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THÈSE EN COTUTELLE PRÉSENTÉE
POUR OBTENIR LE GRADE DE
DOCTEUR DE
L'UNIVERSITÉ DE BORDEAUX
ET DE L'UNIVERSITÉ DU PAYS BASQUE

ÉCOLE DOCTORALE SCIENCES et ENVIRONNEMENTS
ÉCOLE DOCTORALE DE L'UNIVERSITÉ DU PAYS BASQUE
SPÉCIALITÉ GÉOCHIMIE et ÉCOTOXICOLOGIE

Par Ane REMENTERIA UGALDE

**UNDERSTANDING THE IMPACT OF SILVER AS AN
EMERGING CONTAMINANT IN THE IBAIZABAL AND
GIRONDE ESTUARIES**

Sous la direction de Pr. Jörg SCHÄFER (Université de Bordeaux)
et de PhD. Beñat ZALDIBAR (Université du Pays Basque)

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Titre : COMPRÉHENSION DE L'IMPACT DE L'ARGENT COMME CONTAMINANT ÉMERGENT DANS LES ESTUAIRES D'IBAIZABAL ET DE LA GIRONDE

Résumé :

Les estuaires ont été soumis à une forte pression humaine qui a entraîné la présence de polluants dans leurs eaux et sédiments, par exemple les métaux traces. Les estuaires du Golfe de Gascogne n'en sont pas une exception et les estuaires d'Ibaizabal et de la Gironde sont bien connus pour avoir des concentrations élevées de métaux. Dans ce contexte, les programmes de surveillance de l'environnement se sont appuyés sur l'utilisation des moules et des huîtres comme espèces sentinelles, car les analyses chimiques et biologiques de leurs tissus permettent d'évaluer l'état général de santé de leur environnement. Dans le but d'acquérir une meilleure compréhension des interactions entre la bioaccumulation et des effets toxiques du cuivre (Cu) et de l'argent (Ag) dans les huîtres *Crassostrea gigas*, ainsi que l'influence de facteurs environnementaux, le présent travail présente une étude de terrain et trois expériences de laboratoire. Parmi les résultats obtenus, on peut souligner que : (I) même si les huîtres et les moules peuvent indiquer correctement l'état de santé des estuaires du Golfe de Gascogne, une accumulation clairement plus élevée de métaux se produit dans les huîtres que dans les moules, (II) la voie d'exposition (directe ou alimentaire) induit des réponses de différentes intensités dans les huîtres, (iii) la toxicité de l'Ag est renforcée par la présence accrue de Cu dans les huîtres et (IV) la salinité a un faible effet sur la toxicité des métaux dans les huîtres.

Mots clés : *Huîtres, estuaires, argent, cuivre, biomarqueurs*

Title: UNDERSTANDING THE IMPACT OF SILVER AS AN EMERGING CONTAMINANT IN THE IBAIZABAL AND GIRONDE ESTUARIES

Abstract:

Estuaries have been subjected to a high human pressure which has led to the presence of pollutants in their waters and sediments including trace metals. The estuaries in the Bay of Biscay do not constitute an exception of this situation and the Ibaizabal and Gironde Estuaries are both very well known for having high metal concentrations in different compartments. In this context, environmental health monitoring programmes have relied on the use of mussels and oysters as sentinel organisms because chemical and biological analyses of their tissues allow assessing the general health status of their surrounding environment. The present work aims at acquiring a deeper understanding of the interactions between bioaccumulation and the toxic effects of copper (Cu) and silver (Ag) in oysters *Crassostrea gigas*, as well as the effects that environmental factors may have on them. For this, one field study and three laboratory experiments have been carried out. Among the most relevant results obtained it can be highlighted that: (I) both oysters and mussels are useful to properly indicate the health status of estuaries of the Bay of Biscay, yet higher accumulation of metals occurs in oysters than in mussels, (II) the intake pathway (water column and/or food) induces different response intensities in oysters (III) the toxicity of Ag is enhanced in the presence of Cu in oysters and (IV) salinity has a low effect on the toxicity of both metals in oysters.

Keywords: *Oysters, estuaries, silver, copper, biomarkers*

Unité de recherche

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EXTENDED ABSTRACT

Estuarine environments have provided humans food sources and the capacity to exchange goods with the rest of the world through navigation since ancient times. In consequence, many historical main human settlements have occurred around estuaries, leading to their degradation by the modification of their natural functioning and also by increased pollutant inputs due to various anthropogenic sources. In the last centuries, estuaries have become major sites for waste disposal and European estuaries are not an exception. Nowadays, many European estuaries show recovery processes mainly as a result of the general decline on industrial activities and the increased environmental awareness of the society, which has led to the implementation of new policies and regulations. Within the Bay of Biscay two estuaries could be considered as good examples of these recovery processes: the Ibaizabal (Bilbao, Basque Country, Spain) and the Gironde (South-Western France) Estuaries. Despite all the improvements that both estuaries have undergone, pollutant concentrations are still relevant in their waters and sediments. Among the pollutants present in the mentioned compartments (water and sediments), metallic pollution is of special relevance for the scientific community and environmental managers.

In fact, metals can exert toxicity effects on aquatic biota at very low exposure levels and they can bioaccumulate or biomagnify through the food chain, potentially affecting both aquatic environmental and human health. Among the different metals showing anomalies in estuaries silver (Ag) and copper (Cu) are of special interest. Silver is regarded as a very toxic element for aquatic biota, particularly in its ionic form Ag^+ , whereas Cu, is an essential element for living organisms but also toxic once certain exposure levels have been surpassed. These two elements have been extensively used by humans since ancient periods and nowadays they share to some extent similar pollution sources. Mining and metallurgy have been the classical pollution

sources for Ag whereas more recently (during the last decades of the XXth century) main Ag inputs have been related to photographic and electronic industries and also to Ag uses in hospitals. In fact, Ag has been considered as a good tracer for urban pollution during several decades. Nowadays, additional Ag inputs into the environment also originate from domestic and urban wastewaters, being increasingly related with the use of Ag nanoparticles in textiles and personal care products due to the bactericidal properties of Ag. Furthermore, Ag-iodide (AgI) is also used in agriculture to prevent hail storms through networks of cloud seeding devices. In the case of Cu, classical sources for this element have also been related to mining and metallurgy industry. Other Cu sources are fungicides used in agriculture (where Cu appears as CuSO₄) and, more recently, navigation. In fact since the ban of organo-tin compounds, Cu has become a major agent of the antifouling paints avoiding the attachment of biological organisms to the hulls of vessels.

Environmental water monitoring programs have relied on the use of sentinel species, being bivalves such as mussels and oysters widely employed for this purposes since 1976, when “The Mussel Watch” biomonitoring program was first implemented in the USA. Bivalves accumulate pollutants in their tissues with little metabolic transformation and on the other hand, their response in terms of toxicology can reflect the effects caused in the environment by pollutants. Additionally, as they are sedentary organisms, bivalves can record spatial and temporal changes in pollution. In this context, environmental biomonitoring programs rely on the collection of sentinel species for subsequent chemical measurement of pollutants in combination with biological measurements, which include alterations at cell and tissue levels. The latter, known as biomarkers, provide important information not only about individuals’ health status, but also about the surrounding environmental quality offering the perspective of predicting

future changes that may occur at higher biological organisation levels, such as population or ecosystem.

However, for a proper understanding of the information obtained by biomarkers, a battery of them should be applied, otherwise proper understanding of the organisms' response to pollutant stress would not be possible. The battery of biomarkers applied in the present work included the measurements of: Metallothionein (MT) induction, intralysosomal metal accumulation (through autometallography), lipofuscin content determination, neutral lipid content determination, digestive gland atrophy, connective to digestive diverticula ratio, histopathological alterations and measurements of condition index (CI).

Recently, different indices have been proposed for biomarker integration in order to obtain an integrated and simplified view of animals' response. For example, the Integrative Biological Response (IBR) index has been successfully applied in different sentinel organisms under both experimental and field conditions. In fact, biomonitoring studies that have included this index have been able to discriminate between spatial and temporal tendencies.

Since previous works have reported possible interactions between Cu and Ag, affecting their bioavailability and accumulation in oysters, one objective of the present thesis was to acquire a better understanding of such interactions between these metals as well as on the potential of using oysters to understand Ag and Cu behaviour and effects in estuaries. Accordingly, the present work relies on four main approaches: one field study and three laboratory experiments carried out at the Plentzia Marine Station (PiE, UPV/EHU). All biological measurements were carried out at the Cell Biology in Environmental Toxicology (CBET research group, UPV/EHU), whereas chemical measurements were performed in the "Transferts Géochimiques des Métaux à

l'interface continent ocean" research group (TGM, UMR CNRS EPOC 5805, Université de Bordeaux).

The first activity is based on a field study aiming to assess the environmental health status of three estuaries (Ibaizabal, Oka and Gironde) of the Bay of Biscay using mussels and oysters as sentinel organisms. Two of the analysed estuaries (Ibaizabal and Gironde) are well known for chronic metal pollution whereas the Oka Estuary, located within the UNESCO Reserve of the Biosphere of Urdaibai is regarded as a reference site. Collected animals were processed for chemical analysis of Ag and Cu accumulation in tissues and also for biological measurements. Obtained chemical results revealed higher metal concentrations in individuals inhabiting the Gironde Estuary. Moreover, clearly higher metal accumulation values occurred in oysters than in mussels from the same sites. These results were in clear concordance with the obtained values for intralysosomal metal accumulation with higher metal levels in bivalves from the Gironde Estuary. In agreement, the applied battery of biomarkers was able to reflect in both species the higher pollutant pressure in the Gironde Estuary with mussels and oysters showing higher levels of digestive epithelium atrophy and disturbed digestive tissue integrity. Consequently, the IBR index also indicated a more altered health status of oysters and mussels from the Gironde Estuary compared to Ibaizabal and Oka Estuaries.

The second approach of the present work was focused on a laboratory exposure experiment in which oysters *Crassostrea gigas* were exposed through direct pathway to a range of environmentally relevant concentrations of Ag and Cu alone and in combination. In this experiment, stable isotopes (^{107}Ag and ^{63}Cu) were used, allowing using relatively low exposure concentrations (close to environmental levels) of metals and their subsequent quantification in oysters' tissues. The experiment lasted 28 days and the measured biomarkers integrated into the IBR index were able to indicate that

even at very low concentrations Ag and Cu caused alterations on oyster's health status, with oysters exposed to Ag (alone or in combination with Cu) being more affected than those exposed to Cu alone. Metallothionein induction, intralysosomal metal accumulation and digestive tissue integrity were the parameters most affected by the metal exposure.

The third activity consisted in a study of the influence of salinity on the toxicity of Ag and Cu in oysters *Crassostrea gigas*. For this aim, another laboratory experiment was conducted, based on the second activity results exposing oysters, to Cu and Ag at similar maximum concentrations as in the previous experiment, but at lower salinity (S=18) during 14 days. The measured battery of biomarkers integrated into the IBR index, in agreement with the previous activity, indicated higher toxicity of Ag in direct comparison with Cu. Again, the intralysosomal metal accumulation and digestive tissue integrity were the most sensitive parameters.

Finally, the fourth activity aimed to study the effects caused by Cu and Ag in oysters *Crassostrea gigas* after dietary exposure as main route for pollutant entrance. For this, oysters were fed during 21 days with microalgae *Isochrysis galbana* previously exposed (for 24 h) to ^{107}Ag and ^{63}Cu at environmentally relevant concentrations. Metal isotope accumulation was detected in microalgae, although metal accumulation in oysters was not clearly detected. Despite this, the results obtained with the aid of biomarkers, revealed higher toxicity of ^{107}Ag and $^{107}\text{Ag}+^{63}\text{Cu}$ after short exposure time. Furthermore, when exposed either Ag or Cu alone the toxic effects increased through time. In this experiment, the lipofuscin accumulation and the metallothionein induction were the most sensitive parameters.

Overall, the obtained results confirm the suitability of oysters to be employed as sentinel organisms giving accurate biological responses to pollutant exposure.

Moreover, the suitability of integrating all these biomarkers into the IBR index was also confirmed producing a better understanding of these responses. The interactions between Cu and Ag in their bioavailability and bioaccumulation were confirmed, with a synergistic accumulation and toxicity of Ag in the presence of Cu. Furthermore, intralysosomal metal accumulation, lipofuscin accumulation and tissue integrity seem to be the most sensitive measurements in order to determine the toxicity of Ag and to a lesser extent of Cu.

RÉSUMÉ ÉTENDU

Les environnements estuariens ont fourni aux humains des sources alimentaires et la capacité d'échanger des biens avec le reste du monde par la navigation marine depuis l'Antiquité. En conséquence, de nombreuses populations humaines se sont installées autour des estuaires, entraînant leur dégradation par une intense utilisation des terres et une augmentation de la présence de polluants anthropiques. Au cours des derniers siècles, les estuaires sont devenus des sites clés de dépôt de déchets, les estuaires Européens n'en étant pas une exception. Principalement lié à la réduction d'activités industrielles et à la prise de conscience de la société sur la fragilité de l'environnement, de nombreux estuaires européens sont aujourd'hui objet de processus de récupération, répondant à de nouvelles politiques et réglementations mises en œuvre. Dans le cas du Golfe de Gascogne, deux estuaires pourraient être cités comme exemples d'application de ces processus: les estuaires d'Ibaizabal (Bilbao, Pays Basque, Espagne) et de la Gironde (Sud-Ouest de la France). Néanmoins, malgré les améliorations observées dans les deux estuaires, les concentrations de polluants dans les eaux et sédiments y sont encore importantes. Parmi ces polluants, la pollution métallique est particulièrement importante.

La pollution métallique dans les estuaires est considérée comme un problème majeur par la communauté scientifique et les gestionnaires. Les métaux peuvent non seulement être toxiques pour le biote aquatique, mais peuvent aussi être bioaccumulés ou bioamplifiés dans la chaîne alimentaire. Parmi les différents polluants métalliques présents dans les estuaires, l'argent (Ag) et le cuivre (Cu) présentent un intérêt particulier. L'argent est considéré comme un élément très toxique pour le biote aquatique, en particulier quand il est présent sous forme ionique Ag^+ . En comparaison, Cu est un élément essentiel pour les organismes vivants, mais peut aussi devenir très toxique au delà de certaines concentrations. Ces deux éléments ont été largement

utilisés par les humains depuis les périodes anciennes et partagent même de nos jours, des sources de pollution communes. Alors que l'exploitation minière et la métallurgie ont été les principales sources de pollution d'Ag, les sources plus récentes de ce polluant (dernières décennies du XXème siècle), sont liées aux industries de la photographie et de l'électronique ainsi qu'aux hôpitaux. De ces faits, Ag est considérée comme étant un bon traceur de pollution urbaine. De nos jours, des apports supplémentaires d'Ag dans l'environnement à travers les eaux usées domestiques et urbaines, sont principalement liés à l'utilisation croissante de nanoparticules d'Ag dans les textiles et les produits de soin personnels en raison des propriétés bactéricides de cet élément. En outre, l'Ag est également utilisée dans l'agriculture sous forme AgI pour prévenir les tempêtes de grêle. Concernant le Cu, les sources classiques de cet élément sont également liées à l'industrie minière et métallurgique. Le Cu s'utilise aussi en agriculture, essentiellement dans les fongicides sous la forme de CuSO_4 et en navigation, où il remplace les composés organo-stanniques dans les peintures antisalissures évitant la fixation d'organismes vivants sur les coques des vaisseaux.

Dans ce contexte, les programmes de surveillance de l'environnement et de l'eau se sont appuyés sur l'utilisation d'espèces sentinelles, à savoir des bivalves comme les moules et les huîtres utilisés depuis 1976, lors du programme de surveillance «The Mussel Watch» mis en œuvre pour la première fois aux États-Unis. Ces organismes vivants d'accumulent des polluants dans leurs tissus avec peu de transformation métabolique et peuvent ainsi refléter les effets causés par ces polluants dans l'environnement. Le type de vie sédentaire de ces organismes permet la détection de variations spatio-temporelles de la pollution dans l'environnement. Les programmes de biosurveillance environnementale se basent sur la collecte d'organismes sentinelles pour l'analyse chimique des polluants faite en combinaison avec des mesures biologiques, dont des altérations au niveau cellulaire et tissulaire. Ces derniers, connus

sous le nom de biomarqueurs, fournissent des informations importantes non seulement sur l'état de santé des individus, mais aussi sur l'environnement dans lequel ils habitent. Ils permettent également de prédire des changements futurs potentiels à des niveaux d'organisation biologique plus élevés tels qu'à l'échelle de la population ou de l'écosystème.

Toutefois, afin de compléter l'évaluation des effets de polluants sur le biote aquatique, il est conseillé d'étudier une batterie de biomarqueurs ainsi plus adaptée pour décrire les réponses biologiques à l'exposition de polluants. Les biomarqueurs sélectionnés pour l'étude présente permettent de (i) mesurer: l'induction de métallothionéines (MT), l'accumulation intralysosomale de métaux (par la technique d'autométallographie), le contenu en lipofuscines, le contenu en lipides neutres, l'atrophie de la glande digestive, le rapport de tissu conjonctif au diverticule digestif, les lésions histopathologiques et (ii) de calculer l'index de condition (Condition Index, CI).

Afin d'obtenir une vision complète de la réponse des organismes aux polluants, différents indices ont été proposés permettant d'intégrer les différents biomarqueurs étudiés. Parmi eux, l'indice «Integrative Biological Response (IBR)» a été appliqué avec succès dans différents organismes sentinelles dans des conditions expérimentales et sur le terrain. L'utilisation de cet indice dans des programmes de biosurveillance a permis de distinguer les variations spatiales et temporelles.

Des travaux antérieurs ont mis en évidence des interactions possibles entre le Cu et l'Ag, affectant leur biodisponibilité et leur accumulation dans les huîtres. L'objectif du travail présent était d'acquérir une meilleure compréhension de ces interactions entre métaux ainsi que d'évaluer le potentiel d'utilisation des huîtres comme organisme sentinelle pour étudier le comportement d'Ag et Cu dans les estuaires. Pour ce faire, la thèse a été basée sur une étude de terrain et des expériences de laboratoire réalisées

à la Station Marine de Plentzia (PiE, UPV/EHU). Les analyses biologiques ont été effectuées au sein du groupe de recherche de Biologie Cellulaire en Toxicologie Environnementale de l'Université du Pays Basque (CBET research group, UPV/EHU) et les analyses chimiques ont été effectuées dans le groupe de recherche de Transferts Géochimiques des Métaux à l'interface continent océan (TGM, UMR CNRS EPOC 5805, Université de Bordeaux).

