed, using the same protocol for the two studied fractions as for samples, with 18.2 MΩ MQ water (Millipore).

For the sediment cores, the 0.5cm surface layer was carefully collected and sieved with 2 mm nylon mesh sieve. Then, the samples were stored at -18°C in double Ziploc plastic bags.

#### 2.4.2- Sample analysis

- Trace metals

Trace metals determination in marine waters was realized using preconcentration and matrix elimination procedure combined to Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The preconcentration/matrix

elimination procedure was realized in laboratory in April 2002 whereas it was realized directly in the field in May 2003. The complete procedure is described and detailed in Point et al. and Point, 2004.

Briefly, a first UV on-line photolysis was realized using 10ml of acidified dissolved sample to digest the dissolved complexing organic matter. The UV digestion procedure has been validated; detailed operating conditions are available in Point et al. and Point, 2004.

Second, a preconcentration/matrix elimination step was realized using Metpac CC1 column (Dionex) employed in a fully automated preconcentration platform. A five-step treatment was applied consisting in a first manual pH adjustment of the acidified and UV digested sample using a 2M Ammonium acetate buffer solution (pH 5.5). Second, 10ml of sample was loaded on the column, followed immediately by a selective elution of matrix ions ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ) using a 2M Ammonium acetate buffer solution (J.T Baker, Ultrex) (pH 5.5). Finally, the preconcentrated elements trapped in the column were rapidly eluted in a lower volume (5ml) using a 2M sub-boiling  $\text{HNO}_3$  (J.T Baker, Ultrex) solution and stored at 4°C until final analysis.

The preconcentrated and purified samples were then analysed using a Thermo Elemental X7 Inductively Coupled Plasma Mass Spectrometry equipped with an ultrasonic nebulizer (USN AT 5000+, Cetac). The method accuracy was checked with two certified reference materials obtained from NRCC (Canada): SLRS-4 (Freshwater) and CASS-4 (Seawater).

The concentrations of metals in the sediment phase were also determined by ICP-MS (X7, Thermo Elemental) with a 0,3ml.min<sup>-1</sup> nebulizer (Meinhard) and impact bead cooled spray chamber (2°C). The digestion of 100mg of sample was previously performed with a mixture of Nitric Acid (Instra Analysed, J.T. Baker) and Hydrofluoric acid (Instra Analysed, J.T. Baker) with a 5/1 ratio using PTFE flasks in a closed microwave digestion system (Multiwave 3000, Anton Paar). The method accuracy was checked for each extraction procedure with certified reference marine sediment IAEA 405 (IAEA, Monaco).

- Hg/Sn speciation

Mercury species (iHg and MMHg) and organotin compounds (MBT, DBT, TBT) were simultaneously determined by Gas Chromatography (GC)-ICP-MS. For accurate determination of mercury species and TBT, isotope dilution technique was applied to the dissolved water samples. For MBT and DBT, the determination by standard addition was employed. All the detailed aspects of the procedure and operating conditions are available elsewhere (Monperrus et al.). Briefly, a first addition of enriched species ( $^{117}\text{Sn}$ ]TBTCl,  $^{199}\text{Hg}$ ]iHg and  $^{201}\text{Hg}$ ]MMHg) in a flask containing 100 ml of sample was realized. After adjustment to pH 5 with 5ml of acetic acid/sodium acetate buffer (0.1M) and a few drops of concentrated ammonium hydroxide, the derivatization step was achieved using 1 ml of 0.5% (w/v) sodium tetraethylborate followed by the addition of 0.2 ml of isooctane. The sample flask was then immediately capped and vigorously hand shaken for 5 min. All extracts were then analysed by GC (FOCUS GC, Thermo Elemental) separation and the ICP-MS detection (X7, Thermo

Elemental). The mercury species determination in the solid phase was also carried out by GC-ICP-MS with operating conditions described in detail in Monperrus et al. (this issue).

## 2.5-Benthic flux determination

The benthic fluxes determined with the chambers were calculated as follow (Eq.2):

$$F = \frac{\Delta C}{\Delta t} \frac{V}{A} \quad (\text{Eq.2})$$

Where  $\Delta C$  is the difference of concentration obtained between the final and the initial samples for trace metals, mercury species and organotin compounds. In this equation,  $\Delta t$  corresponds to the time of incubation and  $V$  and  $A$  the respective water volume and area integrated by the benthic chamber. Using this approach, a positive flux indicates a transfer from the sediment to the water column whereas the reverse trend is obtained with a negative flux value.

Note that for  $O_2$  fluxes, the complete calculation details are presented in Thouzeau et al. (this issue).

## 3- RESULTS AND DISCUSSION

### 3.1- Trace metals in surface sediments (0-0.5cm) and bottom water

#### 3.1.1 Trace metals in surface sediments (0-0.5cm)

Sediment cores were collected in each benthic chamber allowing to address the spatial variability of the investigated sites. These cores were collected in stations C4, C5 and C7 (Table 1). The spatial variability (%) and the concentrations of trace metals and mercury species at the sediment surface are presented in Table 2.

The spatial variability is particularly high in station C5 with values ranging from 5% (Pb) up to 40% (iHg) and 51% (MeHg). For the other elements (Al, Cu, Co, Mn, Cd and U), it ranges between 13 to 26%. The spatial variability at station C4 ranges between 1 to 20% depending on the element, whereas it is less than 16% in station C7.

The comparison of trace metal levels between sites indicates similar levels between stations C5 and C4 for most of them (Al, Cu, Pb, Co, Mn), except Cd displaying lower concentration in station C5 ( $2.9 \pm 0.8 \text{ nmol.g}^{-1}$ ), compared to C4 ( $6.6 \pm 0.8 \text{ nmol.g}^{-1}$ ). For U, higher levels were also observed in C4 ( $19.2 \pm 3.8 \text{ nmol.g}^{-1}$ ), compared to C5 ( $8.6 \pm 2.1 \text{ nmol.g}^{-1}$ ). For Mercury species, the MeHg levels are similar between the two sites, whereas significant higher levels of iHg are reported in C5 ( $3.0 \pm 1.2 \text{ nmol.g}^{-1}$ ), compared to C4 ( $1.0 \pm 0.1 \text{ nmol.g}^{-1}$ ). The concentrations obtained at station C7 display slightly lower levels for Co, Mn and Cu, compared to C4 and C5.

The concentrations of mercury species obtained in this lagoon are similar to the levels observed in other coastal environments (Choe et al., 2004; Stoichev et al., 2004), except for iHg in station C5 which exceeds the reference level defined by the OSPAR convention (OSPAR, 2000). For the other trace elements, the comparison with the OSPAR values suggests that Pb and Cd levels are similar to the OSPAR levels. For Cu, levels are two times higher in Thau lagoon (for the three stations). The organotin compounds and particularly TBT have not been measured in the sediments. However, the values reported in RNO report (1999), with two stations located close to C4 and C5, were in the range 34-58 pmol.g<sup>-1</sup>. These concentrations in the sediments are similar to the contents measured in low-contaminated coastal environments (RNO, 1999).

| Station |                      | C4        |     | C5       |     | C7        |     |
|---------|----------------------|-----------|-----|----------|-----|-----------|-----|
| Al      | %                    | 5.1±0.3   | 6%  | 4.6±1.6  | 34% | 2.8±0.2   | 8%  |
| Cu      | µmol.g <sup>-1</sup> | 1.76±0.04 | 2%  | 1.4±0.2  | 13% | 1.39±0.01 | 0%  |
| Pb      | nmol.g <sup>-1</sup> | 458±14    | 3%  | 417±21   | 5%  | 246±17    | 7%  |
| Co      | nmol.g <sup>-1</sup> | 195±7     | 3%  | 156±21   | 14% | 116±5     | 4%  |
| Mn      | µmol.g <sup>-1</sup> | 3.9±0.6   | 16% | 4.8±0.6  | 13% | 2.6±0.1   | 5%  |
| Cd      | nmol.g <sup>-1</sup> | 6.6±0.8   | 12% | 2.9±0.8  | 26% | 5.0±0.8   | 16% |
| U       | nmol.g <sup>-1</sup> | 19.2±3.8  | 20% | 8.6±2.1  | 25% | 11.8±0.8  | 6%  |
| Hg(II)  | nmol.g <sup>-1</sup> | 1.0±0.1   | 10% | 3.0±1.2  | 40% | -         | -   |
| MeHg    | pmol.g <sup>-1</sup> | 9.7±2.0   | 20% | 14.3±7.4 | 51% | -         | -   |

**Table 2:** Surface (0-0.5cm) sediment concentrations in metals and within-site variability (%) obtained in April 2002. Sediment concentrations are expressed in µmol or nmol per g sediment dry weight. The variability is expressed as the standard deviation obtained from the triplicate cores at each site.

### 3.1.2- Trace metals in dissolved bottom waters

All concentrations obtained for the different sites and for the two sampling campaigns are presented in Table 3. For trace elements, the ranges of concentrations measured during the two campaigns over the four sites are 0.16 to 0.31 nM for Cd, 0.99 to 3.49 nM for Pb, 6.4 to 21.2 nM for U, around 2.3nM for Co, 7.6 to 18.5 nM for Cu and 0.09 to 0.62 µM for Mn. These concentration ranges are similar to the values usually obtained in coastal environments for Cu, Co, Mn, Cd and U (Point, 2004), whereas Pb displays significant higher levels. The differences in concentration between sites and sampling dates do not usually exceed a factor 2. It is important to note that among sites and dates no strong inter-annual differences in concentrations are reported in general for most elements. A remarkable exception is found at station C4 exhibiting much higher Mn levels (× 6) in May 2003, compared to April 2002. At this station, the highest levels of Pb are also measured. This high Pb level is correlated to the higher sediment contents reported in this station (Table 2).

Mercury species concentrations indicate that iHg ranges between 5.9 nM (C4) and 8.1-9.3 pM (C5-C7). The MMHg levels are similar in C4 and C7 (0.12-0.16pM) and higher in C5 (0.25 pM). Such concentrations are within the range of typical concentrations found for coastal seawater (Mason et al., 1999; Choe et al., 2004). It is important to mention that bottom waters of station C5 display a significant proportion of MMHg (4.2%) compared to the more classical values obtained in station C4 and C7 (1.2-1.9%). This may suggest a significant input of MMHg from the sediment to the water column at this station. Monperrus et al. (this issue) demonstrate that the methylation rate in C4/C5 is maximum at the sediment surface (0-0.5 cm) and correlated to the high sulfate reduction rate ( $31 \pm 0.5 \text{ mmol SO}_4^{2-} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$ , Table 1).

For Organotin species, the TBT dissolved concentrations are similar for the three sites (C4, C5 and C7), ranging between 1.5 and 2.5 pM. These values corresponds to moderate contamination when comparing to ones measured in the Northwestern Mediterranean sea by Michel and Averty (1999), ranging from 0.6 pM in Open Sea to 4 pM in contaminated coastal waters. For MBT and DBT, the concentrations range from 1.3 to 10.6 pM and from 1.35 to 16.09 pM, respectively. The lowest levels were obtained for station C4 located in the center of the lagoon. The median concentrations were measured in station C5 (shellfish farming zone). The highest levels were obtained in station C7 situated in shallow near shore waters (3-4 m depth) on the border of the shellfish farming zone. This concentration gradient along the lagoon follows the possible residual influence of remanent antifouling paint signature (TBT) accumulated in the sediments.

Detail attention of the levels of trace metals, mercury species and organotin compounds in bottom waters indicate that most elements display similar levels to those observed in low-contaminated coastal environments. Significant temporal changes observed for trace elements between April 2002 and May 2003 can be related to the strong hypoxic conditions occurring in the lagoon in May 2003 (Thouzeau et al., this issue).

| Station  |    | C4          | C4          | C5          | C5          | C7            | C7          | C6            |
|----------|----|-------------|-------------|-------------|-------------|---------------|-------------|---------------|
| Campaign |    | April 2002  | May 2003    | April 2002  | May 2003    | April 2002    | May 2003    | April 2002    |
| MMHg     | pM | -           | 0.16±0.03   | -           | 0.249±0.006 | -             | 0.12±0.03   | -             |
| Hg(II)   | pM | -           | 8.1±0.2     | -           | 5.9±0.2     | -             | 9.3±0.1     | -             |
| TBT      | pM | -           | 2.5±0.1     | -           | 1.7±0.1     | -             | 1.5±0.1     | -             |
| DBT      | pM | -           | 1.35±0.16   | -           | 9.43±0.05   | -             | 16.09±0.02  | -             |
| MBT      | pM | -           | 1.3±0.3     | -           | 6.1±0.1     | -             | 10.61±0.09  | -             |
| Cu       | nM | 18.56±0.13  | 7.8±0.1     | 13.3±0.1    | 11.7±0.2    | 14.6±0.2      | 7.6±0.1     | 12.3±0.2      |
| Co       | nM | 2.44±0.06   | -           | 2.31±0.09   | -           | 2.6±0.010.1   | -           | 2.10±0.06     |
| Mn       | μM | 0.098±0.002 | 0.620±0.002 | 0.134±0.001 | 0.325±0.001 | 0.1182±0.0004 | 0.245±0.002 | 0.0902±0.0008 |
| Cd       | nM | 0.212±0.008 | 0.30±0.01   | 0.16±0.01   | 0.29±0.01   | 0.187±0.006   | 0.31±0.01   | 0.132±0.004   |
| Pb       | nM | 3.491±0.006 | 2.080±0.001 | 0.991±0.001 | 2.25±0.02   | 0.3917±0.0001 | 0.903±0.002 | 1.341±0.002   |
| U        | nM | 9.1±0.3     | 21.2±0.6    | 12.1±0.2    | 18.7±0.3    | 8.1±0.010.2   | 16.1±0.7    | 6.4±0.2       |

**Table 3:** Trace metals, organotin and mercury species dissolved levels in bottom waters at stations C4, C5, C6 and C7.



### 3.2- Trace metal benthic flux at the sediment-water interface (SWI)

In the literature, only a few publications have considered mercury species and trace metal fluxes at the sediment-water interface, whereas no data are actually available for organotin compounds. In this section, the significance of the benthic fluxes is first presented (absolute values and variability, comparison to other coastal environments and relative importance compared to the deposition rates). Second, the importance of the diagenetic pathways is discussed. Third, the role of the biological activity on trace metals and organometals benthic flux is presented.

#### 3.2.1 Trace metal benthic flux significance

- Flux variability within sites (%)

The variability of the triplicate incubation fluxes (%RSD) for each station and for the two campaigns allowing to address the spatial heterogeneity of the investigated sites (Table 4). The incubation performed in station C7 in April 2002 was not included in the analysis because of suspicious very high standard deviation between the replicate chambers (Table 4). We can assume that for each station if the average RSD is <50%, it indicates no spatial heterogeneity, ranging between 50%-100%, limited to medium heterogeneity and >100%, important spatial heterogeneity.

The minimum and maximum variability of fluxes over the four stations range between 13-163% for Co, 13-63% for Mn, 23-66% for Cu, 33-72% for Cd, 10-123% for Pb, 23-73% for U, 20-31% for MMHg, 7-65% for iHg, 6-48% for TBT, 9-93% for DBT and 80-112% for MBT. This result first signifies that for MBT no precision better than 80% was obtained. The highest variability ranges were obtained for Co, Mn, Pb, iHg and DBT suggesting important spatial heterogeneity at the sub square meter scale. For these elements, the highest variability ranges were found at station C4 and the lowest at station C5. Detailed attention of the specific biogeochemical processes must be considered to explain the variability. For the other elements (TBT, MMHg, Cd, U and Cu), no significant differences within sites can be established.