Le premier objectif de la présente thèse, basée sur une étude de terrain, visait à évaluer l'état de santé environnementale de trois estuaires (Ibaizabal, Oka et Gironde) du Golfe de Gascogne en utilisant des moules et des huîtres comme organismes sentinelles. Deux des estuaires analysés (Ibaizabal et Gironde) sont connus pour être soumis à une pollution métallique chronique tandis que l'estuaire de l'Oka, situé dans la réserve UNESCO de la biosphère d'Urdaibai est considéré comme un site de contrôle. Les animaux recueillis ont été préparés pour l'analyse chimique de l'accumulation d'Ag et de Cu dans les tissus et aussi pour les mesures biologiques. Les résultats obtenus ont révélé des concentrations plus élevées de métaux chez les individus collectés dans l'estuaire de la Gironde. De plus, les valeurs d'accumulation observées étaient toujours supérieures chez les huîtres que chez les moules. Ces résultats concordent clairement avec les valeurs obtenues pour l'accumulation intralysosomale de métaux avec des teneurs en métaux plus élevées chez les bivalves de l'estuaire de la Gironde. De même, la batterie de biomarqueurs appliquée a démontré un effet des polluants plus marqué dans l'estuaire de la Gironde où les moules et huîtres présentaient des niveaux supérieurs d'atrophie de l'épithélium digestif et une intégrité du tissu digestif perturbée. L'indice IBR indique un état de santé plus altéré chez les huîtres de l'estuaire de la Gironde par rapport à Ibaizabal et Oka.

La deuxième approche était centrée sur une expérience d'exposition en laboratoire dans laquelle les huîtres *Crassostrea gigas* ont été exposées par voie directe à une

gamme de concentrations environnementales d'Ag et de Cu seuls et en combinés. Dans cette expérience, des isotopes stables (^{107}Ag et ^{63}Cu) ont été utilisés, permettant des concentrations d'exposition métalliques relativement basses et leur quantification précise dans les tissus d'huîtres. L'expérience a duré 28 jours. L'intégration des biomarqueurs dans l'indice IBR a permis de démontrer que, même à de très faibles concentrations, les métaux Ag et Cu sont capables d'altérer l'état de santé général des huîtres. Les huîtres exposées à l'Ag (seul ou combiné avec le Cu) ont montré un état de santé plus altéré que les huîtres exposées au Cu seul. L'induction des MTs, l'accumulation intralysosomale de métal et l'intégrité du tissu digestif ont été les paramètres les plus affectés par l'exposition aux métaux.

Le troisième objectif visait à étudier l'influence de la salinité sur la toxicité de l'Ag et du Cu chez *Crassostrea gigas*. Dans ce but et selon les résultats obtenus précédemment, une seconde expérience d'exposition en laboratoire a été réalisée dans laquelle les huîtres étaient exposées au Cu et à l'Ag aux concentrations maximales précédemment utilisées, mais en appliquant des valeurs de salinité plus faibles ($S=18$) pendant 14 jours. La batterie de biomarqueurs mesurés et intégrés dans l'indice IBR indiquent comme précédemment observé, une toxicité plus élevée d'Ag que de Cu. Dans cette expérience, l'accumulation intralysosomale de métal et l'intégrité du tissu digestif étaient à nouveau les paramètres les plus sensibles.

Enfin, la quatrième activité visait à étudier les effets du Cu et de l'Ag sur *Crassostrea gigas* après exposition aux polluants par voie alimentaire. Les huîtres ont été nourries pendant 21 jours avec des microalgues *Isochrysis galbana*, préalablement exposées aux métaux ^{107}Ag et ^{63}Cu (pendant 24 h). L'accumulation d'isotopes métalliques dans les microalgues a pu être détectée avec succès, cependant l'accumulation métallique dans les huîtres n'a pas été significative. L'analyse de biomarqueurs a révélé une toxicité plus élevée des isotopes ^{107}Ag et $^{107}\text{Ag}+^{63}\text{Cu}$ après un court temps d'exposition.

De plus, dans les cas d'une à chacun de ces deux métaux seuls, les effets toxiques augmentaient au cours de l'expérience. Dans cette expérience, l'accumulation de lipofuscine et l'induction des metallothioneins étaient les paramètres les plus sensibles. Dans l'ensemble, les résultats obtenus confirment le grand potentiel des huîtres à être utilisées comme organismes sentinelles montrant des réponses biologiques précises à l'exposition aux polluants. De plus, l'intégration de la batterie de biomarqueurs par l'indice IBR a également permis une meilleure compréhension de ces réponses, soulignant ainsi la validité de cette méthode. Les interactions entre Cu et Ag dans leur biodisponibilité et leur bioaccumulation ont été confirmées, avec une accumulation synergique et une toxicité accrue de l'Ag en présence de Cu. D'autre part, l'accumulation intralysosomique de métaux, l'accumulation de lipofuscines et l'intégrité tissulaire semblent être les mesures biologiques les plus sensibles pour déterminer la toxicité de l'Ag et du Cu.

LABURPEN LUZEA

Antzinarotik, ingurune estuarikoak gizakien elikagai iturri izan dira eta gainera nabigazio bidez, herrialde desberdinen arteko ondasun trukea ahalbidetu dute. Ondorioz, historian zeharreko giza kokaleku garrantzitsuenetakoak estuarioen inguruan ezarri dira, eta gizakiak estuarioen funtzionamendu naturala eraldatuz zein kutsatzaileen isurketaren bidez ingurune hauek degradatu ditu. Hala, azken mendeetan estuarioak hondakinen isurketa leku bilakatu dira, Europako estuarioak barne. Gaur egun, estuario Europear ugari berreskuratze prozesuak erakusten dituzte, batik bat industriaren gainbehera eta gizarteak ingurumenarekiko duen kontzientziaren areagotzearen ondorioz. Azken honek, politika eta neurri berrien inplementazioa ekarri ditu. Bizkaiko Golkoaren barnean badaude berreskuratze prozesu honen adibide on kontsidera daitezkeen bi estuario: Ibaizabal (Bilbo, Euskal Herria, Espainia) eta Gironde (Frantziako hego mendebaldea). Bi estuario hauek hobekuntza ugari izan dituzten arren, oraindik ere euren uretan zein sedimentuetan agertzen diren kutsatzaile kontzentrazioak esanguratsuak dira. Aurretik aipaturiko bi konpartimentu (ura eta sedimentuak) hauetan agertzen diren kutsatzaile guztien artean, kutsadura metalikoak garrantzia berezia du komunitate zientifikoarentzat eta baita ingurumen kudeatzaileentzat ere.

Izan ere, metal esposizio maila baxuek eragin toxikoak sor ditzakete biota akuatikoan, are gehiago kate trofikoan zehar metatu edota areagotu daitezke, bai ingurunearen zein gizakien osasun egoeran eraginez. Estuarioetan ez ohiko balioak aurkezten dituzten metalen artean zilarra (Ag) eta kobrea (Cu) garrantzia berezikoak dira. Zilarra (Ag), oso elementu toxikoa da biota akuatikorako, batik bat bere forma ionikoan Ag^+ agertzen denean. Bestalde, kobrea (Cu) elementu esentziala da izaki bizidunentzat, baina aldi berean toxikoa da esposizio maila jakin batzuk gainditzen direnean. Antzinarotik, gizakiak sarritan erabili izan ditu bai Ag zein Cu-a, eta bi elementuek hein

handi batean kutsadura iturri berdinak partekatzen dituzte. Zilarraren kasuan meatzaritza eta metalurgia izan dira poluzio iturri nagusiak. XX. mendeko azken hamarkadetan aldiz, Ag isurketa gehienak argazkigintza eta elektronikaren industriatik etorri ziren, baita ospitaleetatik ere. Honela bada, Ag hiritar jatorriko kutsaduraren adierazletzat kontsideratu da hainbat hamarkadetan zehar. Gaur egun, ingurumenean gertatzen diren Ag isurketa ugari ere, etxe zein hiritar jatorria dute, izan ere Ag nanopartikulak higiene produktuetan zein ehungintzan erabiltzen dira euren propietate bakterizida dela eta. Gainera, Ag nekazaritzan ere erabilia da txingor ekaitzak ekiditeko horretarako atmosferara zilar ioduroa (AgI) isuriz. Kobrearen kasuan, kutsadura iturri klasikoak meatzaritza eta metalurgiarekin erlazionatuak daude. Gaur egun, Cu isurketek nekazal jatorria ere badute, izan ere, fungizida ugari Cu-a dute euren osagaien artean, nagusiki CuSO_4 eran. Gainera, nabigazioa ere bada Cu isurketen arduradun, itsasontzien kroskoetan izaki bizidunen agerpena ekiditeko organo-eztainu konposatuen erabilpena galarazi zenetik Cu-a "antifouling" deituriko margoen osagaia baita.

Testuinguru honetan, uren kalitatearen jarraipen programak espezie behaleen erabilpenean oinarritu izan dira. Hauen artean, bibalbioak, muskuiluak eta ostrak barne, izan dira animaliarik erabilienak 1976. urtean AEB-etan "The Mussel Watch" deituriko lehen ingurumen biojarraipen programa martxan jarri izan zenetik. Bai muskuiluak zein ostrak, kutsatzaileak euren ehunetan metatzeko gai dira eraldapen metaboliko gutxirekin eta bestalde, kutsatzaileek ingurumenean eragindako efektuak adierazteko ere gai badira. Gainera, organismo sedentarioak izanik kutsatzaileetan gertatutako aldaketa espazio-tenporalak ere adierazteko gai badira. Biojarraipen programak espezie behaleen bilketan oinarritzen dira, ondoren euren ehunetan analisi kimikoak zein biologikoak konbinatzeko. Analisi biologikoen artean zelula eta ehun mailako aldaketak neurtu dira. Azken hauek, biomarkatzaile bezala ezagutzen dira eta

informazio garrantzitsua ematen dute bai indibiduen zein bizi diren ingurumenaren osasunaren inguruan, are gehiago maila biologiko altuagoetan (populazio edota ekosistemak) gerta daitezkeen aldaketak aurreikusteko gai ere badira.

Hala eta guztiz ere, biomarkatzaileek emandako informazioa era egokian interpretatu ahal izateko, biomarkatzaile-bateria baten erabilpena beharrezkoa da, bestela organismoek kutsatzaileen aurrean emandako erantzun biologikoa ondo ulertzea ezinezkoa da. Ikerketa honetan erabilitako biomarkatzaile bateriak hurrengo neurriak biltzen ditu: Metalotioneinen (MT) indukzioa, metalen lisosoma barneko metaketa (autometalografia bidez neurtua), lipofuszinen metaketa, lipido neutroen metaketa, liseri guruinaren atrofia, ehun konektibo eta digestiboaren proportzioaren arteko ratioa, alterazio histopatologikoak eta egoera-indizea (ingelesez Condition Index; CI).

Duela gutxi, indibiduoek emandako erantzunaren irudi integratu eta sinplifikatu bat edukitzeko asmoz, biomarkatzaileak integratzeko indize desberdinak proposatuak izan dira. Horien artean berriki "Integrative Biological Response" (IBR) indizea espezie behale desberdinen artean erabili da, bai landa zein baldintza esperimentaletan emaitza positiboak lortuz. Are gehiago, indize hau erabili izan duten biojarraipen ikerketek tendentzia espazial eta tenporalak diskriminatzeko gai izan dira.

Aurretik burututako ikerlanek ostretan gertatzen diren Cu eta Ag-ren arteko interakzioen berri eman dute, metal hauen bioeskuragarritasunean zein metaketan eragina izanik. Interakzio hauen arteko ulerpen sakonago bat lortzeko asmoz eta baita estuarioetan Ag-ak eta Cu-ak duten jokabidea ulertzeko ostrek eduki dezaketen erabilera potentziala ikertzeko asmoz, ondorengo lau aktibitate nagusiak burutu dira ikerlan honetan: landa jarduera bat eta hiru laborategi-esperimentu Plentziako Itsas Estazioan (PiE, UPV/EHU) aurrera eramandakoak. Ikerlan honetan burututako analisi biologiko guztiak Zelula Biologia Ingurumen Toxikologian (UPV/EHU) ikerketa taldean

aurrera eraman ziren, analisi kimikoak aldiz “Transferts Géochimiques des Métaux à l'interface continent ocean” (TGM, UMR CNRS EPOC 5805, Université de Bordeaux) ikerketa taldean.

Ikerlan honetako lehendabiziko jarduera landa ikerketa batean oinarritua dago, bere helburua Bizkaiko Golkoko hiru estuarioetako (Ibaizabal, Oka eta Gironde) ingurumen osasun egoera ikertzea da horretarako muskuiluak eta ostrak organismo behale gisa erabiliz. Hiru estuarioetako bi (Ibaizabal eta Gironde) kutsadura metaliko kronikoa pairatzeagatik ezagunak dira, Oka Estuarioa aldiz UNESCO-k izendatutako Urdaibai Biosferaren Erreserbaren barnean kokatua dago eta kontrol puntu gisa kontsideratua da. Hiru estuarioetan bildutako animaliak ehunetan metatutako Ag eta Cu balioak ikertzeko erabili ziren eta baita animalien analisi biologikoa burutzeko ere. Lortutako emaitza kimikoek, Girondeko animaliek kontzentrazio metaliko altuagoak zituztela argi utzi zuten. Gainera, metaketa balio altuagoak ikusi ziren ostretan muskuiluetan baino. Are gehiago, emaitza hauek neurtutako metalen lisosoma barneko metaketarekin bat etorri ziren, izan ere metaketa altuagoa ikusi zen Girondeko bibalbioetan. Gainera, erabilitako biomarkatzaile batera gai izan zen Girondeko estuarioan kutsatzaileek eragiten duten presio handiagoa identifikatzeko. Hala, Girondeko muskuiluek eta ostrek liseri guruineko epitelioan atrofia balio altuagoak aurkeztu zituzten eta baita alterazioak ikusi ziren ehunen osotasunean. Ondorioz, IBR indizea ere Gironde estuarioko ostrek eta muskuiluek osasun egoera asaldatuago bat dutela detektatzeko gai izan zen, gainontzeko estuarioekin alderatuz.

Ikerlaneko bigarren jarduera laborategi esperimentu bat izan zen non *Crassostrea gigas* ostrak ingurunean aurki daitezkeen Ag eta Cu kontzentraziopean jarri ziren bai konbinazioan eta bai banaka. Esperimentu honetan Ag eta Cu isotopo egonkorrek erabili ziren (^{107}Ag eta ^{63}Cu), hauei esker esposizio kontzentrazio baxuagoak erabili ahal izan ziren, gainera ostren ehunetan metatutako kopuruak ere kuantifikatu ahal

izan ziren. Esperimentuak 28 egun iraun zituen eta neurtutako biomarkatzaileak IBR indizean integratu ziren. Indize hau Ag eta Cu kontzentrazio maila baxuetan ere ostretan alterazioak sor ditzaketela adierazteko gai izan zen. Horrela, Ag-pean jarritako ostrak (bai Ag soilik zein Cu-rekin konbinatua) kaltetuenak izan ziren. Metalotioneinen indukzioa, metalen lisosoma-barneko metaketa eta liseri guruineko ehunaren osotasuna izanik metal esposizioaren eragin gehien jaso zituzten parametroak.

Hirugarren jarduera, gazitasunak Ag eta Cu-ak ostretan (*Crassostrea gigas*) eragiten duten toxikotasunean izan dezakeen eraginaren ikerketan oinarritu zen. Horretarako, aurreko esperimentuko emaitzetan oinarrituz beste esperimentu bat burutu zen non ostrak aurreko esperimentuko Cu eta Ag kontzentrazio maximoan jarri ziren baina gazitasun balio gutxiagorekin (S=18) eta 14 egunen zehar. Erabilitako biomarkatzaile-bateria eta ondoren IBR indizean integraturik, gai izan zen Ag-aren toxikotasun handiago bat adierazteko Cu-arekin alderatuz, datu hauek aurreko esperimentuarekin bat etorri. Kasu honetan, metal lisosoma-barneko metaketa eta liseri guruinaren ehunaren osotasuna biomarkatzaile sentikorrenak izan ziren.

Amaitzeko, laugarren jarduera bat ere aurrera eraman zen non ostrak (*Crassostrea gigas*) dieta bidez Cu eta Ag-pean jarri ziren esposizio bide honen efektuak ikertzeko asmoz. Helburu hau lortu ahal izateko, ostrak 21 egunez *Isochrysis galbana* mikroalgekik elikatu ziren. Azken hauek, 24 orduz ^{107}Ag eta ^{63}Cu -pean (ingurunemean aurki daitezkeen kontzentrazioetara) jarri zirelarik. Mikroalgek isotopo egonkorak metatu zituzten, ostretan ordea, metal metaketa ez zen horren argia izan. Biomarkatzaileen erabilpenaren bidez lortutako emaitzek ordea, ^{107}Ag eta $^{107}\text{Ag}+^{63}\text{Cu}$ -ren toxikotasun altuagoa erakutsi zuten epe laburrean, bestalde poluitzaileak banaka toxikotasunaren denboran zeharreko emendapen bat aurkeztu zuten. Ikerketa honetan lipofuszen akumulazioa eta metalotioneinen indukzioa izan ziren parametrotik sentikorrenak.

Orokorrean lorturiko emaitzek, ostrek organismo behale gisa erabiliak izateko duten gaitasuna konfirmatzen dute, erantzun biologiko zehatzak aurkeztuz kutsatzaileen presentziaren aurrean. Gainera, biomarkatzaileak IBR indizean integrazeko egokitasuna ere konfirmatu zen, hala erantzunen ulergarritasuna hobetuz. Kobre eta Ag-aren arteko interakzioa euren bioeskuragarritasun eta biometaketan ere konfirmatuak izan ziren, euren artean erlazio sinergiko bat egonik bai metaketan zein toxikotasunean, batik bat Ag-aren kasuan Cu-arekin batera agertzean. Bestalde, metalen lisosoma barneko metaketa, lipofuszinaren metaketa eta ehunaren osatasuna izan ziren Ag eta Cu-aren toxikotasuna determinatzeko neurketarik sentikorrenak.

RESUMEN EXTENSO

Los medios estuarinos han proporcionado a los humanos recursos alimenticios así como la capacidad de intercambiar bienes con el resto del mundo mediante la navegación desde la antigüedad. En consecuencia, muchos de los grandes asentamientos humanos a lo largo de la historia se han ubicado alrededor de los estuarios, provocando su degradación por medio de la alteración de su funcionamiento natural así como por la mayor presencia de contaminantes de origen antrópico. En los últimos siglos, los estuarios se han convertido en uno de los mayores lugares donde depositar los residuos sin que los estuarios Europeos constituyan una excepción. Hoy en día muchos estuarios europeos muestran procesos de recuperación, principalmente como consecuencia del descenso de algunas actividades industriales y de una mayor conciencia ambiental de la sociedad, que a su vez ha propiciado la implementación de nuevas políticas y medidas ambientales. Dentro del Golfo de Bizkaia existen dos estuarios que pueden considerarse como buenos ejemplos de éstos procesos de recuperación: Ibaizabal (Bilbao, País Vasco, España) y Gironde (Sur-Oeste de Francia). Pese a todas las mejoras que han ocurrido en ambos estuarios, las concentraciones de contaminantes en sus aguas y sedimentos son aún relevantes. Dentro de los contaminantes presentes en los compartimentos mencionados anteriormente (agua y sedimentos) la contaminación metálica tiene una relevancia especial para la comunidad científica y los gestores ambientales.

De hecho, los metales pueden causar efectos tóxicos en la biota acuática a valores de exposición bajos, pero también se puede bioacumular o biomagnificar a través de la cadena trófica, afectando potencialmente tanto la salud ambiental acuática como la salud humana. Entre los diferentes contaminantes metálicos presentes en los estuarios la plata (Ag) y el cobre (Cu) son de especial interés. La Ag está considerada como un elemento muy tóxico para la biota acuática, particularmente en su forma

iónica Ag^+ , mientras que el Cu, es un elemento esencial para los seres vivos, pero también es un elemento tóxico una vez que ciertos valores de exposición se han superado. Estos dos elementos han sido ampliamente utilizados por los humanos desde la antigüedad y comparten hasta cierto punto las mismas fuentes de contaminación. La minería y la metalurgia han sido las fuentes más clásicas de contaminación para la Ag, mientras que más recientemente (durante las últimas décadas del siglo XX) los mayores aportes de Ag han estado relacionados con la industria fotográfica y electrónica, así como con los hospitales. De hecho, la Ag ha sido considerada durante décadas como un buen elemento para identificar la contaminación urbana. Hoy en día, los aportes de Ag en el ambiente están también relacionados con las aguas residuales de origen doméstico y urbano, puesto que las nanopartículas de Ag se usan en productos de higiene personal así como en textiles debido a las propiedades bactericidas de la Ag. Además, la Ag también es utilizada en la agricultura en forma de ioduro de plata (AgI) para prevenir las tormentas de granizo. En el caso del Cu, las fuentes clásicas de contaminación también han estado relacionadas con la minería y la metalurgia. Hoy en día, los aportes de Cu también provienen de su uso en la agricultura como parte de los fungicidas (en forma de CuSO_4) y de la navegación. En esta última actividad, el Cu es parte de las pinturas “antifouling” desde que los compuestos de organoestaño fueron prohibidos. Estas pinturas, sirven para evitar que los organismos biológicos se adhieran a los cascos de los buques.

En este contexto, los programas de seguimiento del medio acuático han confiado en el uso de organismos centinelas, siendo los bivalvos como los mejillones y las ostras ampliamente empleados desde que en 1976 se instauró el primer programa de seguimiento en los EEUU conocido como “The Mussel Watch”. Tanto los mejillones como las ostras, son capaces de acumular los contaminantes en sus tejidos con una

mínima transformación metabólica, por otra parte, pueden reflejar los efectos causados en el medio ambiente por estos contaminantes. Además, al ser organismos sedentarios, pueden reflejar los cambios espacio-temporales ocurridos en los contaminantes. Dentro de este contexto, los programas de seguimiento ambiental están basados en la recolección de especies centinelas para su posterior análisis químico de contaminantes en combinación con análisis biológicos, los cuales incluyen alteraciones ocurridas a nivel celular y tisular. Éstos últimos conocidos como biomarcadores, aportan información importante no sólo sobre la salud de los individuos sino que también proporcionan información sobre el medio en el que viven y además son capaces de predecir cambios que puedan ocurrir a niveles más altos de organización biológica tal y como de población o ecosistema.

Aún así, para una correcta comprensión de la información aportada por los biomarcadores, se debe utilizar una batería de éstos, ya que de otro modo no sería posible la correcta comprensión de la respuesta dada por los organismos frente a la contaminación. La batería de biomarcadores aplicada en el presente estudio incluye medidas de: inducción de metalotioneinas (MT), acumulación intralisosomal de metales (por medio de la autometalografía), determinación de los contenidos de lipofuscinas, determinación de los lípidos neutros, atrofia de la glándula digestiva, medida del ratio entre tejido conectivo y digestivo, alteraciones histopatológicas y el índice de condición (condition index, CI).

Más recientemente, con el fin de obtener una imagen integrada y simplificada de las diferentes respuestas proporcionadas por los individuos, diferentes índices han sido propuestos con el fin de integrar los biomarcadores, entre ellos el índice "Integrative Biological Response (IBR)" ha sido empleado satisfactoriamente en varios organismos centinela sujetos tanto a condiciones experimentales como de campo. De hecho, los

estudios de bioseguimiento que han incorporado este índice son capaces de discriminar entre tendencias espaciales y temporales.

Estudios realizados previamente han mencionado la existencia de posibles interacciones entre el Cu y la Ag, afectando en su biodisponibilidad y acumulación en las ostras y con el objetivo de adquirir una mayor comprensión de estas interacciones entre ambos metales así como del uso potencial de las ostras para entender el comportamiento de tanto Ag como Cu en los estuarios, el presente trabajo incluye cuatro actividades principales: una actividad de campo y tres experimentos llevados a cabo en la estación marina de Plentzia (PiE, UPV/EHU). En el presente trabajo, todas las medidas biológicas fueron realizadas en el grupo de investigación Biología Celular en Toxicología Ambiental (UPV/EHU) mientras que las medidas químicas fueron llevadas a cabo en el grupo de investigación "Transferts Géochimiques des Métaux à l'interface continent océan" (TGM, UMR CNRS EPOC 5805, Université de Bordeaux).

La primera actividad del presente trabajo está basada en un estudio de campo con el fin de realizar una evaluación del estado de la salud en el que se encuentran tres estuarios (Ibaizabal, Oka y Gironde) del Golfo de Bizkaia utilizando mejillones y ostras como especies centinelas. Dos de los estuarios analizados (Ibaizabal y Gironde) son muy conocidos por sufrir una contaminación crónica de metales, mientras que el estuario del Oka, está ubicado dentro de la Reserva de la Biosfera de Urdaibai (UNESCO) y es considerado como un punto control. Los animales recolectados fueron procesados para el análisis químico de Ag y Cu acumulada en los tejidos y también para su análisis biológico. Los resultados químicos obtenidos revelaron mayores concentraciones metálicas en los individuos que habitan en el estuario de la Gironde. Además los valores de acumulación fueron mayores en ostras que en mejillones. Estos resultados están en clara concordancia con los resultados obtenidos para la acumulación intralisosomal de metales, con mayores niveles de acumulación en los

bivalvos del estuario de la Gironde. Además, la batería de biomarcadores aplicados fue capaz de reflejar en ambas especies una mayor presión ejercida por los contaminantes existentes en el el estuario de la Gironde, presentando los mejillones y ostras mayores niveles de atrofia en el epitelio de la glándula digestiva y alteraciones en la integridad del tejido. En consecuencia, el IBR index también indicó un estado de salud más alterado en las ostras y mejillones del estuario de la Gironde en comparación con las de los estuarios del Ibaizabal u Oka.

La segunda actividad de este trabajo está centrada en un experimento de laboratorio en el cual las ostras *Crassostrea gigas* fueron expuestas directamente a concentraciones ambientalmente representativas de Ag y Cu en combinación o en solitario. En este experimento se utilizaron isótopos estables (^{107}Ag y ^{63}Cu) que permitieron el uso de concentraciones relativamente bajas de exposición de metales y su posterior cuantificación en los tejidos de las ostras. El experimento duró 28 días y los biomarcadores medidos fueron integrados en el índice IBR, siendo éste capaz de indicar que Ag y Cu son capaces de causar alteraciones incluso a bajas concentraciones. Siendo las ostras expuestas a Ag (únicamente a Ag o en combinación con Cu) las más afectadas. La inducción de metalotioneinas, la acumulación intralisosomal de metales y la integridad del tejido digestivo fueron los parámetros más afectados a la exposición metálica.

La tercera actividad consistió en un estudio de la influencia de la salinidad en la toxicidad de la Ag y Cu en ostras *Crassostrea gigas*. Para poder cumplir este objetivo, se realizó otra experimentación basada en los resultados de la segunda actividad, en la que las ostras fueron expuestas a las mismas concentraciones máximas de Cu y Ag que en el experimento previo pero a una menor salinidad ($S=18$) y durante 14 días. La batería de biomarcadores medidos e integrados en el índice IBR, indicó una mayor toxicidad de la Ag en comparación con el Cu, estando en concordancia con los

resultados del experimento realizado en la segunda actividad. En este caso, la acumulación intralisosomal de metales y la integridad del tejido digestivo fueron los biomarcadores más sensibles.

Finalmente, la cuarta actividad tuvo como objetivo el estudio de los efectos causados por la exposición mediante vía trófica de Cu y Ag en las ostras *Crassostrea gigas*. Para poder alcanzar este objetivo, las ostras fueron alimentadas durante 21 días con microalgas *Isochrysis galbana* previamente expuestas durante 24 h a ^{107}Ag y ^{63}Cu (concentraciones ambientalmente representativas). El diseño experimental indicó una acumulación de isótopos estables en las algas, pero por el contrario la acumulación de metales en las ostras no fue significativa. Los resultados obtenidos mediante los biomarcadores aplicados en las ostras, revelaron una mayor toxicidad de ^{107}Ag y $^{107}\text{Ag}+^{63}\text{Cu}$ tras periodos cortos de exposición, mientras que los efectos tóxicos causados por los contaminantes por sí solos aumentaron durante el tiempo. En este experimento, la acumulación de lipofuscinas y la inducción de metalotioneinas fueron los parámetros más sensibles.

En general, los resultados obtenidos confirman la idoneidad de las ostras para ser empleadas como organismos centinela presentando respuestas biológicas precisas a la exposición de contaminantes. Además, la idoneidad de integrar los biomarcadores en el IBR index fue también confirmada, obteniendo un mejor resultado en la comprensión de las respuestas. Las interacciones entre el Cu y Ag y su biodisponibilidad y bioacumulación fueron confirmadas, con una relación sinérgica en acumulación y toxicidad en el caso de Ag al ser expuesta en combinación con Cu. Por otro parte, la acumulación intralisosomal de metales, la acumulación de lipofuscinas y la integridad del tejido fueron las medidas más sensibles a la hora de determinar la toxicidad de Ag y Cu.

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I. INTRODUCTION

1. THE BAY OF BISCAY

Located in the North Eastern area of the Atlantic Ocean, the Bay of Biscay constitutes an embayment that covers an area located between 0-10°W to 43-48°N. The geographical limits of the Bay are the Cape Ortegal in North-Western Spain (43°N; 8°W) and the Pointe de Pern in the western coast of France Brittany (48°N; 5°W) (Borja and Collins, 2009; Costoya et al., 2015). Thus, the north Iberian Peninsula coast limits on the southern part of the bay with an east-west orientation while the French coast presents a north-south orientation (deCastro et al., 2009) (Fig. 1).



Figure 1: Map of the Bay of Biscay including the main cities located on its coast. Map modified from www.worldatlas.com.

The Bay of Biscay is included within the OSPAR Convention IV Region. Within the bay several areas of The International Council for the Exploration of the Sea (ICES) are also found, for instance: VIIIa, b, c and d2 (Devotes, 2013; OSPAR, 2000).

The Bay of Biscay presents differences in its coastal geography. In this context, the west coast of France is mostly straight until the Basque coast, being only interrupted by

the presence of two estuaries, Loire and the Gironde Estuaries (Lavin et al., 2012). A major change on coastal morphology occurs between the Aquitanian and Basque coasts as this last one is very mountainous and jagged, with the presence of numerous smaller rivers (OSPAR, 2000). This more irregular shape in the southern part continues through the Spanish coast until finally at the west the Galician “Rías” appear (Lavin et al., 2012).

One of the main characteristics of the bay relies on its oceanography, as it contains three different shelves: Armorican, Aquitanian and Cantabrian shelf. The continental shelf on the south of the bay is very narrow (7-20 km), however it gets wider along the French coast and finally at the northern part of the Bay of Biscay it reaches a width of 60 to 200 km (Costoya et al., 2015; deCastro et al., 2009). On the other hand, the bay is considered as a deep sea, with maximum depth values of 5000 m (Devotes, 2013). Water circulation at the oceanic part of the bay is characterised by being anticyclonic and weak (1-2 cm/s) (Costoya et al., 2015; deCastro et al., 2009; OSPAR, 2000). Conversely, at the continental shelf water circulation is seasonally dependant because of the influence of semidiurnal tide cycles (tidal amplitude is 3-6 m), winds and density gradients (Costoya et al., 2015; deCastro et al., 2009; Rubio et al., 2013). These density gradients are caused by freshwater inputs into the ocean and are mainly observed in the surrounding areas of estuaries (Rubio et al., 2013). The main freshwater inputs into the bay come from the following rivers: Vilaine, Loire, Gironde and Adour. It should be mentioned that the Loire and Gironde Estuaries both together with an annual mean discharge of 900 m³/s, contribute almost 80% of the total freshwater inputs into the bay (Lavin et al., 2012; Costoya et al., 2015). On the other hand, due to the proximity of mountains to the ocean in the Cantabrian Coast (northern Spain coast), rivers are very small and their total freshwater input into the ocean is very

low in comparison with the Loire and Gironde Estuaries. The main estuaries in the Cantabrian coast are: Bidasoa, Ibaizabal, Navia, Nalón and Eo (Lavin et al., 2012).

In the Bay of Biscay, several antropogenic pressures of different nature are present. along the coasts of the bay several cities have historically settled up including: Brest, Nantes, La Rochelle, Bordeaux, Biarritz, Donostia-San Sebastián, Bilbao, Santander and Gijón. Among them Nantes, Bordeaux and Bilbao have the peculiarity of being next to the main estuaries of the bay: Loire, Gironde and Ibaizabal, respectively, which are considered as some of the most human impacted estuaries of the bay. On the other hand, the harbours of Nantes, Bordeaux, Bilbao and Gijón present a key role in Atlantic marine transport (Devotes, 2013; OSPAR, 2000). The last report of the OSPAR commission has identified several human impacts on the bay, among them, the most relevant are: tourism, agriculture, fishing, aquaculture (especially in Galicia and some areas of France), sand and gravel extraction, dredging activities, coastal pollutant discharges and maritime transport. In this context, anomalous levels of pollutants occur in some parts of the bay. The presence of metals in the Bay of Biscay has been classified in the “other important issues” category by the OSPAR commission for Region IV.

1.1 Ibaizabal Estuary

The Ibaizabal Estuary is located on the Basque coast (Northern Spanish coast), more precisely in the province of Biscay (43-23'-43°14'N, 3°07'-2°55'W). The estuary has a length of ~23 km, being the largest of the Basque Country and it is considered a small macro-mesotidal system (Borja et al., 2004; Cajaraville et al., 2016). Although it was the most extensive estuarine area in the Cantabrian coast, the estuary has been transformed during the last 150 years by urban, industrial and port activities (Cearreta et al., 2004). As a consequence, the estuary conserves the 68.7% of its original surface

(100% of subtidal surface and 0% of intertidal surface is maintained) (Rivas and Cendrero, 1992).

Nowadays, two clear zones can be distinguished in the estuary: a first channelised zone between the tidal limit and the mouth of the estuary (~15.2 km) and a second zone that is formed by a funnel shaped embayment called “Abra of Bilbao”. Here the port of Bilbao is located and its last dock acts as the end of the estuary (in a line that extends from Punta Galea to the outset dock of the port in Punta Lucero) (Borja et al., 2004; Cajaraville et al., 2016). The width of the estuary ranges from 25 to 270 m in the inner part of the estuary while maximum width occurs in the Abra with 3.8 km. The depth of the estuary also changes along the river, thus, minimum depth values are found at the head of the estuary (0.5 m) while at the outer part, the estuary reaches a maximum depth of 32 m, being the deepest one in the Basque Country. However, this depth can vary significantly depending on the location (Borja et al., 2004; Cajaraville et al., 2016). Moreover, the tidal amplitude in the Abra can vary from 4.6 m (in spring tides) to 1.2 m (in neap tides). Overall the basin of the estuary has a surface of 1755 km² and a mean river flow of 3.6 m³s⁻¹ (Valencia et al., 2004). Major freshwater inputs in the estuary originate from the Nerbioi-Ibaizabal River (68%) and other tributary rivers (Kadagua 27%, Galindo 4%, Asua 0.7% and Gobela 0.3%) (Gredilla et al., 2013). Most of the estuary is euhaline (>30) due to the relative low river flow compared with the estuary water volume, and the estuary is characterised by strong stratification of the waters, especially in the channelised zone (Iriarte et al., 2010) (Fig. 2).

As mentioned above, the estuary has been dramatically modified in the last 150 years by urban, industrial and port settlement. Major physical changes were caused by making the estuary navigable directly from Bilbao to the open sea, while chemical pollution of anthropogenic origin started in 1854 with the implementation of the first iron and steel industry. The following industrial development and the consequent impact

(including mineral sluicing, industrial wastes and urban effluents) ended up into the environmental collapse of the estuary by the mid XXth century (Cearreta et al., 2004; Gredilla et al., 2013). Thus, water and sediments presented low concentrations of dissolved oxygen and high concentrations of metals and organic matter (Cearreta et al., 2004). Although during last decades the environmental status of the Ibaizabal Estuary has greatly ameliorated due to the decline of industrial activity (including closure of major pollutant sources), the improvement of waste water-treatment systems and the implementation of new environmental policies (Cearreta et al., 2004; Gredilla et al., 2013), metals are still present in sediments and biota, being the estuary regarded as chronically polluted (Cajaraville et al., 2016).