| Site              | Date         | Co                                   | Mn          | Cu           | Pb            | U         | Cd                                 | TBT         | DBT        | MBT       | iHg         | MMHg                               | References                                                                     |
|-------------------|--------------|--------------------------------------|-------------|--------------|---------------|-----------|------------------------------------|-------------|------------|-----------|-------------|------------------------------------|--------------------------------------------------------------------------------|
|                   |              | $\mu\text{M.m}^{-2}.\text{day}^{-1}$ |             |              |               |           | $\text{nM.m}^{-2}.\text{day}^{-1}$ |             |            |           |             | $\text{pM.m}^{-2}.\text{day}^{-1}$ |                                                                                |
| C4-1              | April 2002   | -0.35±0.31                           | -11±1       | -11.4±3.8    | 1.5±0.5       | 1.7±1.0   | 151±59                             | -           | -          | -         | -           | -                                  |                                                                                |
| C4-2              | April 2002   | -1.1±2.6                             | -11±17      | -4.6±2       | 0.5±1.5       | 1.8±0.9   | 69±11                              | -           | -          | -         | -           | -                                  |                                                                                |
| C4                | May 2003     | -                                    | 63±12       | 7.3±3.2      | 3.0±0.2       | -4.8±3.2  | -60±26                             | -0.5±0.1    | 25.4±23.7  | 4.3±4.8   | -3.2±2.1    | 300±59                             |                                                                                |
|                   | Mean RSD (%) | 163%                                 | 63%         | 41%          | 123%          | 57%       | 33%                                | 26%         | 93%        | 112%      | 65%         | 20%                                |                                                                                |
| C5                | April 2002   | 3.44±0.44                            | 115±5       | 11.1±1.4     | 3.8±0.4       | -2.7±0.5  | 110±61                             | -           | -          | -         | -           | -                                  |                                                                                |
| C5                | May 2003     | -                                    | 160±35      | 7.7±2.6      | 4.2±0.4       | -7.5±2.1  | -130±98                            | 2.1±1.0     | 37.0±4.3   | 3.7±3.1   | 2.6±1.0     | 94±23                              |                                                                                |
|                   | Mean RSD (%) | 13%                                  | 13%         | 23%          | 10%           | 23        | 66%                                | 48%         | 12%        | 84%       | 38%         | 25%                                |                                                                                |
| C6-1              | April 2002   | 1.7±1.2                              | 125±49      | 10.4±4.7     | 3.2±1.3       | 9.8±6.2   | 286±103                            | -           | -          | -         | -           | -                                  |                                                                                |
| C6-2              | April 2002   | 4.76±3.53                            | 246±165     | 30.8±26.9    | 3.0±1.4       | 20.4±14.7 | 264±225                            | -           | -          | -         | -           | -                                  |                                                                                |
|                   | Mean RSD (%) | 72%                                  | 53%         | 66%          | 44%           | 68%       | 61%                                |             |            |           |             |                                    |                                                                                |
| C7                | April 2002   | 0.49±4.51                            | -3±204      | 2.1±21.2     | 0.9±1.2       | 1.2±13.6  | 64±299                             | -           | -          | -         | -           | -                                  |                                                                                |
| C7                | May 2003     | -                                    | -97±42      | -5.5±1.9     | -0.5±0.2      | 4.3±2.6   | 109±114                            | 11.6±1.7    | 111.8±30.6 | 27.3±24.1 | -6.8±2.8    | -147±74                            |                                                                                |
| C7                | May 2003     | -                                    | 47±33       | -1.9±0.6     | 0.9±0.9       | -2.4±2.0  | -71±29                             | 2.1±0.5     | 51.6±21    | 17.4±15.4 | -4.2±2.2    | 334±37                             |                                                                                |
|                   | Mean RSD (%) |                                      | 57%*        | 34%*         | 67%*          | 73%*      | 72%*                               | 106%        | 34%        | 89%       | 47%         | 31%                                |                                                                                |
| Bay of Brest      | Oct 2002     | -1.7 to 1.2                          | -49 to 111  | -2.6 to 3.2  | -1.5 to 2.6   | -         | -256 to 226                        | -8.5 to 4.5 | -          | -         | -12 to 14   | -304 to 315                        | Amouroux et al., unpublished results using same benthic chambers and protocols |
| Gulf of Trieste   |              | -                                    | -           | -            | -             | -         | -                                  | -           | -          | -         | 0.13 to 32  | 523 to 11800                       | Covelli et al. 1999                                                            |
| Lavaca Bay        |              | -                                    | -           | -            | -             | -         | -                                  | -           | -          | -         | -           | 1.6 to 4.3                         | Gill et al. 1999                                                               |
| San Francisco Bay |              | -                                    | -           | -            | -             | -         | -                                  | -           | -          | -         | -1.2 to 2.6 | -92 to 850                         | Choe et al. 2004                                                               |
| River Po          |              | -                                    | -           | -3.5 to -0.6 | -             | -         | -225 to 163                        | -           | -          | -         | -           | -                                  | Zago et al. 2000                                                               |
| Gullmarsfjorden   |              | -                                    | -           | 0.03 to 0.12 | -             | -         | 11 to 13                           | -           | -          | -         | -           | -                                  | Westerlund et al. 1986                                                         |
| Galveston Bay     |              | -                                    | 100 to 2300 | -0.7 to 3.6  | 0.006 to 0.05 | -         | -14 to 65                          | -           | -          | -         | -           | -                                  | Wamken et al. 2003                                                             |
| California        |              | -                                    | -           | -            | -             | -         | -212 to 118                        | -           | -          | -         | -           | -                                  | Colbert et al. 2001                                                            |
| Ansedonia Bay     |              | 0.10 to 0.34                         | 240 to 322  | -2.4 to 9.1  | -0.9 to -1.9  | -         | 8 to 194                           | -           | -          | -         | -           | -                                  | Ciceri et al. 1992                                                             |

\*April 2002 Not included, because of anormal high standard deviation observed during the incubation procedure

**Table 4 :** Benthic fluxes in the Thau Lagoon: Comparison with other documented ecosystems. The flux values correspond to the average obtained for the three benthic chambers deployed at each station. The error corresponds to the “sd” determined from the three replicates.

- Benthic fluxes in the Thau lagoon: comparison with reference values

The fluxes obtained in April 2002 and May 2003 at the four stations are presented and compared to literature reference values in Table 4. Data obtained in the Bay of Brest (Atlantic Ocean, France) in October 2002 with the same benthic chambers and sampling procedure are also included.

For trace metals, the flux ranges are  $-130$  to  $286 \text{ nmol.m}^{-2}.\text{day}^{-1}$  for Cd,  $-0.5$  to  $4.2 \text{ } \mu\text{mol.m}^{-2}.\text{day}^{-1}$  for Pb,  $-0.35$  to  $4.76 \text{ } \mu\text{mol.m}^{-2}.\text{day}^{-1}$  for Co,  $-7.5$  to  $20.4 \text{ } \mu\text{mol.m}^{-2}.\text{day}^{-1}$  for U,  $-11.4$  to  $30.8 \text{ } \mu\text{mol.m}^{-2}.\text{day}^{-1}$  for Cu and  $-11$  to  $246 \text{ } \mu\text{mol.m}^{-2}.\text{day}^{-1}$  for Mn. The comparison with the Bay of Brest gives similar fluxes ranges for all elements except Cu, for which higher flux are obtained in the Thau lagoon on average. When comparing to other documented coastal environments such as the Po River (Italy), California continental margin (USA), the Ansedonia Bay (Italy), the Gullmarsfjorden (Sweden) and Galveston Bay (USA), the fluxes determined in this study are similar for most elements except for Cu presenting the highest fluxes published (Table 4). In the case of Mn benthic flux, any previous study has reported influx to the sediment such as in the Thau Lagoon.

For mercury species (iHg, MMHg), the fluxes obtained in May 2003 are in the ranges  $-6.8$  to  $2.6 \text{ nmol.m}^{-2}.\text{day}^{-1}$  for iHg and  $-147$  to  $334 \text{ pmol.m}^{-2}.\text{day}^{-1}$  for MMHg. These values are consistent with values reported in San Francisco Bay by Choe et al. (2004), but significantly lower compared to the Gulf of Trieste (Covelli et al., 1999). The comparison with the Bay of Brest indicates similar flux span for the two species.

For organotin species, the fluxes obtained in May 2003 for TBT, DBT and MBT are in the ranges  $-0.5$  to  $11.6 \text{ nmol.m}^{-2}.\text{day}^{-1}$ ,  $25.4$  to  $111.8 \text{ nmol.m}^{-2}.\text{day}^{-1}$ , and  $3.7$  to  $27.3 \text{ nmol.m}^{-2}.\text{day}^{-1}$ , respectively. The study realized in the Bay of Brest in October 2002 displayed similar range for TBT.