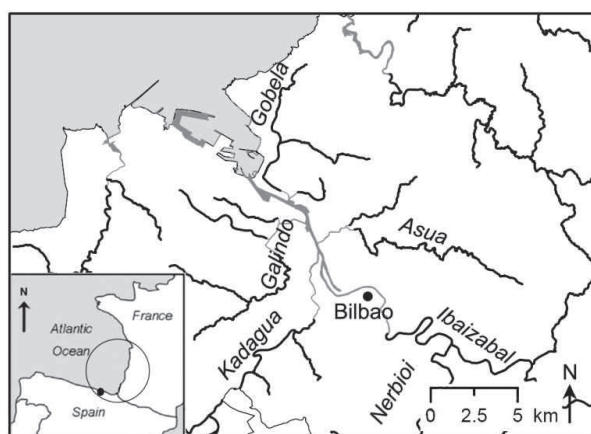


Figure 2: Ibaizabal Estuary and the main tributary rivers.

The main pressures on the estuary as identified by the Basque Government were the high industrial development, the port activity (with 5 ports within the Abra) and the high population of the area (Borja et al., 2004). Since 2004, the most noticeable pressures that have occurred in the estuary have been the construction of an outer dock, some new industrial discharges in the outer part of the estuary and an important dredging carried out in the mouth of the estuary (Borja et al., 2014). Overall, the last annual report from the Basque Government indicated that neither the outer nor the inner part of the estuary achieved good environmental status (Borja et al., 2014). In particular,

metal concentration appeared to be higher in the inner part of the estuary but they did not exceed the limit values established by the Basque Government in any case (Borja et al., 2014).

1.2 Oka Estuary

The Oka Estuary is located in the South-Eastern Bay of Biscay (43°22'N, 2°40'W) in the Basque Coast, within northern Spanish coast (Sarobe, 2009) and inside the so-called Urdaibai Biosphere Reserve declared by UNESCO in 1984.

The main tributary river, the Oka, gives the name to the estuary and crosses the region of Busturialdea which is composed by 20 towns with 45.705 inhabitants (Raposo et al., 2009; Eustat 2015), where Gernika (16.493 inhabitants) and Bermeo (16.752 inhabitants) are the most populated villages. The main economical activities from the area are: agriculture, industry (particularly food, metallurgy and forest industry) and tourism.

The Oka Estuary it is a drowned valley type estuary (Monge-Ganuzas et al., 2013) that covers a length of 12.5 km from the tidal limit point located in Gernika to the lower boundary of the estuary in Mundaka. At this last point, the estuary also reaches its maximum width of 1.2 km (Villate et al., 1989). Moreover, the extension of the estuary is estimated to 1.89 km² (Villate et al., 1989). Considered as a meso-macrotidal type estuary with semidiurnal tides (Monge Ganuzas., 2013), the mean tidal range is 2.5 m. The maximum variation of this tidal range occurs on spring tides (4.5 m) while during neap tides variation falls to minimum values (1.5 m) (Monge-Ganuzas et al., 2013). Regarding the salinity gradient of the estuary, at the inner part of the estuary fresh and salt waters appear well mixed during high precipitation periods but in low hydrological regimes this water mixture becomes partial. Finally, at the mouth of the estuary waters are always well mixed because of tidal influence (Raposo et al., 2009) (Fig. 3).

The depth of the Oka Estuary varies according to the tide and zone ranging from 0 to 10 m (Borja et al., 2004). The inner channel presents a maximum depth of only 3 m. However, this depth is periodically augmented due to the periodical dredging and dumping activities carried out by the shipyard company “Astilleros Murueta” established in 1943 in Murueta on the left bank of the river (Monge-Ganuzas et al., 2013; Rodriguez-Iruretagoiena et al., 2016). This is the main human activity (that has occurred) located inside the estuary during the last 50 years (Monge-Ganuzas et al., 2013). The estuary conserves the 71.2% of its original surface, more precisely 30.1% of the original subtidal surface and the 69.9% of the original intertidal surface are conserved (Rivas and Cendrero, 1992). These data reveal that the antropogenic pressure suffered by this estuary has been lower than in other Basque estuaries (Borja et al., 2004).

Although considered as a reference estuary in many scientific studies because of the relatively low concentrations of organic and heavy metal pollutants measured in bivalves (Lekube et al., 2013; Ortiz-Zarragoitia and Cajaraville, 2010), the presence of other human activities in the estuary apart from dredging activities cannot be excluded. Industrial and tourism activities are considered among the potential sources of pollution (Rodriguez-Iruretagoiena et al., 2016). Industrial activities are mainly located in the inner part of the estuary in Gernika (Ortiz-Zarragoitia and Cajaraville, 2010; Raposo et al., 2009) whereas villages near the seashore such as Mundaka, Sukarrieta and Ibarrangelu concentrate tourist activities, including two marinas and a port (Rodriguez-Iruretagoiena et al., 2016). Moreover in 2004, the Basque Government identified the following main threats for the estuary (Borja et al., 2004):

1. Inputs of non treated sewage waters (accidental and irregular ones).
2. Inefficient functioning of the waste water treatment plant in Gernika.
3. Industrial discharges.
4. The presence of periodical dredging activities.
5. The presence of the alien specie *Baccharis halimifolia*.

Indeed, the last report from the Basque Government for transitional and coastal waters established an overall not good environmental health status for the estuary because of the inappropriate functioning of the waste water treatment plant and industrial discharges. However, heavy metals concentrations did not present especially high concentrations (Borja et al., 2014). It should also be mentioned that in the last decade, an unusually high number of intersex individuals for mussels and fish has been reported, in agreement with the high concentrations of endocrine disruptors found in water (Bizarro et al., 2014; Puy-Azurmendi et al., 2013). In this context, a recent study carried out in the Oka Estuary has concluded that this estuary nowadays should not be considered as a pristine area (De los Ríos et al., 2016).

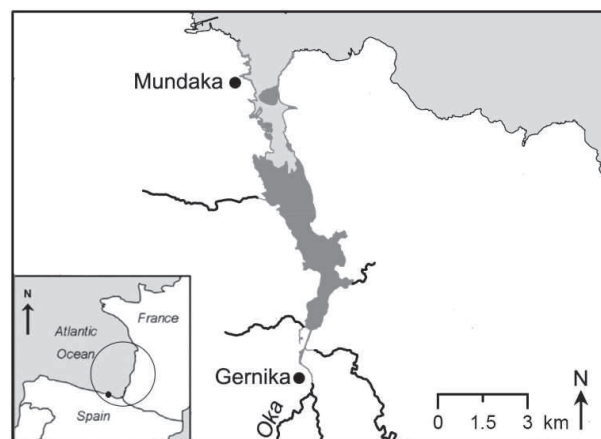


Figure 3: Oka Estuary and main tributary rivers.

1.3 Gironde Estuary

The Gironde Estuary is located in the South-Western coast of France (45° 16' 1" N, 0° 42' 39" W), thus, on the Aquitanian coast of the Bay of Biscay. It is the largest estuary in South West Europe and it has also been widely studied by the scientific community. Indeed, it is considered as a model estuary for geochemical studies of trace element transport and reactivity (Lanceleur et al., 2011b).

It is a coastal plain type estuary (Salomon, 2002) of ~170 km long and covers at high tide a total surface area of 635 km² (at low tide 450 km²) (Salomon, 2002) draining a watershed of around 80 000 km². It is regarded as a hypersynchronous meso-macrotidal type estuary (5.5 m in spring tide, increases towards upper estuary) (Salomon, 2002; Sottolichio and Castaing, 1999) with a semidiurnal tidal cycle (period of 12.5 h). Due to its funnel shape, the seawater volume that enters the estuary can be 40 or 80 times the volume introduced by rivers (Lanceleur, 2011).

Three main contributory rivers are present in this estuarine system: the Garonne (length=647 km; Q=594 m³/s), Dordogne (length=490 km; Q=318 m³/s) and Isle Rivers (length=255 km; Q=64 m³/s) (which drain on of Europe's least industrialised/urbanised regions with only two major agglomerations: Bordeaux and Toulouse (each one with approximately 1 million inhabitants) (Deycard et al., 2014; Schäfer et al., 2002). The main basin of the estuary is divided into 3 main sub-bassins: Garonne (51 500 km²), Dordogne (15 000 km²) and Isle (7000 km²) (Schäfer et al., 2002). The geographical limit of the estuary is located at the confluence of the Garonne and Dordogne Rivers but tidal influence can be detected almost 180 km upstream from the estuary mouth at La Réole (Garonne River), Pessac sur Dordogne (Dordogne River) and at Guîtres (Isle River) (Lanceleur, 2011). The depth of the estuary varies between 10 and 30 m depending on the zone (Lanceleur, 2011) (Fig. 4).

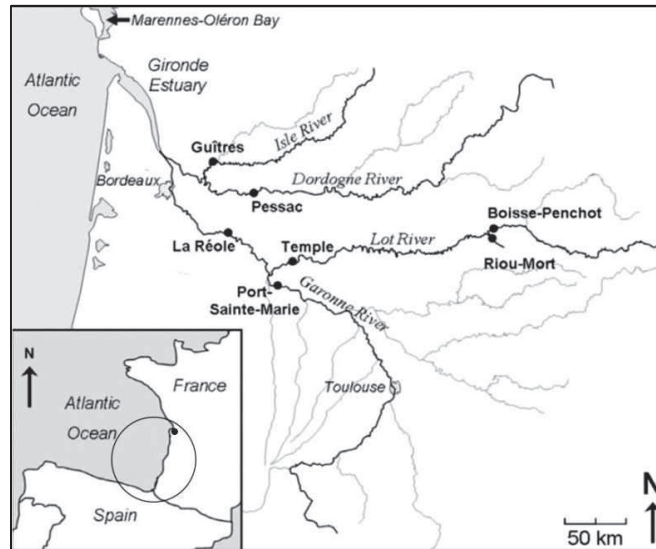


Figure 4: Gironde Estuary and its tributary rivers adapted from Lancelleur et al., 2011a.

The mean annual freshwater flux into the estuary is $27 \text{ km}^3/\text{year}$ (1990-2007) (Dabrin et al, 2009). The hydrological regime varies along the year, during floods river flow can reach $5000 \text{ m}^3/\text{s}$ whereas in dry periods it can be as low as $200 \text{ m}^3/\text{s}$ (Castaing and Jouanneau, 1979). Water residence time is highly influenced by the hydrological regime, in dry periods it can be estimated to be 86 days while in high rainfall periods it can decrease to 20 days (Castaing and Jouanneau, 1979).

The seasonal hydrological regime also influences other important parameters of the estuary such as the salinity gradient which depends among others on: sea water and fresh water mixture, tidal cycle, tidal coefficient and river flow. In general the estuary is regarded as being partially mixed, however 3 types of salinity gradients occur: longitudinal salinity gradient (increases towards the ocean with maximum values at high tide and low river flow), transversal salinity gradient (left banks of the river have higher salinity values because of Coriolis force) and a vertical gradient that implies a low mixture of fresh and sea water (stratification, only appears during floods or neap tides) (Lancelleur, 2011).

One of the main characteristics of the estuary is the presence of a Maximum Turbidity Zone (MTZ) known as “Bouchon Vaseux”. The presence of high turbidity zones near sea water and freshwater confluence is typical for macrotidal estuaries and are characterised by the presence of high concentrations of Suspended Particulate Matter (SPM). In the case of the Gironde Estuary, the SPM concentration at MTZ can be higher than 1 g/L and the average particle residence time is 1-2 years (Sottolichio and Castaing, 1999). As the MTZ generally is in the low salinity region, it moves through the estuary up and down depending on hydrological regime (with marked seasonality) and tides (Sottolichio and Castaing, 1999). In fact, during drought periods, the MTZ can move upstream and can reach the city of Bordeaux (Sottolichio and Castaing, 1999), while under high river discharges and high tidal coefficient, the MTZ can almost be flushed out of the estuary (Castaing and Allen, 1981). This MTZ plays a key role in metal speciation as it will be explained later in the case of Cu. On the other hand, the fluvial (upstream) estuary seasonally suffers from hypoxic situation with dissolved oxygen concentrations next to 2 mg/L and 7 day-long hypoxic events (Lanoux et al., 2013).

Although being considered as one of the morphologically best preserved estuaries in Europe, this system is also very well known for important historical polymetallic pollution (Cd, Zn, Cu, Pb, Hg and Ba) mainly originating from the former mining and ore-treatment activities carried out in Decazeville in the Riou-Mort watershed (Masson et al., 2011; Schäfer et al., 2002). The factory was installed in 1871 and for more than one century, main activities were focused on Zn extraction and purification, producing 3 kg of cadmium (Cd) per obtained tonne of zinc (Zn) (Strady, 2010). This Cd release into the Lot and Garonne Rivers and the Gironde Estuary, has caused the interdiction of oysters production after the detection of extremely high Cd values in wild oysters

from the estuary in 1979 (Baudrimont et al., 2016; Lanceleur et al., 2011a; Mikolaczyk et al., 2016; Salles et al., 2013).

However, a significant decrease in Cd concentration in oysters inhabiting the mouth of Gironde Estuary has been recorded during the three past decades, mainly reflected by the cessation of Zn production in 1987 and the implementation of a remediation plan in the estuary (2007) (Lanceleur et al., 2011a). Indeed, recent studies have suggested the possibility to harvest again oyster spats in the salt marshes of the Gironde Estuary, since the accumulated Cd, Pb and Hg values were below human safety consumption levels (Baudrimont et al., 2016).

Finally, it should be mentioned that as a consequence of these metal inputs into the estuary, important stocks of polluted sediments have accumulated, particularly in the hydroelectric reservoirs located along the Lot River (Lanceleur et al., 2011b). Hence, these elements could constitute an important source of metallic pollution in the case of their remobilisation (caused by: construction works, inundations or dredging activities...), ending up again in the estuary (Coynel et al., 2007; Lanceleur et al., 2011b).

2. Metal pollution in Estuaries

For many centuries, aquatic environments all over the world have suffered from different anthropogenic pressures, leading to an increased presence of pollutants in estuaries (Millward and Turner, 2001). Estuaries have traditionally been subjected to intense human activity, and main human settlements have occurred along estuaries, since these environments have provided food sources for humans and allowed communication and commerce activities through navigation. However, estuaries have also been main recipients of waste waters, leading to relevant deterioration of their ecological functions. Increased release of pollutants into the environment, has led to a

situation, where estuaries are among the most metal polluted environments (de Souza Machado et al., 2016). Accordingly, the scientific community has paid special attention to this issue, with more than 5000 scientific articles on pollution in estuaries published between 1991-2010, many of them focusing on metal pollution (Sun et al., 2012).

Metallic pollutants are of special interest since some of them are essential for many biological/biochemical processes whereas others are highly toxic elements for aquatic biota. Furthermore, some of them can be bioaccumulated and magnified through the food chain, hence metals may become a potential threat for both human and environmental health (Zhou et al., 2008). In this sense, the European Pollutant Release and Transfer Register (E-PRTR, 2013) has included trace metals in their key list of pollutants (Jimeno, 2014). The increased awareness of metal pollution in developed countries, together with (i) the implementation of new policies and regulations and (ii) the decline of major source industries has led into a general decrease of metal concentrations in European estuaries. However, the presence of metals in some estuaries is still high enough to cause deleterious effects on the biota (Lanceleur et al., 2011a; Leorri et al., 2008).

Sources of metals in aquatic environments include both natural and anthropogenic origins. Among natural sources the soil/rock leaching to water, wind-blown dust, volcanic activity and forest fires contribute to metal inputs (Schindler et al., 1991; Zhou et al., 2008). Anthropogenic metal inputs into estuaries are mainly related to industrial activities (sewage waters and effluents from: mining, mill run, metallurgy, plating, chemical plants, paper industry...) and agricultural activities (Zhou et al., 2008), but urban inputs (from urban surface runoff, urban sewage inputs and harbours) should also be taken into account. Once metals are released into the environment, they will arrive to estuaries and the coastal zones through river inputs and atmospheric deposition (de Souza Machado et al., 2016). According to the review by de Souza

Machado et al., (2016) metals in estuaries undergo several complex physico-chemical processes (Fig. 5) that can affect their bioavailability and hence their toxicity and/or effects caused on the biota.

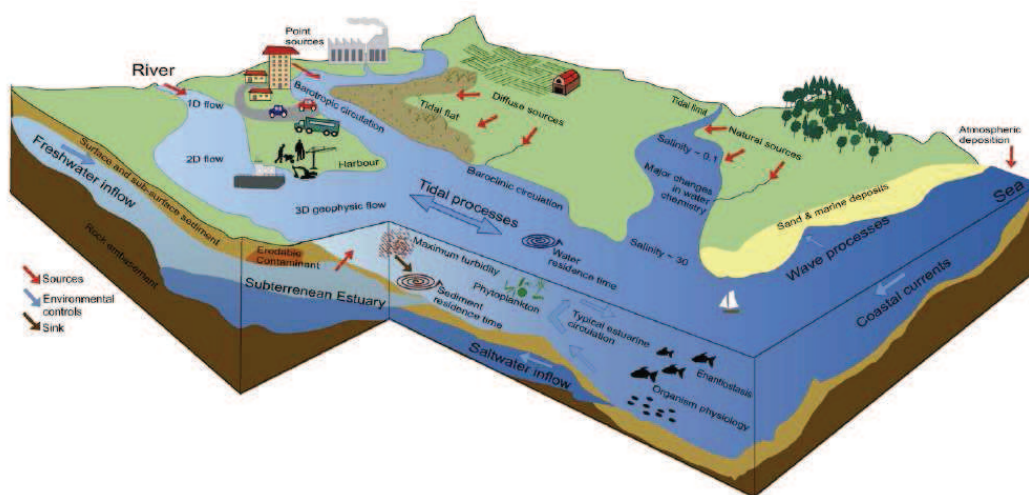


Figure 5: Conceptual model physical, chemical and biological variables and processes for behavior and toxicity of metals adapted from Bianchi (2007) by de Souza Machado et al., (2016).

Regarding the metals that may interact with organisms inhabiting estuaries, copper (Cu) and silver (Ag) are regarded as some of the most toxic elements for aquatic biota. Once these elements enter into the estuary, they are dissolved and present an additive behaviour. In the case of Cu estuarine dissolution can occur because of organic matter degradation (Masson et al., 2011; Petit et al., 2013). In the case of Ag, the salinity gradient drives Ag desorption from the particulate phase due to chloride complexation (Lanceleur et al., 2011b).