- Comparison of benthic fluxes to apparent sedimentation

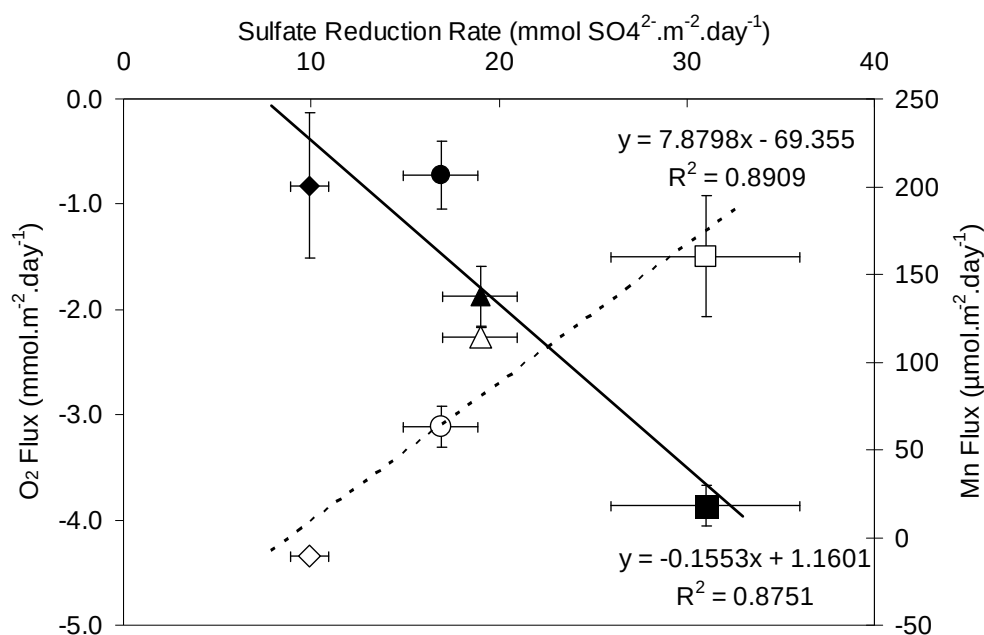
Trace metals deposition rate in the surface sediment layer can be estimated from the sediment accumulation rate of  $0.25 \text{ cm.yr}^{-1}$  in station C4 and  $0.15 \text{ cm.yr}^{-1}$  in station C5 based on  $^{210}\text{Pb}$  activity (Schmidt et al., this issue), the respective surface sediment dry concentrations (Table 2) and the dry density of surface sediments estimated to  $500 \text{ Kg.m}^{-3}$ . On average and for the two stations, the deposition rate was estimated to be  $2 \text{ pmol.m}^{-2}.\text{day}^{-1}$  for MMHg,  $0.2 \text{ nmol.m}^{-2}.\text{day}^{-1}$  for iHg,  $1.3 \text{ nmol.m}^{-2}.\text{day}^{-1}$  for Cd,  $4 \text{ nmol.m}^{-2}.\text{day}^{-1}$  for U,  $40 \text{ } \mu\text{mol.m}^{-2}.\text{day}^{-1}$  for Co,  $94 \text{ } \mu\text{mol.m}^{-2}.\text{day}^{-1}$  for Pb,  $0.4 \text{ } \mu\text{mol.m}^{-2}.\text{day}^{-1}$  for Cu and  $0.8 \text{ } \mu\text{mol.m}^{-2}.\text{day}^{-1}$  for Mn. For TBT, the deposition rate was evaluated to be  $10 \text{ pmol.m}^{-2}.\text{day}^{-1}$ .

The comparison of the deposition rates with the mean benthic flux values obtained in this study indicates that the sedimentation rate is limited for all elements ranging between 0.04% to 7% of the benthic flux for U and iHg respectively. For the other elements (Cu, Cd, Pb, Mn, Cd, MMHg and TBT), the sedimentation represent 0.2-3% of benthic flux values. These results suggests that for all elements and organospecies the process controlling the exchanges at the sediment water interface is solution driven (not particles).

### 3.2.2 Influence of diagenetic processes on trace metal and organometal fluxes

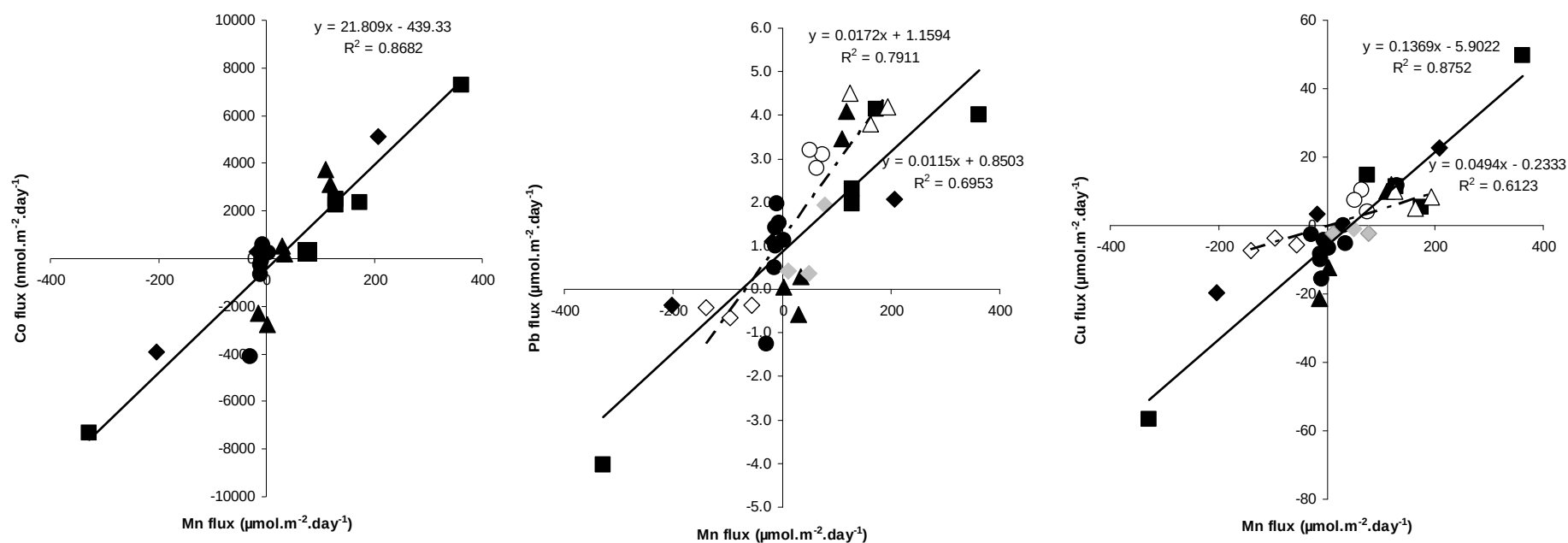
The fluxes database (Table 4) demonstrates with evidence an important variability for the different fluxes measured with the benthic chambers. This variability is expressed on both temporal scale (differences between the sampling campaigns) and spatial scale (differences between stations). This variability, existing at all sites, could be also related to different redox conditions at the sediment surface (Dedieu et al., this issue). Special attention to the redox tracers (Fe/Mn) must be considered (Ciceri et al., 1992; Tseng et al., 2001; Chaillou et al., 2002). In this study, Mn was employed as a tracer of the redox conditions. Mn is remobilized under suboxic conditions and the slow dissociation kinetics of Mn makes the reduction of the Mn oxides to be detected up to 20 cm over the sediment surface (height of SWI integration in the benthic chamber). No Fe determination was performed, and reduced Fe has a much shorter lifetime in the water column and can be rapidly reoxidized (Yao and Millero, 1995).

Figure 4 displays the distribution of Mn and O<sub>2</sub> fluxes at the sediment-water interface for stations C4 and C5 as a function of the sulfate reduction rate at the sediment surface (0-0.5cm). The opposite trend shown by O<sub>2</sub> and Mn indicates that Mn oxides dissolution rate increases as a function of the decrease of O<sub>2</sub> flux in the chambers. It also suggests that the strong modification of the O<sub>2</sub> balance (i.e penetration) (Dedieu et al., this issue) at the sediment surface is directly reflected in the benthic Mn flux. The reverse linear correlation between the O<sub>2</sub> flux and the sulfate reduction rate is particularly marked in May 2003 with warmer conditions (Table 1). The redox gradient is driven by the degradation of organic matter. However, the concomitant macrobenthic community respiration rate has also to be considered in the O<sub>2</sub> budget, as observed for stations C4 and C5 (Thouzeau et al., this issue). For the two stations, the contribution of macrobenthic communities respiration to the O<sub>2</sub> budget was assumed to be lower in May 2003 compared to April 2002. Duport et al (this issue) and Thouzeau et al. (this issue) indeed found an important decrease of biomass in stations C4 and C5 (for small size organisms <1mm). These results lead us to conclude that the higher O<sub>2</sub> uptake flux observed in May 2003 compared to April 2002 in the two stations (Figure 4) is essentially attributable to the increased microbial heterotrophic activity (the latter controlling organic matter remineralization rate).



**Figure 4:** Influence of sulfate reduction activity on Oxygen (black) and Mn fluxes (white) at the sediment-water interface in stations C4 and C5. (circle: C4 May 2003, diamond-shaped: C4 April 2002, square : C5 May 2003, triangle: C5 April 2002)

Fluxes of most metals and organometals were plotted versus the Mn flux for all incubation series (Figure 5). A significant full-range correlation between Mn and Co, iHg, Pb, Cd, U, Cu and MMHg fluxes is observed ( $R^2 > 0.68$ ), suggesting a strong control of Mn oxides in trace elements mobility. By contrast, no links with organotin compounds (TBT, MBT and DBT) were observed. This low affinity of Mn oxides for organotin compounds illustrates other mobilization processes for these organic species, such as hydrophobic absorption/desorption interactions (Amouroux et al., 2000). The specific association of Mn and Co observed in this study ( $R^2 = 0.86$ ) has been suggested to be a tracer of porewater diffusion within the water column (Chiffoleau et al., 1994; Zwolsman and Van Eck, 1997). For Pb and iHg, the significant correlation with Mn reflects their strong affinity for oxides (Benoit et al. 1994; Tseng et al. 2001). For U, the connection to Mn oxides dissolution at the hypoxic/oxic interface has been already proposed (Swarzenski et al., 1992; Swarzenski et al., 1999). For Cu and Cd, the influence of Mn oxides is also suggested in April 2002.



**Figure 5:** Influence of Mn flux on trace metals and mercury fluxes at all stations. In black :April 2002 flux; in white:May 2003 flux. (circle: C4, triangle: C5, square: C6, Diamond-shaped: C7). Note that the dark incubation at station C7 in May 2003 is in Grey.