Aquatic organisms possess several strategies to face metal exposure which may vary according to the metal element but also according to the organism facing metal pollution. Therefore different mechanisms exist for the uptake, storage and excretion of metals in aquatic biota (Marigómez et al., 2002). In general, once metals are inside organisms, they can interact with proteins such as metallothioneins, metalloproteins or

metal chaperons (Deeds et al., 1999; Amiard et al., 2006; Kozłowski et al., 2013) and be transferred to specific organelles (for instance the lysosomes of digestive gland cells; in the case of molluscs) for their detoxification and subsequent excretion or to be bioaccumulated in specific compartments (Marigómez et al., 2002).

Since the present work is focused on the effects exerted by Cu and Ag in oysters, a more detailed description of possible pollution sources for these elements are described below.

2.1 Copper

Copper (Cu) has been a pivotal element during the history of humanity, since it was the first metal used by humans. Indeed, the discovery of Bronze (an alloy between Cu and tin) was so important for humanity that this period was called the Bronze Age. Nowadays, this element is mainly used in wiring and motors due to its excellent properties as heat and electricity conductor.

Copper generally occurs in mineral form such as chalcopyrite (CuFeS_2) and bornite (Cu_5FeS_4), although it can also be found as covellite (CuS), chalcocite (Cu_2S) or cuprite (Cu_2O). Therefore, Cu is extracted from these ores and minerals, being Chile, Peru and China the major countries producing this element (Royal Society of Chemistry, 2016).

Although Cu is an essential element for living organisms, it can become toxic once certain values are exceeded particularly in aquatic animals (Lee et al., 2010; Levy et al., 2007; Raftopoulou and Dimitriadis, 2011). Copper is a transition metal that appears as co-factor in proteins such as hemocyanin among others (Santovito et al., 2015). However, when Cu overexposure occurs deleterious effects on biota are induced including: the formation of reactive oxygen species (ROS), lipid peroxidation, enzymatic inactivation and even changes in the structure of DNA (Santovito et al., 2015).

Apart from mining, ore extraction and industry, major Cu sources to estuaries are (i) erosion of contaminated agricultural soil (CuSO_4 is the component of many fungicides) (Masson et al., 2011) and (ii) the use of antifouling paints on vessels to prevent the attachment of aquatic organisms (Gamain et al., 2016). In the marine environment, Cu undergoes several biogeochemical processes that change the speciation of the element (Petit et al., 2013). Because, Cu, Cu^{2+} , Cu^+ and $\text{Cu}(\text{OH})^+$ are considered the most toxic species (Lee et al., 2010) the main speciation process affecting Cu in terms of toxicology is the organic complexation, which binds Cu to dissolved organic matter and reduces the toxicity (Money et al., 2011). In estuarine environments, a decrease in the toxicity of Cu is found with increasing salinities, due to the increasing concentration of sodium (Na) ions that directly compete with the most toxic ionic forms of Cu for binding sites (Lee et al., 2010).

2.2 Silver

Silver (Ag) like Cu has also been employed by humans since ancient times: Ag mining started by Greeks around 3000 BC (Royal Society of Chemistry, 2016).

Although the natural occurrence of the element is rare (Howe and Dobson, 2002), it is usually present in combination with other elements in sulphide ores as argentite (Ag_2S) or halogenides chlorargyrite (AgCl). Silver is mainly extracted from Cu, gold (Au) and nickel (Ni) ores. The world production of this metal is around 20 000 tonnes per year (Royal Society of Chemistry, 2016).

It should be noted that Ag is regarded as one of the most toxic elements for aquatic biota (Ratte, 1999; Tappin et al., 2010). Silver pollution sources in the environment have been related to its use by humans since ancient times. Pollution sources for this element have changed through history, particularly in the last century. Historically, main pollution sources were metallurgy and mining industry but, during the last decades of

XXth century, other industrial activities (jewellery, electronic, photographic) together with hospitals disposals have become the main pollution sources for this metal (Barriada et al., 2007; Lanceleur et al., 2013, 2011a; Tappin et al., 2010). Moreover, Ag release into the environment was also produced by urban run-off, sewage treatment plants and because of the use of cloud seeding devices which released AgI into the atmosphere to prevent hails storms (Lanceleur et al., 2011b). Currently, new technical applications and forms applied (nano-materials), usually employed due to bactericidal properties, have made Ag an emerging metallic contaminant (Fabrega et al., 2011; Royal Society of Chemistry, 2016).

Like Cu, when Ag enters into the aquatic environment it also undergoes several speciation process, mainly influenced by changes in salinity enhancing Ag desorption from the particulate phase by chloride complexation (Lanceleur et al., 2011b). In this context, Ag accumulation in organisms depends on the speciation (Tappin et al., 2010), and the AgCl⁰ lipophilic molecules are the ones that enter easily into organisms. (Barriada et al., 2007; Tappin et al., 2010). However, Ag⁺ is regarded as the most toxic form of Ag and it belongs to the highest toxicity class elements (Lanceleur et al., 2011a; Ratte, 1999).

3. Bivalves as sentinel organisms

Bivalves have been proposed as ideal surrogates to assess the environmental health status of aquatic ecosystems, because they can accumulate pollutants with little metabolic transformation and reflect their deleterious effects on the environment (Farrington et al., 2016; Goldberg., 1986; Ortiz-Zarragoitia and Cajaraville, 2010).

Since in 1976 the Mussel Watch biomonitoring programs was implemented in the USA, bivalves have been employed as sentinel organisms in several biomonitoring programs around the world such as in the Oslo Paris (OSPAR) convention (OSPAR, 2005).

These programs rely on the ability of bivalves to accumulate and concentrate pollutants in their tissues (Phillips and Rainbow 1993; Rainbow 1995). Moreover, bivalves are the most used sentinel organisms over the world because they also present the following advantages (Farrington, 1983) recently reviewed (Farrington et al., 2016):

1. They have a cosmopolite distribution; same or similar species are widely distributed. Moreover, as they do not show significant differences in terms of metabolic activity, it is possible to make comparisons among them.
2. They are sedentary and hence they can represent one location. Moreover they are filter feeders, thus they have to face environmental stressors including pollutants developing pertinent biological adaptations. Therefore, pollutant concentrations in their tissues can reflect the pollution levels of the ecosystem where they inhabit.
3. Populations are big and locally stable. Moreover, these animals are present during the whole year, allowing repeated samplings.
4. They are resistant to pollutant presence, but at the same time they are sensitive to them.
5. They do not have a high capability to metabolise xenobiotic compounds, thus they tend to accumulate them.
6. The analysis of pollutant compounds in sentinel species is easier than directly collecting sediment and water samples since these animals can accumulate pollutant compounds by factors of 100 to 100 000 and also over longer periods.
7. Bivalve species (for instance mussels and oysters) can be translocated to other locations and be used for monitoring/assessments purposes.
8. Some bivalves are of interest for sea food industry, being commercially important.

However, the use of sentinel species also presents the following constraints that need to be taken into account (Farrington et al., 2016):

1. Some chemicals of environmental concern are soluble in seawater or are not sufficiently bioconcentrated by sentinel organisms.
2. The differences on bioconcentrations of some chemical compounds between species are not yet well documented and comparisons between them are difficult.
3. The chemical measurements in bivalves' tissues and their relationship with biological effect measurements in sentinel organisms are not easy to extrapolate to the health assessment of the entire ecosystem.
4. The accumulated contaminant body burdens in bivalves cannot be extrapolated to other organisms. Moreover, the assessment of bivalve consumption risk for human health must be done with great care.

Despite the above mentioned limitations and constraints, mussels and oysters have been widely employed in biomonitoring programmes. As an example, three of the main monitoring programs that are carried out in the Bay of Biscay rely on the use of these organisms. In the northern Spanish coast the Spanish Monitoring program is carried out since 1991 by the Spanish Institute of Oceanography (IEO) based on the periodical (every 5 years) collection of mussels *Mytilus galloprovincialis* for subsequent chemical measurements (Besada et al., 2014). More specifically, in the Spanish Basque coast, another biomonitoring program is carried out since 1990 by the Department of Environment, Territorial Planning, Agriculture and Fishing of the Basque Government and the Basque Water Agency (URA). This program mainly collects mussels and occasionally oysters, for their accumulated metal and PAH content analysis (Borja et al., 2014; Solaun et al., 2013). Finally, along the French Atlantic and Mediterranean coasts, a biomonitoring programme has been carried out since 1979 by the National

Network for the Observation of Marine Environment Quality (RNO/ROCCH; i.e the French Mussel-Watch Program) which is based on the collection of mussels and oysters for metal and PAH measurements among others (Lanceleur et al., 2011b).

3.1 Chemical measurements: Stable isotopes application in ecotoxicology

Metal accumulation in bivalves and the effects exerted after metal exposure have largely been studied in biomonitoring programs and ecotoxicological studies (Díez, 1996; Farrington et al., 2016; Strady et al., 2011; Xie et al., 2016; Zorita et al., 2006). In particular, the accumulation and effects produced in oysters after Ag and Cu exposure has also been a research subject (Amiard-Triquet et al., 1991; Géret et al., 2002), although it should be noted that classical ecotoxicological studies required in many cases relatively high pollutant exposure concentrations, i.e. many orders of magnitude higher than their environmentally realistic concentrations. Such high exposure concentrations were necessary to assure both the detection of biological responses and also to quantify pollutant accumulation in the tissues of sentinel species (Strady et al., 2011). In this context, Strady et al., (2011) have developed a technique based on stable isotopic spikes which allowed tracing metal bioaccumulation in bivalves simultaneously for different Cd isotopes in seawater and algae at environmentally realistic exposure levels. This technique using Cd stable isotope spikes has revealed to be a powerful tool for the detection and quantification of metal accumulation in oysters (Mikolaczyk et al., 2016).

Different isotopes occur for most elements naturally, each isotope differs among them in the number of neutrons they have and thus in their atomic mass. Stable isotopes are those isotopes that do not decay into other elements through time. In the case of Ag and Cu, both elements present 2 isotopes, each. In the case of Ag they are: ^{107}Ag

(51.839% of abundance) and ^{109}Ag (48.161% of abundance). For Cu they are: ^{63}Cu (69.17% of abundance) and ^{65}Cu (30.83% of abundance) (IUPAC, 1997).

Therefore, as the abundance ratios between isotopes is constant, the technique developed by Strady et al., (2011) allows metal accumulation measurements in animals based on the change of these ratios after spiking one of the stable isotopes into the experimental units.

3.2 The Cu and Ag interactions in oysters

In oysters, strong correlations between Ag and Cu accumulation have been previously reported (Daskalakis, 1996; Giltrap et al., 2013). Although the cause of this relationship remains unclear, it has been attributed to the bound of Cu and other metals into haemocytes (Roesijadi, 1996).

Moreover, under experimental conditions synergistic and antagonistic effects in the accumulation of both metals (Ag and Cu) have been detected. In fact, Ag accumulation in oysters increases under Cu exposure whereas Cu accumulation is reduced under Ag exposure. Accordingly, Cu produces a synergic effect on Ag accumulation and Ag has an antagonistic effect on Cu accumulation (Amiard et al., 2004; Ettajani et al., 1992; Mikolaczyk et al., 2016).

Recently, Mikolaczyk (2016) has reported a relative constant Cu/Ag accumulation ratio in wild oysters coming from different estuaries along the French Atlantic coast. Indeed, the use of the Cu/Ag ratio appears as an additional tool to be used in environmental water quality programs, since it can add information on the temporal and spatial pollution trends (Mikolaczyk, 2016).

3.3 Biomarkers

Living organisms tend to develop abnormal biological responses when they are subjected to stress conditions such as pollutant exposure. These responses occur at different levels, among them, there are some responses that occur at sub-individual level or that are reflected in the products that the organism produces (Martín-Díaz et al., 2004). The biomarker concept is based on the measurement of these changes (Van Gestel and Van Brummelen, 1996). Thus biomarkers are defined as the measurements of body fluids, cells, or tissues at cellular, biochemical and molecular levels that indicate the presence of pollutants (exposure biomarkers) or the magnitude of the organism response (effect biomarkers) (McCarthy and Shugart, 1990).

Measurements of biomarkers at molecular or cell level have been proposed as early warning tools in order to assess the biological impact in environmental health assessments (McCarthy and Shugart, 1990), since biomarkers help predicting future changes that can occur at higher biological levels (population, community, ecosystem) (Cajaraville et al., 2000).

During the last decades there has been an increasing tendency for using biomarker application together with physic-chemical measurements (Au, 2004). In fact, it is nowadays generally accepted that in order to perform accurate environmental health assessments, both biological and chemical data are necessary, since chemical analysis alone cannot give any information on the impact that pollutants may exert on the biota. Thus, measurement of biological impacts has become crucial for environmental health assessments (Bayne, 1989; Gray, 1992; Vethaak et al., 2017).

3.3.1 Metal exposure biomarkers

Metal exposure biomarkers are those biomarkers that are able to reveal that an organism has been exposed to metallic pollutants. Among them, induction of

metallothioneins (MTs) and measurements of Black Silver Deposites (BSDs) through autometallography have been used in the present study.

3.3.1.1 Metallothioneins

The synthesis of metallothioneins (MTs) is considered as one of the main reactions that can occur after metal exposure and thus it is recognised as a biomarker for metal exposure in bivalves (Le et al., 2016; Marigómez et al., 2013b; Viarengo et al., 1997). This biomarker has been included in the *Action Plan of the United Nations Environment Program* (UNEP) (Amiard et al., 2006; Le et al., 2016).

Metallothioneins are cysteine rich non enzymatic proteins (Amiard et al., 2006; Giltrap et al., 2013; Viarengo et al., 1997) which are able to bind to metals (Ag, Cd, Co, Cu, Hg, Ni, Pb, Pd and Zn) (Le et al., 2016) and thus participate in their regulation and detoxification (Roesijadi, 1996; Viarengo et al., 1997; Zorita et al., 2005). Indeed, this metal binding capacity of MTs is caused by the thiol groups (-SH) which are cysteine residues (Amiard et al., 2006). Thus, MTs take part in the detoxification of excessive metal concentrations reducing the amounts of metals in the cells (Roesijadi., 1992; 1996) through their binding and subsequent transport into lysosomes for their storage and posterior degradation in this organelle (Viarengo and Nott,1993). Moreover MTs are known to participate in other biological processes such as cell protection against oxidative stress (Le et al., 2016) or taking part in the homeostatic control of metals, particularly essential metals (Cu and Zn) to be further used in other metabolic activities (Roesijadi, 1996; Viarengo and Nott, 1993).

Metallothioneins are cytosolic proteins and they are usually present in this compartment, although they can also occur in the nucleus of the cell or in lysosomes. During the present study, the digestive gland has been chosen as target organ for MTs content determination because of the involvement of this organ in: digestion,

detoxification and accumulation processes (David et al., 2012). Moreover this organ is known to be a major site for MT accumulation (Amiard et al., 2006; Strady et al., 2011) and it has been widely employed as a target tissue for the use of MT as metal exposure biomarker (Le et al., 2016).

3.3.1.2 Intralysosomal metal accumulation

The intralysosomal metal accumulation in the digestive epithelium of molluscs has been widely used as metal exposure biomarker through the quantification of the Black Silver Deposits (BSDs) after application of the autometallography (Marigómez et al., 2002; Soto and Marigómez, 1997). The autometallography (AMG) is a histochemical technique which allows the detection of ionic metals that are not strongly attached to proteins, as BSD form (Marigómez et al., 2002; Soto et al., 2002). This technique is based in the autoinduced amplification of Ag, which applying the basic principles of photography, allows the amplification of the atoms and molecules present in biological samples (Danscher, 1984).

Due to its high sensitivity, although not metal specific, autometallography has become an extendedly used technique in field and laboratory experiments for detecting metals such as Cu, Zn, Hg and Cd in mollusc tissues through light microscopy (Marin et al., 2006; Raftopoulou and Dimitriadis, 2011; Rementeria et al., 2016; Rodríguez-Iruretagoiena et al., 2016; Soto et al., 2002). These elements were amplified by Ag ion amplification and finally they were deposited as BSD, which are detectable under light microscope and measurable through image analysis systems.

3.3.2 Effect biomarkers

3.3.2.1 Lipofuscin accumulation

Lipofuscins are lipopigments produced after lipid peroxidation, when polyunsaturated fatty acids are damaged by free radical/reactive oxygen species (Au, 2004). These

lipofuscins accumulate in lysosomes after pollutant exposure or other stresses (Marigómez et al., 2013b) and are able to bind cations such as: Cu, Zn, Fe, Mg and Mn (Zorita et al., 2006). Previous works have reported an enhanced presence of lipofuscins after pollutant exposure in mollusc tissues, including oysters *Crassostrea virginica* exposed to metals (Ringwood et al., 1998). The presence of lipofuscins is generally related with the impairment of the correct lysosomal functioning (Moore, 1990), it is also regarded to contribute to the decrease in the degradative capacity of the digestive system (Moore et al., 2006).

3.3.2.2 Intracellular accumulation of neutral lipids

The accumulation of intracellular neutral lipids in the lysosomes of digestive cells has been classically considered as an exposure biomarker to organic compounds, however it has also been linked to the presence of other non identified stressors (Marigómez et al., 2013b). This increase of neutral lipids in the lysosomes can also occur because of an autophagy of the excess of lipid droplets (Krishnakumar et al., 1994; Lowe, 1988; Moore et al., 1987; Moore, 1988).

Neutral lipids constitute an important storage of nutrients related to growth and reproduction (Holland, 1978). In this context, oysters are known to possess a connective tissue composed by vesicular cells that store glycogen and act as an energy reservoir for energy demanding processes (Jouaux et al., 2013; Thompson et al., 1996). During spawning months neutral lipids are known to be transferred from connective tissue to gonads (Cancio et al., 1999; Dridi et al., 2007) whereas the presence of stress factors can induce their depletion from connective tissue, altering the total energy budget of molluscs (Guerlet et al., 2006).

3.3.2.3 Tissue level biomarkers

As mentioned above, the digestive gland of bivalves has been usually selected as a target organ for the analyses of tissue level biomarkers, since this organ is known to participate in: food digestion, metabolism processes, reserve storage and in the detoxification and elimination of pollutants among others (Marigómez et al., 2002; Moore and Allen, 2002).