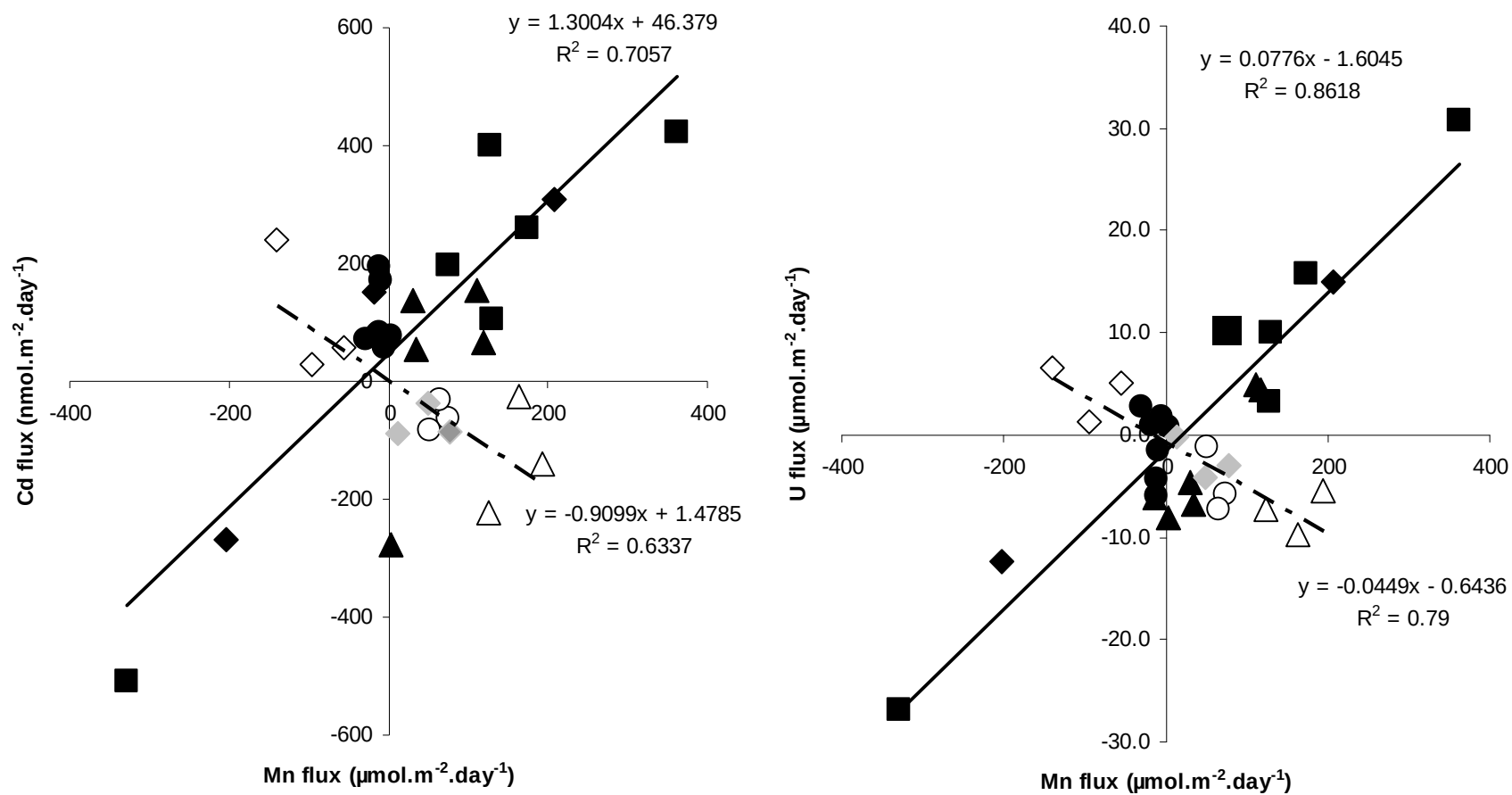


Figure 5: Continued

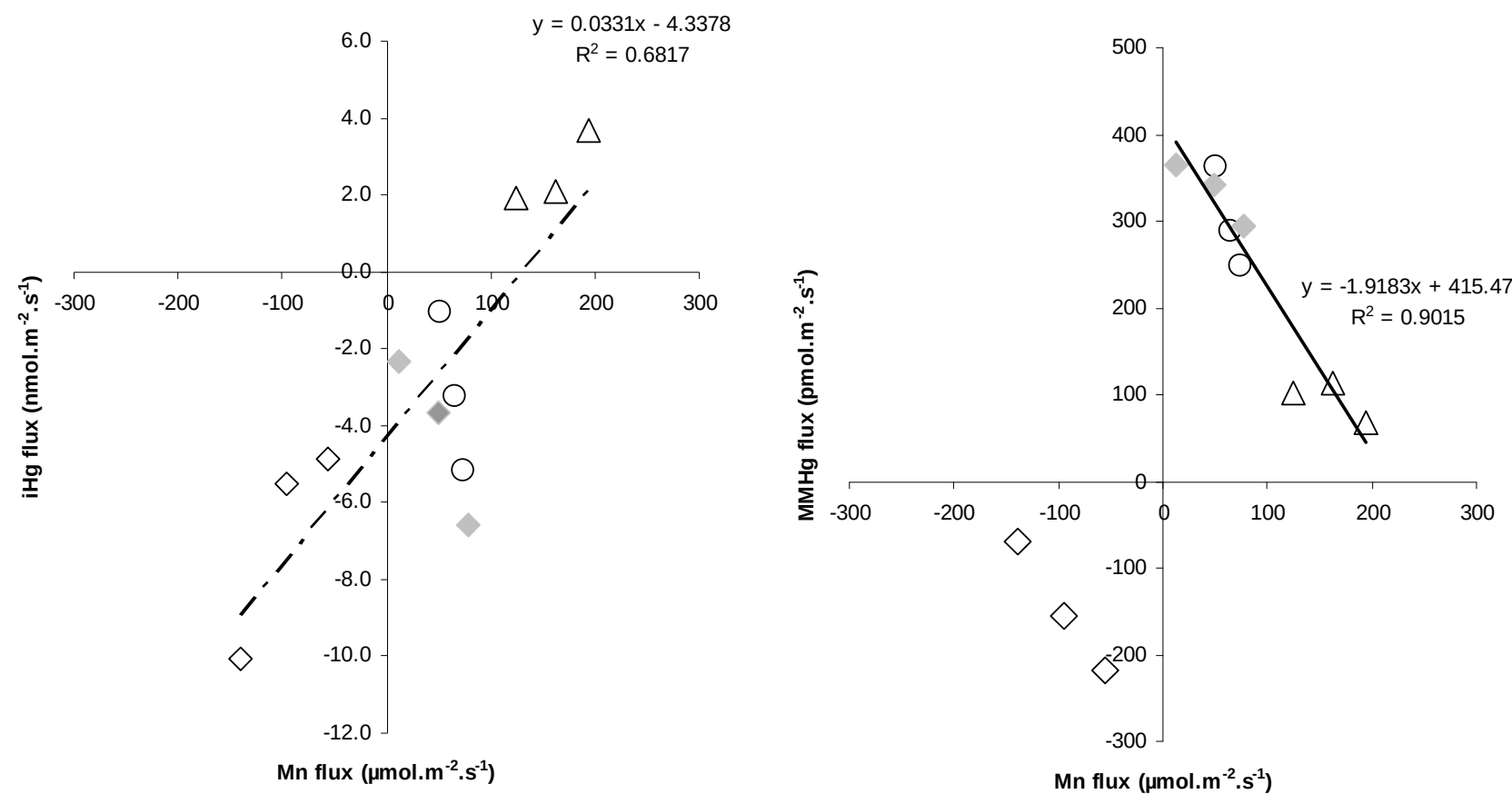
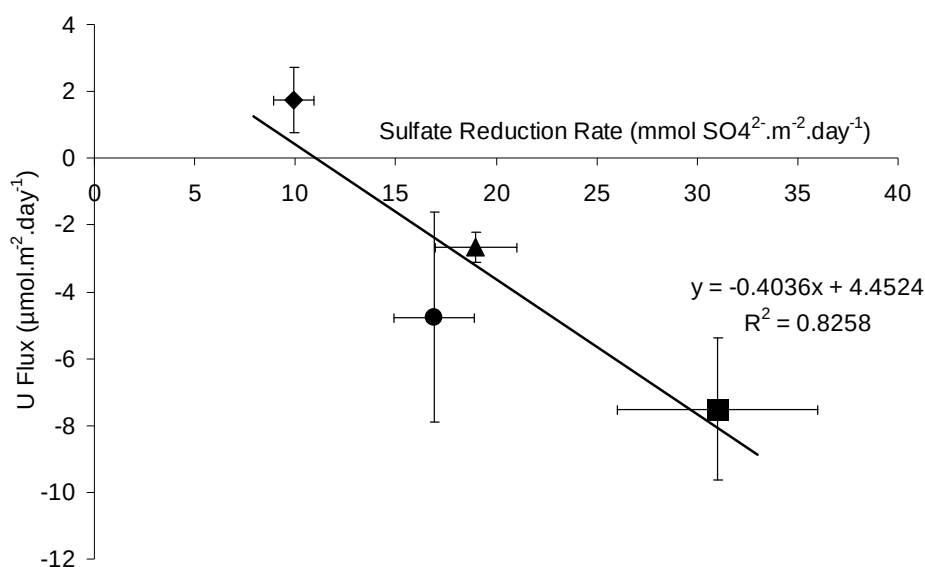


Figure 5: Continued



In May 2003, a shift of hypoxic/oxic conditions near the sediment surface has been observed (Dedieu et al., this issue) in C4 and C5. These authors have reported a higher  $O_2$  penetration depth of 3.9 mm in C4 and 1.4 mm in C5 in April 2002 compared to 0.9 mm and 0.8 mm in May 2003, respectively. This shift up to the surface does not affect Pb and Cu that display similar behaviour with Mn in May 2003. For Cd and U, an inverse relationship with Mn is observed, suggesting a dynamic control of the hypoxic/oxic interface with the highest sulfate reduction rate measured at the sediment surface (Momperrus et al., this issue). The shift of this interface to the sediment surface might enhance the role of dissolved sulfides in Cd removal from bottom water (Gerringa et al., 2001; Chaillou et al., 2002). This hypothesis is consistent with the negative Cd flux reported in stations C4 and C5. For U, the same pathway can be considered as documented in other coastal environments (Church et al., 1996; Chaillou et al., 2002). However, mobility of U at the sediment surface has been for long considered as purely chemical (reduction and precipitation, Bonati et al., 1971; Yamada and Tsunogai, 1984), whereas a more biologically driven pathway is observed here. Recent studies have suggested the possible role of anoxic bacteria (Barnes and Cochran, 1993) and particularly sulfate reducing bacteria (Lovely et al., 1991; Francis et al., 1994). Indeed, sulfate reduction rate correlates with U influx to the sediment (Figure 6). The maximum activity of this group of bacteria is correlated to the strongest removal of U in bottom waters, suggesting that the flux of U removal (precipitation) is biologically driven.



**Figure 6:** Influence of sulfate-reducing bacteria activity on U flux in stations C4 and C5.  
(circle: C4 May 2003, diamond-shaped: C4 April 2002, square: C5 May 2003, triangle: C5 April 2002)

The global decreasing trend of MMHg with Mn flux observed in May 2003 emphasizes a complete opposite behaviour compared to iHg, without considering the C7 data (sunlight incubation of the macroalgae cover). The dark control at this station correlates with the general trend observed for MMHg and Mn. The opposite behaviour of iHg and MMHg suggests that the increasing iHg flux with Mn oxides dissolution corresponds to a simultaneous decrease of MMHg flux. Monperrus et al. (this issue) have demonstrated that the methylation rate was maximum at the sediment surface, while the flux of MMHg to the water column was relatively limited and corresponds to the potential formation rate. The possible significant demethylation rate located just above the sediment surface is suggested in this case (Oremland et al., 1991).

This section demonstrates the importance of Mn oxide dissolution as a control of trace metal mobility at the sediment-water interface. In the Thau lagoon, this relationship might explain the variability of trace elements and mercury species fluxes on both temporal (April 2002 and May 2003) and spatial scales.

### 3.2.3 Influence of biological activity on metal and organometal fluxes

- Importance of biorrigation/bioturbation processes on metal and mercury species fluxes

- o Comparison of benthic chamber fluxes and estimated diffusive fluxes for, Cd, Mn, iHg and MMHg

The differences between benthic fluxes and diffusive fluxes allow to determine the magnitude of the advective fluxes, directly connected to biorrigation and bioturbation (Warnken et al., 2001; Berelson et al., 2003; Choe et al., 2004). This phenomenon has been clearly established for U (Zheng et al., 2002). We compare here the measured benthic flux to the calculated diffusive flux evaluated by Metzger et al. (this issue) at the same station during the same sampling period. As it is difficult to compare the fluxes direction because of different spatial and temporal integration scale, the comparison of fluxes intensity can be discussed.

The Cd diffusive flux in C4 was  $-0.98 \text{ nmol.m}^{-2}.\text{day}^{-1}$  in May 2003, whereas in station C5 it was established to 0.11 and  $0.17 \text{ nmol.m}^{-2}.\text{day}^{-1}$  in Avril 2002 and May 2003, respectively (Metzger et al., this issue). The comparison with the benthic fluxes measured in this study indicates that the diffusive component represents less than 1%. For Mn, the diffusive flux in station C4 was estimated to  $10.4 \text{ nmol.m}^{-2}.\text{day}^{-1}$  in May 2003. At Station C5, the value reported in April 2002 was  $33 \text{ nmol.m}^{-2}.\text{day}^{-1}$  and  $0.05 \text{ nmol.m}^{-2}.\text{day}^{-1}$  in May 2003 (Metzger et al., this issue). For Mn, the diffusive component represents also less than 1%. Finally, for mercury species, the diffusive fluxes were estimated to  $0.039 \text{ nmol.m}^{-2}.\text{day}^{-1}$  for total dissolved Hg and  $4 \text{ pmol.m}^{-2}.\text{day}^{-1}$  for MMHg (Muresan et al., this issue), representing less than 1.5% of iHg and 5% of MMHg benthic fluxes respectively.