The digestive gland of oysters is composed by several alveolo-tubular units, with secondary ducts connected to primary ducts that finally end up into the stomach (Galtsoff 1964; Langdon and Newell., 1996). In these digestive alveolo-tubular units two main types of cells are present: digestive and basophilic cells. The first ones are in charge of the intracellular digestion of food and materials through their well developed endo-lysosomal system. Once the intracellular digestion has finished waste products are released into the lumen of the tubules as residual bodies. The second type of cells, basophilic cells, are secretory cells related with the synthesis and excretion of proteins (Langdon and Newell., 1996) that could be involved in extracellular digestion and metabolic regulation (Marigómez et al., 2002; Izagirre et al., 2009).

The digestive gland of oysters is a very plastic organ that suffers cyclic changes, even under normal environmental conditions, when bivalves have to face stress conditions these changes can be enhanced (Marigómez et al., 1990; Zaldibar, 2006). Among the observed changes, the most relevant ones are the following: reduction on the epithelium thickness that conform digestive gland tubules (atrophy), changes on proportional ratio among digestive and basophilic cells (Cajaraville et al., 1991; Marigómez et al., 2006) and the increase of connective tissue with respect to digestive tubules, because the latter reduce their amounts or shrink, being surrounded by larger amounts of connective tissue (Brooks et al., 2012, 2011; Garmendia et al., 2011b).

Digestive gland tubule atrophy is caused by several factors including nutrient scarcity or pollutant exposure. Although the atrophy in digestive gland tubules is a reversible

pathology (Kim et al., 2006), the epithelial thickness of digestive tubules has been previously used among other tools to determine mussel's health status in environmental monitoring programs (Garmendia et al., 2011b). In this context, planimetric methods have provided valuable information about mollusc health status, (Garmendia et al., 2011c). In fact, the ICES comparative report includes these measurements as valuable tools for ecosystem health assessments, concluding that the MLR/MET ratio is a more sensitive biomarker for digestive gland atrophy than MET alone (Garmendia et al., 2011c; ICES 2012).

On the other hand, the Connective to diverticula ratio (CTD ratio), corresponds to the relative proportion of connective tissue in front of digestive tubules, has been proposed as a successful biomarker in the digestive gland of molluscs exposed to different stress sources including PAHs or metals (Brooks et al., 2012, 2011; Garmendia et al., 2011b; Múgica et al., 2015).

3.3.2.4 Histopathology

Histopathological changes have been previously used (including bivalves) in order to assess the individual health status as a sensitive and reliable indicator in several studies (Bignell et al., 2011; Garmendia et al., 2011a).

In oysters as in other bivalve molluscs, the immune system is based on haemocytes (Ottaviani et al., 2010; Renault, 2015) and their main function is to phagocytise biological pathogens, cell debris and foreign particles (De Vico and Carella, 2012). Moreover, haemocytes also participate in other processes such as: tissue reparation (for instance in gonadal follicles after spawning), nutrient digestion, transport and excretion (Cheng, 1981) but also in pollutant detoxification processes through the accumulation of metal or organic compounds in their endolysosomal system (De Vico and Carella, 2012).

The presence of haemocytic infiltrations in bivalves is closely related to the presence of stress conditions (Bayne et al., 1985) including exposure to organic xenobiotics (Auffret, 1988; Bignell et al., 2011; Sunila, 1984), metals or pathogens (Lowe and Moore, 1979; Rasmussen, 1986). In some cases, other types of inflammatory responses known as granulocytomes can appear (Neff et al., 1987; Svärth and Johannenson, 2002).

Parasite presence in oysters also affects their health status. Gills and digestive gland are the most exposed organs to be parasited due to the filter feeding behaviour of oysters, these organs being almost constantly in contact with sea water (Bignell et al., 2008; Villalba et al., 1993). The impacts produced by these parasite infestations in oysters are multiple and vary according to the pathogen. Some of them, i.e. trematods, cestods or copepods can alter oyster growth. Other pathogens such as protozoans are known to cause severe alterations including even death of oysters.

During the last decades, special attention has been paid to the relationships between infectious diseases and pollutant exposure. In fact, the measurement of parasite prevalence has been included in biomonitoring programs as they can change the sensitivity level to pollutants and endanger host health status (Kim et al., 2008). Thus, the analysis of histopathological alterations provides a non despicable amount of information about individual health status (Bignell et al., 2011; Knowles et al., 2014; Renault, 2015).

3.3.3 The integrative Biological Response Index (IBR)

It is generally accepted that biomarkers used individually do not provide an overall picture of the organism health status and thus the application of several biomarkers, in a battery, is needed to acquire a proper understanding of biological responses to stressors. Consequently, in order to obtain a better understanding of these responses,

biomarkers should be integrated. Different indexes have been proposed for this purpose (Broeg and Lehtonen, 2006; Sanchez et al., 2013). The integration of biomarkers measured at different organisational levels into the Integrative Biological Response (IBR) index has been demonstrated to be a powerful tool that allows obtaining an overall view of the health status of sentinel organisms after pollutant exposure (Beliaeff and Burgeot, 2002; Broeg and Lehtonen, 2006; Serafim et al., 2012). The IBR index was first proposed by Beliaeff and Burgeot, (2002) and it was described as the area of a star plot that allowed the visual integration the biomarker responses.

Since then, the index has been successfully applied in different sentinel species including oysters (Brooks et al., 2011; Cravo et al., 2012; Garmendia et al., 2011a; Marigómez et al., 2013a; Xie et al., 2016). This index has demonstrated its validity in biomonitoring programs being able to discriminate between less and higher impacted sites (Broeg and Lehtonen, 2006; Marigómez et al., 2013a; Serafim et al., 2012; Xie et al., 2016) whereas it has also demonstrated its validity under controlled exposure to pollutants (Asensio et al., 2013; Brooks et al., 2011; Rementería et al., 2016).

However, caution should be taken when applying and interpreting the IBR index. Indeed two main weak points have been identified for this index. The first one is related to the dependency of the obtained final IBR value to the arrangement of measured biomarkers in the calculation formula. The second weakness, relies on the interpretation of biological responses since only up or down regulation of measured biomarkers are considered (Sanchez et al., 2013). In order to overcome this problems, the calculation of the IBR index has been developed and improved into a new version (Devin et al., 2014; Sanchez et al., 2013) which now includes a simpler formula for its calculation and also a permutation procedure in order to diminish the influence of the biomarker arrangement in the obtained final IBR value.

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II. STATE OF THE ART, HYPOTHESIS AND OBJECTIVES

STATE OF THE ART

Estuaries have been one of the most impacted aquatic environments, since historically main human settlements have been located around estuaries. Estuaries from the Bay of Biscay have also suffered these pressures, leading to the enhanced presence of pollutants in their waters and sediments. In this context, Ibaizabal and Gironde Estuaries are both good examples of chronic metal pollution related to their past industrial history. Although the implementation of new policies and regulations together with the closure of major historical sources have resulted in a considerable decrease of pollutant concentrations, some elements such as copper (Cu) and silver (Ag) are still present in high enough concentrations to provoke deleterious effects on aquatic biota, indeed both elements are regarded as very toxic.

On the other hand, biomonitoring programs for assessing coastal and estuarine health status have considered oysters good sentinel organisms because of their sedentary way of life and ability to accumulate pollutants with little metabolic transformation. Although biomonitoring programs have provided reliable information, there are still some knowledge gaps that need to be resolved. Furthermore, the presence of new contaminants and the possible interactions between them in terms of bioaccumulation and toxicity are also needed for a deeper understanding together with the influence that environmental factors may have. Recent studies have mentioned a possible relationship on Cu and Ag bioavailability and accumulation in oysters and synergistic effects on Ag accumulation when Cu is present have been recorded, as well as antagonistic effects in Cu accumulation when Ag is present.

Biomarkers are defined as the measurements of body fluids, cells, or tissues at cellular, biochemical and molecular levels that indicate the presence of pollutants (exposure biomarkers) or the magnitude of the organism response (effect biomarkers). However, these responses are sometimes difficult to be interpreted,

thus the integration of the obtained data through biological indexes such as the IBR index, presents an alternative tool for obtaining a better understanding of both the severity of the toxicity exerted by the pollutant and the biological processes involved.

HYPOTHESIS

Metals present in estuarine environments such as Cu and Ag can provoke deleterious effects in the environment, including oysters (*Crassostrea gigas*). Interactions between these two metals can provoke differences in the bioaccumulation and biological responses in oysters. Moreover, different biological response in oysters may be expected depending on the environmental factors in which pollutant insult has occurred and on the major uptake pathway. Produced effects can be assessed through a battery of cell and tissue level biomarkers through their integration into the Integrative Biological Response (IBR) index.

OBJECTIVES

In order to prove this hypothesis true and also for completing the understanding of the effects exerted by Ag and Cu in oysters using biomarkers the following general objectives were defined:

1. To study the different accumulation patterns and biological responses produced by Cu and Ag in oysters and mussels collected in three different estuaries from the Bay of Biscay with different pollution history and levels.
2. To study possible interactions on Cu and Ag bioaccumulation and biological responses caused in oysters *Crassostrea gigas* after direct exposure in natural sea water to a range of environmentally relevant concentrations of both elements.
3. To identify the possible influence of salinity in the biological response in oysters after Cu and Ag pollutant exposure at environmentally relevant levels.

4. To determine the bioaccumulation and biological responses of Cu and Ag in oysters *Crassostrea gigas* after dietary exposure to Cu and Ag alone and in combination at environmentally relevant concentrations.

III. RESULTS AND DISCUSSION

Chapter I

Environmental health assessment of 3 estuaries from the Bay of Biscay using cell and tissue level biomarkers in mussels (*Mytilus galloprovincialis*) and oysters (*Crassostrea gigas*)

This chapter has been presented in:

Rementeria A., Mikolaczyk M., Lanceleur L., Blanc G., Soto M., Zaldibar B., Schäfer J., 2013. Environmental health assessment of 3 estuaries using cell and tissue level biomarkers in oysters (*Crassostrea gigas*). *9th Iberian and 6th Iberoamerican Congress on Environmental Contamination and Toxicology, 1-4 july 2013, Valencia (Spain)*. Poster presentation.

Rementeria A., Zaldibar B., Schäfer J., 2014. Understanding the impact of silver as an emerging contaminant in the Ibaizabal and Gironde estuaries. Bordeaux-Euskampus Symposium, 19-20 november 2014, *Bordeaux (France)*. Best poster presentation.

Rementeria A., Zaldibar B., Schäfer J., 2015. Understanding the impact of silver as an emerging contaminant in the Ibaizabal and Gironde estuaries. Bordeaux-Euskampus Symposium, 26-27 november 2015, *Donostia-San Sebastián (Basque Country, Spain)*. Best poster presentation.

ABSTRACT

Environmental health assessment of 3 estuaries from the Bay of Biscay using cell and tissue level biomarkers in mussels (*Mytilus galloprovincialis*) and oysters (*Crassostrea gigas*).

Aquatic bivalves such as mussels and oysters have been widely considered as good surrogates for monitoring environmental health assessment because contaminant levels in their soft tissues respond to changes in the environment and are accumulated with little metabolic transformation. Moreover, due to their sessile way of life they are useful monitors of spatial and temporal changes in pollutant loads in coastal and estuarine ecosystems. Together with the chemical measurement of pollutants, cell and tissue level alterations have been considered as early warning signals of overall health status of organisms and predictive indicators of changes at higher and more relevant biological organisation levels such as population or ecosystem level. Among contaminants present in the estuaries, copper (Cu) and silver (Ag) are of special interest since they are present at increasing concentrations in several estuaries in Europe and are known for high toxicity in aquatic organisms. In the present study, Ag and Cu accumulation values together with the integration of biomarkers into the Integrative Biological Response (IBR) index was carried out in oysters and mussels collected in February 2013 in 3 estuaries (Ibaizabal, Oka and Gironde) of the Bay of Biscay. The Ibaizabal and Gironde Estuaries are well known for a long history of metal pollution, whereas the Oka Estuary is located in the UNESCO Reserve of the Biosphere of Urdaibai. Results from chemical analyses showed higher Ag and Cu concentrations in individuals from the Gironde Estuary, in good agreement with the metal distribution in bivalves' tissues obtained by autometallography. Moreover, this technique also pointed to clearly higher metal accumulation in oysters than in mussels. Different biomarkers in sentinel organisms reflected the higher pollutant accumulation in bivalves from the Gironde Estuary, however their response intensity varied depending on the sentinel specie.

RÉSUMÉ

Evaluation de la santé environnementale de 3 estuaires du Golfe de Gascogne par l'utilisation de biomarqueurs cellulaires et tissulaires chez les moules (*Mytilus galloprovincialis*) et huîtres (*Crassostrea gigas*).

Les bivalves aquatiques comme les moules et les huîtres sont largement utilisés dans l'évaluation de la santé environnementale. En effet, la proportion de contaminants accumulés dans leurs tissus rend compte des changements environnementaux car ils y sont peu transformés par leur métabolisme. De plus, ces bivalves ont un mode de vie sessile, c'est pourquoi ils sont aussi des moniteurs utiles pour indiquer les changements de niveaux de contamination des écosystèmes côtiers et estuariens. L'analyse chimique des polluants ainsi que l'étude des altérations cellulaires et tissulaires, ont été utilisées en parallèle comme pré - marqueur d'alerte, afin d'évaluer l'état de santé général des organismes en vue de leur utilisation comme indicateurs précoces de changements au niveau biologique d'une population ou d'un écosystème. De tous les polluants présents dans les estuaires, le cuivre (Cu) et l'argent (Ag) suscitent un grand intérêt parce que leurs concentrations sont en augmentation dans différents estuaires européens ainsi que par leur haute toxicité pour les organismes aquatiques. Dans cette étude, des moules et des huîtres ont été collectées en février 2013 dans trois estuaires (Ibaizabal, Oka and Gironde) du Golfe de Gascogne. Les niveaux d'accumulation Ag et de Cu dans les huîtres et moules ont été évalués et différents biomarqueurs ont été mesurés et intégrés dans l'index IBR (Integrative Biological Response Index). Les estuaires d'Ibaizabal et de la Gironde sont connus pour leur pollution en métaux. L'estuaire d'Oka quant à lui est placé dans une Réserve de la Biosphère UNESCO Urdaibai. Les résultats à partir d'analyses chimiques ont montré de fortes concentrations en Ag et Cu dans les organes des individus provenant de l'estuaire de la Gironde. Les données obtenues sont en concordance avec la distribution des métaux dans les tissus observée par autométallographie. Cette technique a aussi clairement souligné une accumulation de métaux plus importante dans les huîtres que dans les moules. Par ailleurs, les différents biomarqueurs appliqués aux organismes sentinelles ont révélé la présence d'une plus forte accumulation des polluants métalliques dans les bivalves de l'estuaire de la Gironde, avec une intensité de la réponse variant selon l'espèce sentinelle.

LABURPENA

Bizkaiko Golkoko 3 estuarioen ingurumen osasunaren ebaluazioa zelula eta ehun mailako biomarkatzaileak erabiliz muskuilu (*Mytilus galloprovincialis*) eta ostretan (*Crassostrea gigas*).

Muskuiluak eta ostrak bezalako bibalbioak ingurumen biojarraipen programetan ordezkari egoki gisa kontsideratzen dira, izan ere beraien ehun bigunetako kutsatzaile-kontzentrazio aldaketa ingurumenean gertatzen diren aldaketekin bat datozen eta kutsatzaileek aldaketa metaboliko gutxi pairatzen dituzte bibalbioen ehunetan. Gainera beraien bizitza sesilari esker kostalde eta estuarioetako ekosistemen aldaketa espazial eta tenporalak monitorizatzeko gai dira. Kutsatzaileen neurketa kimikoekin batera, zelula eta ehun mailetan gertatzen diren aldaketak organismoen osasun egoeraren abisu goiztiarreko seinale gisa kontsideratzen dira eta maila biologikoki altuagoetan eta esangarriagoetan (populazio edo ekosistema) gertatu daitezkeen aldaketak aurrerako indikatzaile egokiak kontsideratzen dira. Estuarioetan ager daitezkeen kutsatzaile desberdinen artean, zilarrak (Ag) eta kobreak (Cu) berebiziko garrantzia dute, izan ere Europako zenbait estuariotan beraien kontzentrazioa emendatzen ari da eta gainera oso toxikoak dira organismo urtarrentzat. Ikerlan honetan, Ag eta Cu-aren metaketalbioak eta hainbat biomarkatzaile neurtu dira, azken hauek "Integrative Biological Response (IBR)" indizean integraturik. Horretarako Bizkaiko Golkoko 3 estuariotan (Ibaizabal, Oka eta Gironde) ostrak eta muskuiluak hartu ziren 2013ko Otsailean. Ibaizabal eta Gironde Estuarioek kutsadura metalikoaren historia luzea dute, eta Oka Estuariora Urdaibai-ko UNESCO Biosferaren Erreserban dago kokatua. Lan honetako emaitzek erakutsi dute Gironde Estuarioko indibiduoetan Ag eta Cu kontzentrazioak altuagoak direla autometalografia bitartez lorturiko metalen banaketarekin bat etorriz. Azken teknika honen emaitzen arabera, ostretan metal metaketa handiagoa behatu da muskuiluetan baino. Beste alde batetik erabilitako biomarkatzaileek Gironde Estuarioko kutsatzaileen metaketa altuagoa zela erakutsi zuten bai ostra zein muskuiluetan, baina erantzunaren intentsitatea aukeratutako espeziearen arabera izan zen.

RESUMEN

Evaluación de la salud ambiental de 3 estuarios del Golfo de Bizkaia utilizando biomarcadores celulares y tisulares en mejillones (*Mytilus galloprovincialis*) y ostras (*Crassostrea gigas*).

Los bivalvos marinos tales como las ostras y los mejillones han sido ampliamente reconocidos como buenos organismos en los programas de monitoreo de la salud ambiental puesto que la concentración de contaminantes en sus tejidos blandos responden a los cambios producidos en el medio y acumulan los contaminantes con poca transformación metabólica. Además, debido a su forma de vida sésil, son útiles para monitorizar los cambios espaciales y temporales en la carga de contaminantes en los ecosistemas marinos y estuarinos. Las medidas químicas de los contaminantes junto con las alteraciones celulares y tisulares pueden considerarse como señales de aviso tempranas del estado general de salud de los organismos y pueden ser indicadores predictivos de cambios que pudieran ocurrir a mayores y más relevantes niveles de organización biológica como puede ser el nivel población o el nivel ecosistema. De entre los contaminantes presentes en los estuarios, la plata (Ag) y el cobre (Cu) son de especial interés, ya que sus concentraciones están aumentando en algunos estuarios europeos y son altamente tóxicos para los organismos acuáticos. En el presente trabajo, se ha estudiado la acumulación de Ag y Cu junto con varios biomarcadores integrados en el índice denominado "Integrative Biological Response (IBR)" en ostras y mejillones recolectados en Febrero de 2013 en 3 estuarios, (Ibaizabal, Oka y Gironde) del Golfo de Bizkaia. Los estuarios de Ibaizabal y Gironde tienen una larga historia de contaminación por metales y por otra parte el estuario del Oka está situado en la Reserva de la Biosfera de la UNESCO de Urdaibai. Los resultados de los análisis químicos muestran una mayor concentración de Ag y Cu en los individuos del Estuario del Gironde, además éstos resultados concuerdan con los resultados de distribución de metales en los tejidos obtenidos mediante autometalografía. Los resultados de la autometalografía, además, muestran una mayor acumulación de metales en ostras comparando con mejillones. Por otra parte, los biomarcadores empleados en los organismos centinela fueron capaces de reflejar una mayor acumulación de los contaminantes en el estuario del Gironde, pero la intensidad de la respuesta varía dependiendo del organismo utilizado.

INTRODUCTION

Aquatic environments in general and estuaries in particular, have been historically subjected to different types of anthropogenic perturbations. In fact, until the early 2000's many of the European estuaries were still highly polluted (Leorri et al., 2008).

Estuaries have a recognised ecological and economical value (de Souza Machado et al., 2016) and environmental policies (including the improvement on treatments for both urban and industrial waste waters) carried out in the area of the Bay of Biscay during the past decades together with a general decline of industrial activity have lead to a significant decrease of the amount of discharged pollutants (Leorri et al., 2008). However, pollutant concentrations in some estuaries in the Bay of Biscay are still high, with industrial and urban activities, sewage treatment plants, land run-off and atmospheric deposition being considered as common sources of metallic pollution inputs in the estuaries (Gredilla et al., 2013). Metal pollution is an issue of major concern, because metallic elements do not degrade and hence they can accumulate through the food chain (Waykar and Deshmukh, 2012).

In this context, silver (Ag) and copper (Cu) are considered as some of the most relevant elements for marine biota due to their toxicity even at low exposure levels (especially in the case of Ag) (Lee et al., 2010; Ratte, 1999; Santovito et al., 2015; Tappin et al., 2010). Although Cu is an essential micronutrient for living organisms, it becomes strongly toxic when the tolerable exposure is surpassed (Debelius et al., 2009; Foster et al., 2011; Gamain et al., 2016; Raftopoulou and Dimitriadis, 2011).

Silver uses have varied through history resulting in its release into aquatic environment by mining, acid mine drainage and metallurgy from pre-Roman periods until the XXth century and, more recently, by modern technologies implying increasing urban sources (jewellery, electronic, photography, hospital disinfectants) (Barriada et al., 2007; Lanceleur et al., 2011a, 2013; Tappin et al., 2010).

Nowadays, Ag is increasingly present as nanoparticles in a wide range of products (plastics, textiles, medical and personal care products) due to its bactericidal properties and relatively low cost of production (Fabrega et al., 2011). As a consequence, overall Ag concentration levels in coastal and estuarine waters have clearly increased around urban settlements, making Ag a good tracer for urban wastewater inputs (Barriada et al., 2007; Lanceleur et al., 2011a, 2013; Deycard et al., 2016). In the case of Cu, the main pollution sources to coastal water bodies apart from mining and metallurgic industry are related to (i) the release of domestic sewage water effluents, (ii) the use of Cu in fungicides in agriculture (especially in vineyards and fruit production) and (iii) to the recent introduction of Cu containing anti-fouling paints in vessels, in order to avoid the attachment of aquatic organisms into boats (Gamain et al., 2016; Lee et al., 2010). This context highlights the necessity to continuously monitor the environmental health status of aquatic environments through biomonitoring programs, often relying on the systematic collection of bivalves (usually mussels and/or oysters) and subsequent analyses for various priority contaminants. These aquatic molluscs are able to rapidly record environmental changes (Goldberg, 1986) as a consequence of their capability to accumulate and concentrate pollutants in their tissues with little metabolic transformation (Phillips & Rainbow 1993; Rainbow 1995). However, their responses depend on the sentinel species, reproduction cycle, and differences between individuals and local bivalve populations (Farrington et al., 2016). Species dependent responses and the fact that there are very few systematic samplings/comparisons of oysters and mussels from the same sites, i.e. facing identical environmental conditions, limit the assessment of the actual overall environmental status of coastal areas. In addition to chemical measurements in bivalves' tissues, European and American marine pollution monitoring programs have incorporated biomarker measurements in bivalves (Cajaraville et al., 2000) as a powerful tool to gain information on pollutant effects in biota (Au., 2004; Bayne,

1989) and predict related future changes at higher biological structures (population, community, ecosystem).

The present study is focused on three estuaries (Ibaizabal, Oka and Gironde) of the Bay of Biscay (Fig. 1), this bay extends from 43°48'N and from 0-10°W (Borja and Collins, 2009; Costoya et al., 2015). Two of the analysed estuaries, Ibaizabal (43°23'–43°14'N, 3°07'–2°55'W) and Oka (43°22'N, 2°40'W) are located on the Basque coast, whereas the Gironde Estuary, is situated in the southwest of France (45°12'N, 0°42'W). Biomonitoring programs in the Bay of Biscay including the presently studied estuaries have routinely been carried out by the respective administrations. The Ibaizabal and Gironde Estuaries are chronically metal polluted (Gredilla et al., 2013; Lanceleur et al., 2011a, 2011b; Leorri et al., 2008; Ortiz-Zarragoitia and Cajaraville, 2010; Petit et al., 2013), whereas the Oka Estuary, is generally considered as a low polluted estuary (Rodríguez-Iruretagoiena et al., 2016). During decades, the Ibaizabal Estuary has been impacted by various urban and industrial inputs, with mining activity being the dominant source of metal pollution (Leorri et al., 2008). Although the industrial activity has strongly decreased, and in spite of great efforts to restore water quality levels (Lekube et al., 2013; Leorri et al., 2008), the Ibaizabal Estuary still faces strong human pressure due to high population density (Gredilla et al., 2013; Leorri et al., 2008). In contrast, the Oka Estuary is located in one of the most important wetlands of the Basque Country, which was declared a Biosphere Reserve by UNESCO in 1984. Several studies have considered this estuary as a reference site due to relatively low concentrations of organic pollutants and heavy metals in bivalves (Lekube et al., 2013; Ortiz-Zarragoitia and Cajaraville, 2010). However, this estuary is not free of anthropogenic disturbances with several metal sources in the area: (i) periodical dredging of the estuary due to a shipyard located in Murueta, (ii) industrial activities near to Gernika and (iii) leisure activities including recreational and fishing boats (Rodríguez-

Iruretagoiena et al., 2016). Finally, the Gironde Estuary is the largest estuary of the European Atlantic Coast with approximately 170 km length, draining a 80 000 km² watershed including two main French urban agglomerations: Bordeaux and Toulouse (both together 2.7 million inhabitants) (Lanceleur et al., 2013). Past mining activities (Au, Ag, Pb, Zn, As and coal) and metallurgy have led to historical metal pollution which is still persistent in sediments and sentinel organisms, although generally decreasing. Intensive agriculture (wine farming, fruit production) in the watershed also contributes to these metal loads (Lanceleur et al., 2011a). Indeed, for more than 3 decades, wild oysters from the Gironde Estuary have shown the highest Ag and Cu concentrations of all the sites of the French national biomonitoring program (RNO/ROCCH 1979-2015; Lanceleur et al., 2011a, 2011b).

In this context, the present study aims to (i) assess the current environmental health status of three south-eastern Bay of Biscay estuaries using wild bivalves and (ii) compare the responsiveness of oysters *Crassostrea gigas* and mussels *Mytilus galloprovincialis*, the sentinel species used in the different biomonitoring programs (Spanish, Basque and French) carried out in the area. Accordingly, this work addresses (i) Cu and Ag levels in bivalve tissues, and the resulting Cu/Ag ratio, which has been recently proposed as an additional tool for biomonitoring programs (Mikolaczyk, 2016) and (ii) a battery of biomarkers integrated through the use of the Integrative Biological Response Index (IBR Index). This index has already demonstrated to be a powerful tool for assessing the health status of sentinel species and subsequently of the ecosystem (Beliaeff and Burgeot, 2002; Broeg and Lehtonen, 2006; Marigómez et al., 2013a; Rementería et al., 2016).

MATERIALS AND METHODS

1. Sampling sites

Three estuaries from the Bay of Biscay (Ibaizabal, Oka and Gironde Estuaries) were selected for the present study, based on their respective properties (see above). One sampling point per estuary was established: Arriluze (Ibaizabal Estuary), Murueta (Oka Estuary) and La Fosse (Gironde Estuary) (Fig. 1).

The Ibaizabal Estuary sampling point, Arriluze, is a chronically polluted marina located in the right bank of the estuary mouth ($43^{\circ}20'N$, $3^{\circ}087'W$) (Lekube et al., 2013; Marigómez et al., 2013). The Oka Estuary sampling point, Murueta ($43^{\circ}21'N$, $2^{\circ}40'W$), represents the mid part of the estuary and it is situated on the left bank close to a shipyard (Raposo et al., 2009; Sarobe et al., 2009), considered a threat for the estuary due to periodical dredges (Borja et al., 2004). The sampling point in the Gironde Estuary is La Fosse ($45^{\circ}28'N$, $0^{\circ}59'W$) located on the left bank of the estuary mouth.

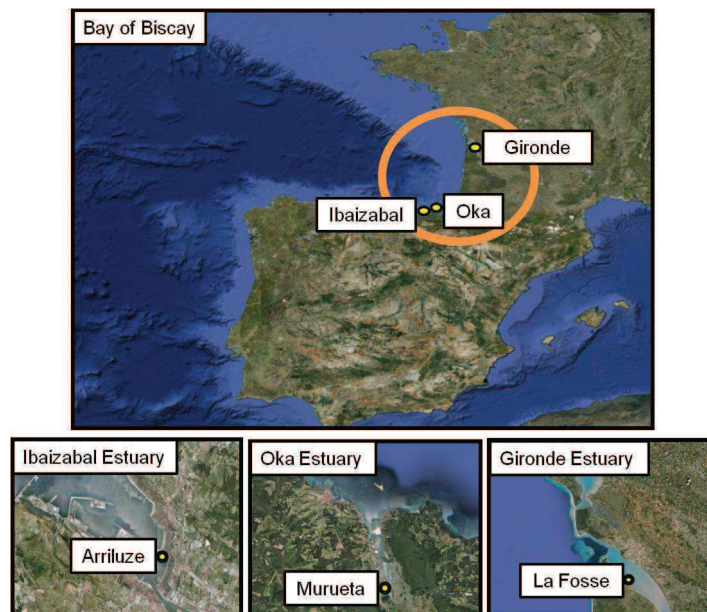


Figure 1: The three selected estuaries from the Bay of Biscay: Ibaizabal, Oka and Gironde Estuary. The selected points were Arriluze (Ibaizabal Estuary), Murueta (Oka Estuary) and La Fosse (Gironde Estuary).

2. Sample collection and processing

In February 2013 oysters *Crassostrea gigas* (6 - 8 cm long) (Thunberg, 1793) and mussels *Mytilus galloprovincialis* (Lamarck, 1989) (3.5 - 5.5 cm long) were collected at the three sampling points. Thirty individuals per specie were obtained at each sampling point following OSPAR's commission guidelines for monitoring contaminants in the biota (<http://www.ospar.org>) (Lanceleur et al., 2011b). All animals were immediately brought to the laboratory facilities, half of them ($n=10$) were immediately dissected for biological measurements carried out at the "Plentzia Marine Station" (PiE, UPV/EHU, Plentzia, Basque Country, Spain) whereas the rest ($n=15$) were left in natural filtered sea water 24 h until processing for chemical measurements at the TGM-EPOC laboratory (UMR EPOC 5805, Bordeaux, France).

Oysters from the Oka Estuary designated to chemical measurements were accidentally lost during processing and therefore no chemical measurements were carried out. Moreover, additional sets of oysters *Crassostrea gigas* for biological measurements were obtained in the same three sampling points in June 2012 and October 2012 (see Appendix I).

2.1 Chemical measurements

All the labware used for chemical analyses was decontaminated as follows: (i) soaking in acid (HNO_3 10%) during 72 h, (ii) thorough (3 times) rinsing with de-ionised water, (iii) thorough (3 times) rinsing with Milli-Q® water and (iv) drying under a laminar flow hood. Once the cleaning process finished, all the labware was stored in double sealed polyethylene bags until use to prevent contamination. Fifteen individuals were opened, the soft bodies were carefully separated from the shells using Teflon scissors in order avoid any metal contamination. Shell and flesh weights were individually stored for Condition Index (CI) calculation (see section

2.2.1). Each soft body was introduced into previously weighed and clean (see above) polyethylene tubes, deep-frozen (-80°C), freeze-dried, weighed and homogenised with an agate mortar obtaining a homogeneous powder.

Aliquots of each sample (200 mg nominal weight) were precisely weighed in clean polyethylene tubes for acid digestion with 1.4 mL HNO₃ (14 M, PlasmaPur) and 2 mL HCl (12 M, PlasmaPur) following the procedure described by Daskalakis, (1996). In summary, closed tubes were digested during 3 h in a heating block at 90°C (DigiPREP MS; SCP SCIENCE). Once digestates cooled, they were diluted with Milli-Q® water and analysed for Cu and Ag concentrations by Inductively Coupled Plasma Mass Spectrometry (ICP-MS; Thermo, X Series II) with external calibration (made from commercially available standard solutions PLASMACAL, SCP Science). At each analytical session, accuracy and precision were controlled by parallel analysis of international referenced certified materials (TORT 2, NIST Oyster Tissue 1566b and IAEA 407), and were respectively >90% and 5% (r.s.d.).

2.1.1 Bioconcentration factor

Metal bioconcentration factor (BCF) is defined as the uptake of a chemical substance from water via respiratory surface and/or skin (Arnot and Gobas, 2006). The BCF was estimated as follows: $BCF = C_B / C_{WD}$, where: C_B is the chemical concentration in the organism and C_{WD} the freely dissolved concentration in water (Arnot and Gobas, 2006).

2.2 Biological measurements

For biological measurements, all oysters and mussels were opened and dissected. Animals were opened and shell and flesh weights were individually measured for the calculation of CI (see section 2.2.1). For histology, a ~5 mm dorsoventral cross-section which included all main organs and tissues (at least gills, digestive gland and gonad) was obtained per animal ($n=10$). Each section was introduced into

histological cassettes and left in formalin (4% formaldehyde buffered in natural sea water) for 24 h. Then fixative was removed and samples were stored in 70% ethanol until being dehydrated and embedded into paraffin following the standard histological procedure.

2.2.1 Condition Index

Oyster's Condition Index (CI) was calculated according to the formula: $CI_{\text{oyster}} = (\text{WW visceral content} / \text{WW shell} \times 100)$ (in grams) (Strady et al., 2011). For mussels the Flesh CI was calculated according to the formula: $CI_{\text{mussel}} = (\text{flesh weight (mg)} / \text{shell weight})$ (Lobel and Wright, 1982).

2.2.2 Digestive gland and gonad histology

Digestive gland and gonad tissues were sliced to 4 μm thick sections in a microtome from paraffin embedded samples and stained with haematoxylin-eosin. Afterwards, microscopic slides were analysed under a light microscope (Olympus BX 61) (Olympus, Japan).

2.2.3 Gamete developmental stage

Animals sex and gamete developmental stage as well as Gonad Index (GI) were determined for each individual following the procedure described by Kim et al., (2006), which is based on a subjective scale of the developmental stage of follicles and gametes after examination under the light microscope. For oysters 8 different developmental stages were defined, from sexually undifferentiated to spawned, with their corresponding values ranging from 1 to 8. In the case of mussels, 4 reproductive stages (Resting/Spent gonad, Developing gonad, Ripe gonad and Spawning gonad) were established with a Gonad Index value varying from 0 to 5. Finally in both cases, a mean gonad index value (GI) was calculated for the pool of individuals from each sampling point and season.

2.2.4 Histopathological alterations

Histopathological analyses through oyster's and mussel's whole body transections were performed at different microscopical magnifications. Different pathologies indicative of bivalve's health status were identified including: Granulocytomes (GRN), Haemocytic infiltrations (HAE), Haemocytic infiltrations in Gonads (HAE gn), Inflammations (INF) and Parasites (PAR) (among them: *Mytilicola intestinalis*, *Nematopsis* and *Marterlia* and copepods). Prevalence values for each alteration were obtained in terms of percentages.

2.2.4.1 Digestive gland atrophy

A planimetric procedure was followed in order to determine changes in the morphology of digestive tubules (Garmendia et al., 2011a). Micrographs of digestive gland tubules were obtained with a digital camera attached to an Olympus BX-61 microscope (Olympus, Japan). Five fields of digestive glands were recorded per oyster, and 2 tubule profiles per field were transferred into an image analysis system (Visilog 5.4 Noesis) to determine the following parameters and ratios: MET (Mean Epithelial Thickness, μm), MLR (Mean Luminal Radius, μm) and MLR/MET ratio.

2.2.4.2 Tissue integrity in digestive gland (CTD ratio)

The integrity of the digestive gland tissue was determined by calculating the connective-to-diverticula (CTD) ratio, i.e. the extent of the interstitial connective tissue relative to the area occupied by digestive diverticula. For this, 5 fields of digestive gland tubules were obtained per individual with a digital camera attached to an Olympus BX-61 microscope (Olympus, Japan) and processed through Image J program (Image Processing and Analysis in Java, Maryland, USA). The following formula was applied to obtain CTD ratio: $\text{CTD ratio} = c / (b + d + l)$. Where: (c) interstitial connective tissue, (b) basophilic cells, (d) digestive cells and (l) diverticular lumen (Brooks et al., 2011).

2.3 Intralysosomal metal accumulation

Histochemical detection of metals was carried out in 4 µm thick sections of paraffin embedded samples ($n=10$ per treatment). After embedding, sections were dewaxed in xylene and hydrated, then, the slides were left at 37°C oven during at least 24 h for drying. Samples were covered with the commercial silver enhancement kit (Silver enhancing kit for light and electron microscopy, BB International) at room temperature; development was checked under light microscope and stopped after 15-20 min. Slides were washed with tap water and metals were developed as black silver deposits (BSD). Finally, slides were mounted in Kaiser's glycerine gelatine and volume density of BSDs ($V_{V_{\text{BSD}}}$) in the lysosomes of digestive gland cells was measured with an image analysis system (Soto et al., 2002).