For Cd, Mn and mercury species, the relative low contribution of the diffusive flux compared with the benthic flux is similar to the contributions documented in other coastal environments (Ciceri et al., 1992; Gill et al., 1999; Warnken et al., 2001; Berelson et al., 2003; Choe et al., 2004). These large differences between fluxes

emphasize the fact that bioturbation and biorrigation are strong active pathways that facilitate the exchanges of metals between the sediment and overlaying waters.

o Biologically-transported metal flux at the sediment-water interface

The macrofaunal abundance and biomass measured in the benthic chambers in station C4 are presented in Thouzeau et al. (this issue). For station C5, no individual > 1 mm was collected in the benthic chambers during the two sampling periods (April 2002 and May 2003). These observations are confirmed by Duport et al. (this issue) indicating the presence of small-sized species at this station. At station C4, the abundance and characteristics of the macrofaunal population indicate that in April 2002 and May 2003, the deposit feeders and the suspension feeders were representing the majority of the individuals found in this station (Thouzeau et al., this issue). In this section, we propose to evaluate the relative proportion of the metallic and organometallic fluxes mobilized/transported by the deposit feeders and suspension feeders, compared to the benthic fluxes. The details of the calculation of the biological fluxes with respect to the two trophic groups are presented in Table 5.

First, the volume of water filtered by the suspension filters was estimated to  $160 \text{ l.m}^{-2}.\text{day}^{-1}$  in station C4 in April 2002, considering the species (Thouzeau et al., this issue) and bottom-water temperature (Schwinghamer et al., 1986). The biologically-mediated flux (Table 5) was estimated for trace metals to range between  $34 \text{ nmol.m}^{-2}.\text{day}^{-1}$  for Cd and  $16 \text{ } \mu\text{mol.m}^{-2}.\text{day}^{-1}$  for Mn. For mercury species, the flux was  $26 \text{ nmol.m}^{-2}.\text{day}^{-1}$  for MMHg and  $1296 \text{ nmol.m}^{-2}.\text{day}^{-1}$  for iHg. Finally, for the organotin compounds, the fluxes ranged between 208 and  $400 \text{ nmol.m}^{-2}.\text{day}^{-1}$ . The comparison of the metal flux mediated by the suspension feeders with the benthic flux indicates first that the biological flux is negligible for DBT and MBT (1% and 5%) and low for MMHg (9%). Second, for the other elements, the flux mobilized by this trophic group is estimated to range between 35% (Pb) and 80% (TBT) of the benthic flux. The similar orders of magnitude of the fluxes for Cu, Co, Cd, Pb, U, Mn, TBT and iHg indicate that a large proportion of the benthic flux measured with the benthic chambers would be due to the biorrigation processes. This result confirms the predominance of this advective component for metals exchanges at the sediment-water interface, compared to diffusive flux.

Second, the deposit feeder ingestion activity was estimated to be  $180 \text{ g.m}^{-2}.\text{day}^{-1}$  of surface sediments in April 2002. This estimation was obtained using the species abundance presented in Thouzeau et al. (this issue) and specific species/ingestion coefficients published by Fauchald and Jumars (1979) and Jorgensen et al. (1991). For trace elements, the biological ingestion flux (Table 5) was evaluated to range between  $1188 \text{ pmol.m}^{-2}.\text{day}^{-1}$  (Cd) and  $702 \text{ } \mu\text{mol.m}^{-2}.\text{day}^{-1}$  (Mn). For mercury species, it was estimated to  $1746 \text{ pmol.m}^{-2}.\text{day}^{-1}$  of MMHg and  $180 \text{ nmol.m}^{-2}.\text{day}^{-1}$  of iHg. Finally for TBT, the flux determined using the sediment concentration reported in RNO report (1999) was  $8280 \text{ pmol.m}^{-2}.\text{day}^{-1}$ . The comparison of the order of magnitude of the biological flux associated to deposit feeders with the benthic flux indicates strong differences, except for U. For

MMHg, Cd, TBT and Mn, the ingested metal flux is respectively 6, 13, 17, and 25-fold higher. For Cu, Pb, Co and iHg, the ingested flux is on average 50 times higher than respect to the benthic fluxes. These estimations indicate that the biological metal fluxes ingested directly from the sediment by the deposit feeders are significantly more important than the benthic fluxes, except for U. However, deposit feeders ingestion will mostly affect trace metal mobilization within the sediment (reworking), most of these metals being not uptaken into these organisms.

| Element | Flux                                    | C4 Average<br>Benthic flux<br>April 2002 | Suspension<br>Feeder<br>Filtered metal Flux | Filtered metal Flux /<br>Benthic flux | Deposit Feeder<br>sediment Ingested<br>metal Flux | Ingested metal Flux /<br>Benthic flux |
|---------|-----------------------------------------|------------------------------------------|---------------------------------------------|---------------------------------------|---------------------------------------------------|---------------------------------------|
| MMHg    | pmol.m <sup>-2</sup> .day <sup>-1</sup> | 300                                      | 26 <sup>a</sup>                             | 9%                                    | 1746                                              | 582%                                  |
| Hg(II)  | pmol.m <sup>-2</sup> .day <sup>-1</sup> | 3000                                     | 1296 <sup>a</sup>                           | 43%                                   | 180000                                            | 6000%                                 |
| TBT     | pmol.m <sup>-2</sup> .day <sup>-1</sup> | 500                                      | 400 <sup>a</sup>                            | 80%                                   | 8280                                              | 1656%                                 |
| DBT     | pmol.m <sup>-2</sup> .day <sup>-1</sup> | 25400                                    | 216 <sup>a</sup>                            | 1%                                    | -                                                 |                                       |
| MBT     | pmol.m <sup>-2</sup> .day <sup>-1</sup> | 4300                                     | 208 <sup>a</sup>                            | 5%                                    | -                                                 |                                       |
| Cu      | nmol.m <sup>-2</sup> .day <sup>-1</sup> | 8000                                     | 2970                                        | 37%                                   | 316000                                            | 3950%                                 |
| Co      | nmol.m <sup>-2</sup> .day <sup>-1</sup> | 724                                      | 390                                         | 54%                                   | 35100                                             | 4849%                                 |
| Cd      | nmol.m <sup>-2</sup> .day <sup>-1</sup> | 93                                       | 34                                          | 36%                                   | 1188                                              | 1275%                                 |
| Pb      | nmol.m <sup>-2</sup> .day <sup>-1</sup> | 1600                                     | 559                                         | 35%                                   | 82440                                             | 5153%                                 |
| U       | nmol.m <sup>-2</sup> .day <sup>-1</sup> | 2800                                     | 1456                                        | 52%                                   | 3456                                              | 123%                                  |
| Mn      | μmol.m <sup>-2</sup> .day <sup>-1</sup> | 28                                       | 16                                          | 56%                                   | 702                                               | 2492%                                 |

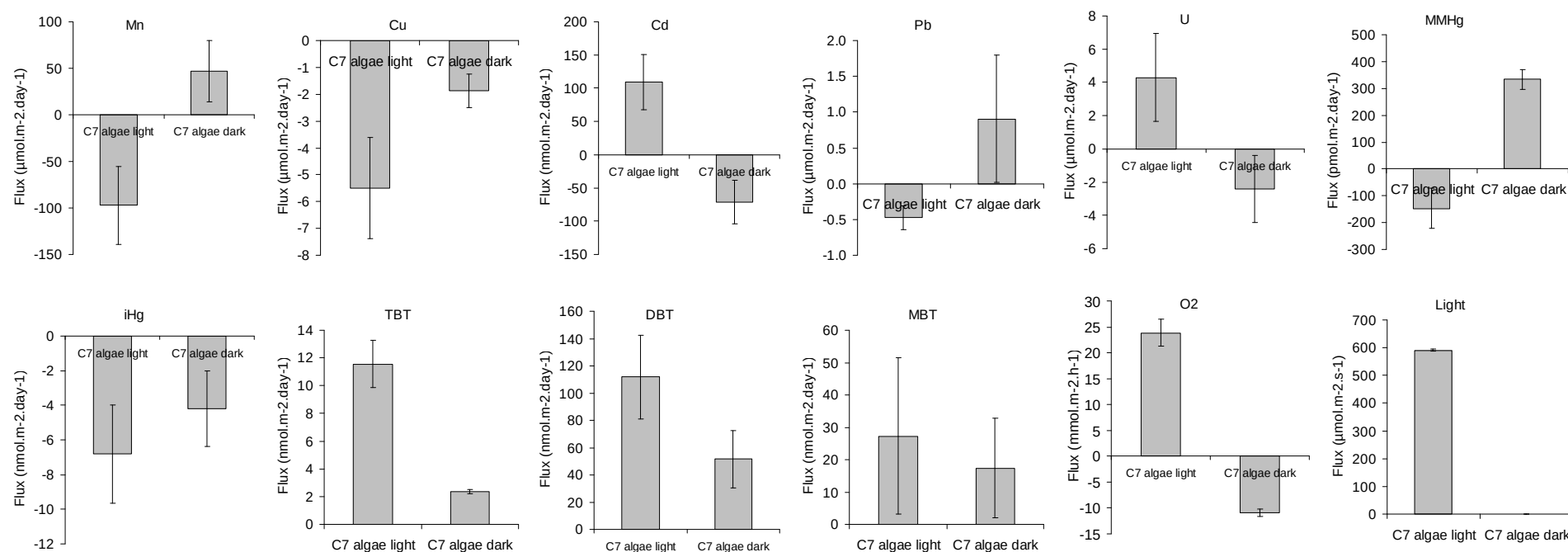
**Table 5:** Estimation of the biologically-mediated flux in Station C4: influence of suspension and deposit feeders. The estimation of the suspension feeder-related flux was determined using the dissolved concentration in bottom waters in April 2002 for trace metals and in May 2003 for Organotin and mercury species<sup>a</sup>. The volume of filtered seawater was calculated considering the species (Thouzeau et al, this issue) and bottom-water temperature (Schwinghamer et al., 1986). The estimation of the digested flux of metal by deposit feeders was determined considering the different macrofaunal species (Thouzeau et al, this issue) and the digestion coefficients proposed by Jorgensen et al. (1991) and Fauchald and Jumars (1979).

These two quantitative aspects, illustrated for station C4, indicate that the sediment-water interface must be considered as a dynamic biological layer transporting continuously an important amount of sedimented particles and facilitating the water flow to the sediment. The biological activity mediated by the different trophic groups located at the sediment surface appears to be the dominant pathways of trace metal cycling in the Thau lagoon SWI.

- Influence of the macroalgal cover on benthic metal fluxes

At station C7, the influence of a dense macroalgal cover at the sediment surface on trace metal benthic fluxes was studied in May 2003. This algae cover was mostly composed of the nitrophilous opportunistic seaweeds *Ulva spp.* and *Gracilaria spp.*, characteristic of eutrophicated conditions. The total macroalgal cover reached  $463.55 \pm 225 \text{ g AFDW.m}^{-2}$  (Ash Free Dry Weight) in the benthic chambers (Thouzeau et al., this issue). A series of triplicate incubations was performed successively in both sunlight and dark conditions to focus on the importance of the photosynthetic activity on metal and organometal exchanges. In sunlight conditions ( $590 \pm 1 \mu\text{M quanta.m}^{-2}.\text{h}^{-1}$ ), the photosynthetic biological flux of  $\text{O}_2$  is clearly enhanced ( $23.9 \pm 2.6 \text{ mM.m}^{-2}.\text{h}^{-1}$ ) compared to dark conditions for which benthic respiration occurred ( $-11.0 \pm 0.7 \text{ mM.m}^{-2}.\text{h}^{-1}$ ). The influence of the photosynthetic activity on the benthic fluxes of elements and metal species is presented in Figure 7.

First, for most trace elements (Mn, Pb, U, iHg and Cu), the influence of photosynthesis is obvious. The comparison of the sunlight incubation with the dark control indicates drastic changes in flux direction for Mn, Cd, Pb and U. The sunlight incubation changes the flux direction to the water column for Mn and Pb, whereas opposite trend is observed for U and Cd. For Cu and iHg, no change in flux direction was observed during the sunlight incubation. However, the uptake rates were enhanced. For all these elements, the flux values in both sunlight and dark conditions fit properly with the correlations obtained with Mn for the other stations (Figure 5). This result suggests that the same redox process controlling trace metal exchanges at the sediment-water interface is involved. At station C7, we assume that the redox gradient occurring at the sediment surface is mainly driven by the biological production/consumption of  $\text{O}_2$ .



**Figure 7:** Influence of macroalgae photosynthetic activity (C7) on benthic trace metals, mercury species and organotin compounds fluxes. Comparison of light and dark incubations.

Second, a strong influence of the macroalgal cover on MMHg mobility is observed. Under sunlight conditions, the MMHg flux is  $-334 \pm 37$  pmol.m<sup>-2</sup>.day<sup>-1</sup> whereas the dark control indicates a flux of  $+147 \pm 74$  pmol.m<sup>-2</sup>.day<sup>-1</sup>. This significant decrease in MMHg can be linked first to a direct uptake of MMHg, already observed for green algae (Miles et al., 2001; Moye et al., 2001). At this station, the redox gradient at the sediment-water interface would not affect the MMHg flux because the values of the sunlight incubation do not follow the relationship with Mn described previously (Figure 5). Second, the decrease of MMHg levels in the overlaying water during the sunlight incubations could be also related to abiotic demethylation as proposed by Monperrus et al. (this issue).

Third, for organotin compounds, we observe that both DBT and TBT fluxes towards the water column are enhanced during sunlight incubations with a 2-fold and 5-fold increase, respectively. No specific process can be actually considered to explain this mechanism.

These incubation experiments demonstrate with evidence the active role of the macroalgal cover on the mobility of trace elements and organometals at the sediment-water interface. The respective differences in flux direction and magnitude between the sunlight and dark incubations suggest a diurnal variability scale for the fluxes that can be also extended to a seasonal scale with maximum macroalgal proliferation when eutrophicated conditions occur.

The comparison of the flux orders of magnitude emphasizes that most of the total fluxes are directly connected to the biological activity of the different macrofaunal trophic groups and to the photosynthetic activity of the macroalgal cover. The comparison of the total flux budgets makes consider the diffusive component to be negligible. This scenario highlights the influence of spatial heterogeneity and variability occurring at the sediment surface on metal and organometal fluxes. Taking spatial heterogeneity into account requires to consider the specific distribution and abundance of benthic species in each site. The temporal scale must also be considered with both seasonal and diurnal cycles.

#### 4.- SUMMARY AND CONCLUSIONS

In situ benthic flux studies were conducted at four stations in the Thau lagoon. The development of a robust integrating incubation procedure combined to sensitive analytical techniques allowed for the first time the simultaneous determination of trace metals, mercury species (iHg, MMHg) and organotin compounds (MBT, DBT, TBT) fluxes. This study documents also for the first time the fate of organotin compounds at the sediment-water interface. Using this technique, significant, accurate and reproducible fluxes were obtained. This study demonstrates that the sediment-water exchange processes in the Thau Lagoon are highly dynamic and variable



in terms of intensity and direction for most elements or organometallic species. The flux variability depends mainly on the combined influence of diagenetic processes suggested by the role of Mn oxides in trace metal mobility, and biological activity (microbial activity and bioturbation processes). Mn has been demonstrated to be an important element to identify microscale redox gradient changes. The microbial activity does control the O<sub>2</sub> balance at the sediment-water interface. The macrobenthos activity stimulates the exchanges between porewater and bottom water through bioturbation and bioturbation processes. These combined processes induce variability on both spatial and temporal scales. A seasonal cycling of fluxes at the sediment-water interface must also be considered with first the influence of macroalgae (*Ulva spp.* and *Gracilaria spp.*) present at the sediment surface when eutrophicated conditions occur in the lagoon. Second, the role of sulfate-reducing bacteria, particularly important during warm conditions, is demonstrated through changes in the redox conditions at the sediment-water interface. The latter modify metal and organometal fluxes. A diurnal periodicity of the fluxes was also demonstrated, resulting from the macroalgae present on the sediment. The photosynthetic activity of the algal cover stimulates the O<sub>2</sub> budgets at the sediment-water interface, thus influencing strongly the redox-sensitive trace component fluxes.

Finally, these results demonstrate the strong linkage between diagenetic processes and the biological activity. We can assume that the biofilm present at the sediment surface enhances O<sub>2</sub> circulation and renewal, promoting thus the diagenetic processes.

The final consideration and comparison of all flux categories and pathways indicate with evidence that the biological activity drives most of the exchanges at the sediment-water interface. Then, the pure physicochemical diffusive fluxes are negligible when considering the total budgets of the different compounds.

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