2.4 Integrative Biological Response (IBR) index

Integrative Biological Response (IBR) index was calculated according to Devin et al., (2014). This procedure is based on the method described by Beliaeff and Burgeot, (2002). A total of 4 biomarkers were used for this purpose ordered from less biological complexity level to highest: cellular level (intralysosomal metal accumulation), tissue level (MLR/MET), organ level (CTD index) and individual level (Condition Index). Finally, as the IBR value depends on the number of applied biomarkers, the IBR/n was obtained dividing IBR by the number of biomarkers applied ($n=4$) (Brooks et al., 2011; Marigómez et al., 2013a,b).

2.5 Statistics

All data was statistically analysed with SPSS v 22.0 statistical package (SPSS Inc., Chicago, Illinois, USA). Results were presented as mean values \pm standard deviations (SD). Homogeneity of variances (Levene's test) and data normality (Kolmogorov-Smirnov test) were checked before performing one-way analysis of variance (ANOVA) or student's-t comparison (in the case of comparisons between

two sampling sites), which determined significant differences between sampling points and months. Differences were considered statistically significant when $p < 0.05$ and Duncan's test was used for multiple range comparison between pairs after performing ANOVA. In those cases where data was not normally and homogeneously distributed, the Kruskal Wallis non parametric test was used as an equivalent of ANOVA, whereas Mann-Whitney's U non parametric test was used as the equivalent of student's-t test.

RESULTS

1. Metal concentrations in oysters

Metal accumulation values for Ag and Cu were obtained in oysters and mussels collected in February 2013 in the Ibaizabal and Gironde Estuaries (Tables 1 and 2). Higher metal contents were observed in oysters than in mussels and differences between sites also occurred, with generally the highest values present in individuals from the Gironde Estuary. Indeed, Ag contents were significantly higher in oysters from the Gironde Estuary than in those from Ibaizabal Estuary. Although not statistically significant, Cu concentrations also tended to be greater in oysters from the Gironde Estuary (Tables 1 and 2). In the case of mussels, again Ag contents were clearly higher in individuals from the Gironde Estuary, whereas higher Cu contents occurred in mussels from the Ibaizabal Estuary.

Regarding Cu/Ag ratio (Tables 1 and 2) significant differences between sampling sites were found both in oysters and mussels, with values in bivalves from the Gironde Estuary being clearly lower than those in the Ibaizabal Estuary. On the other hand, Cu/Ag ratios were lower in mussels than in oysters in all sampling points.

Tables 1 and 2: Ag and Cu concentrations and Cu/Ag ratios measured in oysters and mussels from the Ibaizabal and Gironde Estuaries in February 2013. Statistically significant differences ($p < 0.05$) between sampling points for the same specie (*).

Oysters	Estuary	Min	Max	Mean±SD	R ²
Ag (mg.kg ⁻¹)	Ibaizabal	1.47	10.2	5.4±2,0	
	Gironde	0.49	99.1	36.5±31.9*	
Cu (mg.kg ⁻¹)	Ibaizabal	76.5	1290	268±340	
	Gironde	5.06	1670	692±612	
Cu/Ag	Ibaizabal	22.8	190	51.0±55.8*	0.3256
	Gironde	7.2	39.8	16.5±7.53	0.8634

Mussels	Estuary	Min	Max	Mean±SD	R ²
Ag (mg.kg ⁻¹)	Ibaizabal	0	0.08	0.04±0	
	Gironde	0	0.42	0.10±0.1	
Cu (mg.kg ⁻¹)	Ibaizabal	0.14	0.81	0.46±0.2	
	Gironde	0.05	0.73	0.35±0.2	
Cu/Ag	Ibaizabal	7.4	91.9	23.5±28.3*	0.7778
	Gironde	1.7	17.4	7.17±5.15	0.6098

The bioconcentration factor (BCF) for oysters and mussels was also calculated (Tables 3 and 4). In general, higher bioconcentration was observed in oysters than in mussels in both sites, whereas between sites higher BCF values were found in bivalves coming from Gironde Estuary. However, BCF of Cu in oysters from Ibaizabal Estuary showed higher values than Gironde Estuary.

Tables 3 and 4: Bioconcentration factor (BFC) for oysters and mussels from the Ibaizabal and Gironde Estuaries. C_B is the chemical concentration in the organism (ng.kg⁻¹) and C_{WD} the freely dissolved metal concentration in water (ng.L⁻¹). Values for C_{WD} for Ag in the Ibaizabal Estuary were not available (n.a), whereas C_{WD} for Cu were obtained from Borja et al., (2014). Values for C_{WD} Ag and C_{WD} Cu in the Gironde Estuary were respectively obtained from (Audry et al., 2007; Lanceleur et al., 2013).

Oysters	Estuary	C_B (ng.kg ⁻¹)	C_{WD} (ng.L ⁻¹)	BCF
Ag	Ibaizabal	5.43E+06	n.a	n.a
	Gironde	3.65E+07	8,00	4.56E+06
Cu	Ibaizabal	2.68E+08	2000	1,34E+05
	Gironde	6.92E+08	635	1.09E+06

Mussels	Estuary	C_B (ng.kg ⁻¹)	C_{WD} (ng.L ⁻¹)	BCF
Ag	Ibaizabal	4.40E+04	n.a	n.a
	Gironde	9.87E+04	8.00	1.23E+04
Cu	Ibaizabal	4.61E+05	2000	2.31E+02
	Gironde	3.51E+05	635	5.53E+02

2. Condition Index (CI)

Statistically significant differences between groups were obtained in oysters (Fig. 2 A). Lowest values were recorded in oysters coming from the Ibaizabal Estuary, whereas oysters located in the Oka and Gironde Estuaries displayed similar values. In mussels, no statistically significant differences in the condition index occurred (Fig. 2 B), however mussels from the Oka Estuary had the lowest values while the highest values were found in mussels from the Gironde Estuary.

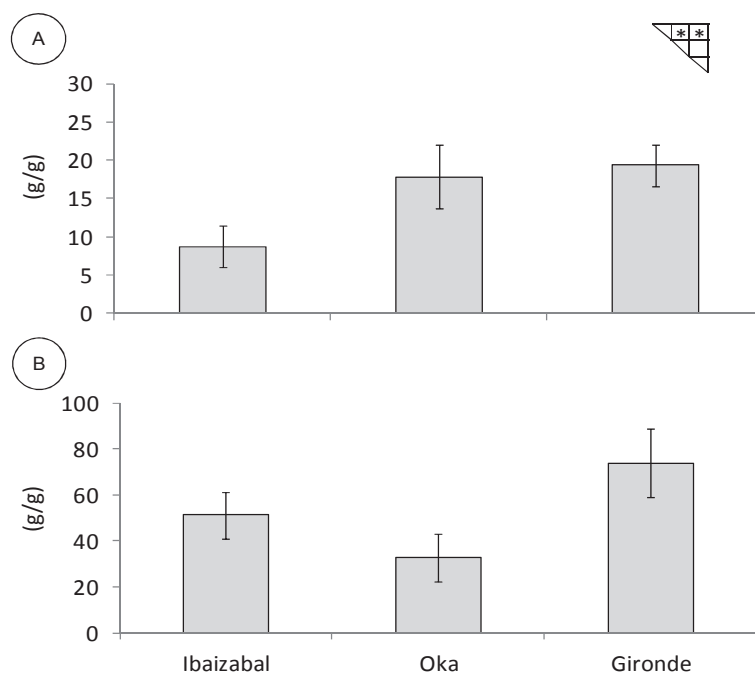


Figure 2: Mean values (\pm standard deviations) of the Condition index for oysters (A) and flesh Condition Index for mussels (B) from three different estuaries in the Bay of Biscay in February 2013. Vertical segments indicate standard deviations.

3. Gamete developmental stage

Oysters from the three estuaries were mainly in early gamete developmental stage (60% in the Oka and Ibaizabal Estuaries and 55% in the Gironde Estuary) in February 2013 (Fig. 3 A) (Fig. 4 A, B and D), but some oysters in the Oka Estuary (10%) were in spawned phase. For mussels a similar gamete development pattern was observed, whatever the site. The majority of the individuals of this specie were

in developing phase (100% in the Gironde Estuary and 90% in the Oka and Ibaizabal Estuaries) (Fig. 3 B). Some individuals (10%) in the Oka Estuary presented ripe gonads (Fig. 4 E), while in the Ibaizabal Estuary some mussels (%10) were in spawning phase. As observed for oysters, individuals from the Oka Estuary had a higher mean GI value, followed by individuals coming from the Ibaizabal and Gironde Estuaries (Fig. 3 B).

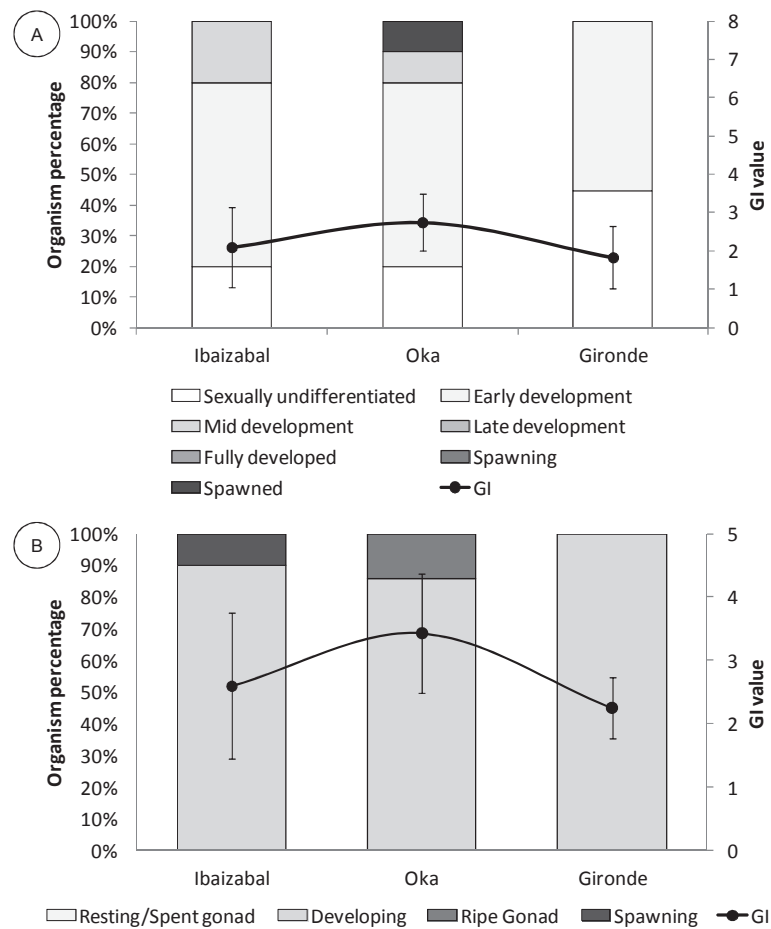


Figure 3: Percentages of the gamete developmental stage in (A) oysters and (B) mussels from three different estuaries from the Bay of Biscay in February 2013 and mean values (\pm standard deviations) of the Gonad Index.

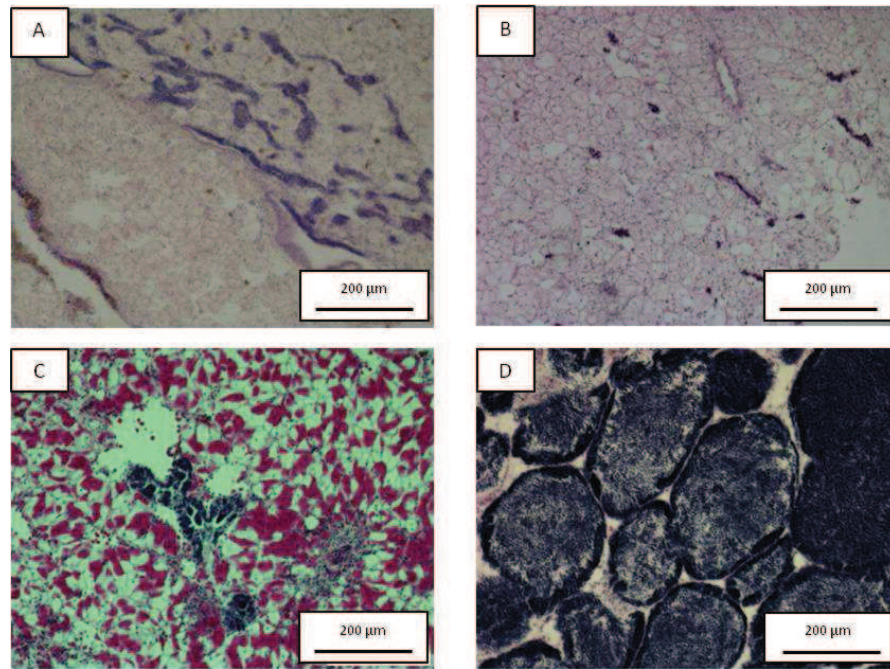


Figure 4: Gonad developmental stages in oysters (A and B) and mussels (C and D) from three different estuaries: (A and C) Ibaizabal Estuary, (D) Oka Estuary and (B) Gironde Estuary. Note that oysters from the three sites showed similar gamete developmental stages. (C) Female mussel from the Ibaizabal Estuary in the first phases of gametogenesis. (D) Ripe male gonad in a mussel from the Oka Estuary.

4. Digestive gland atrophy

In February 2013 no statistically significant differences between sampling points in both oysters and mussels were recorded regarding digestive gland atrophy (Fig. 5 A and B). Oysters from the Oka Estuary showed slightly higher values than those from the Gironde and Ibaizabal Estuaries, whereas values for mussels were almost similar for the three estuaries.

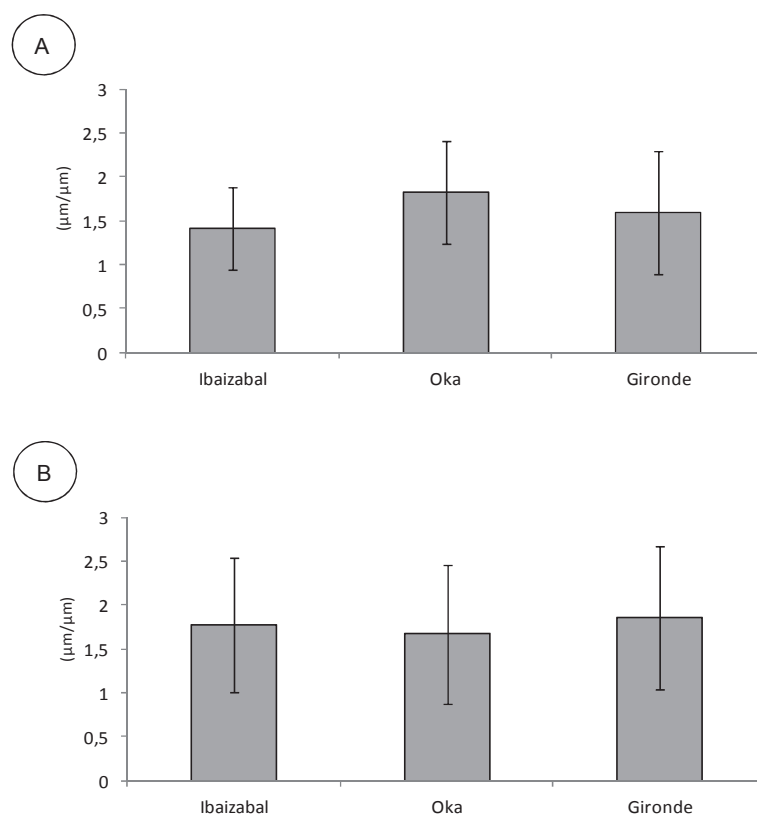


Figure 5: Mean values (\pm standard deviations) for MLR/MET of oysters (A) and mussels (B) from three estuaries in February 2013.

5. Tissue integrity in digestive gland (CTD ratio)

The obtained CTD ratio in oysters and mussels from February 2013, followed the same pattern for both species (Fig. 6 A and B). In both bivalves, the highest values occurred in the Gironde Estuary followed by the Oka and Ibaizabal Estuaries. Indeed, values obtained in oysters from the Gironde Estuary were significantly higher than in the other estuaries.

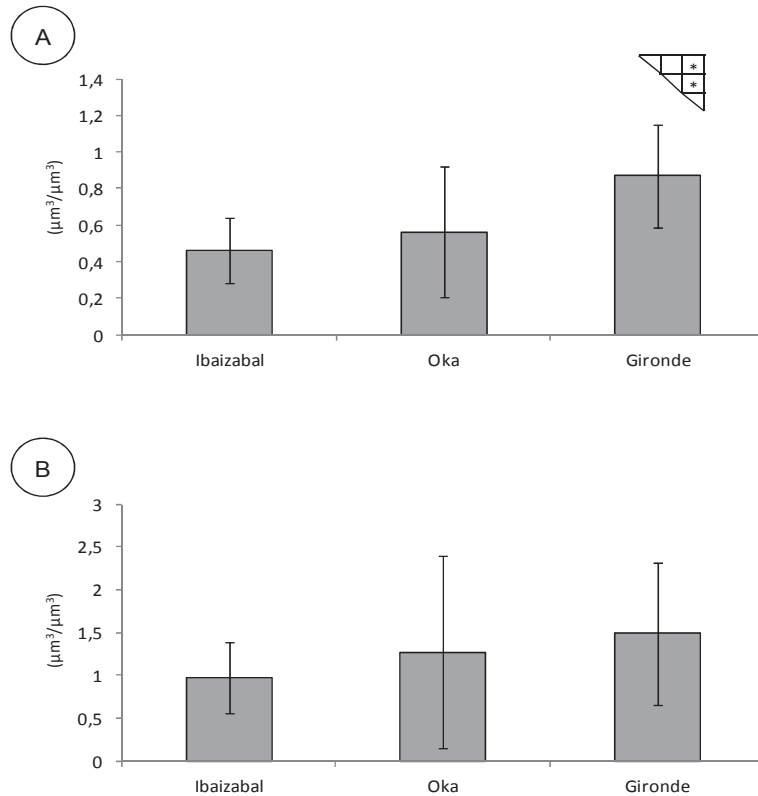


Figure 6: Mean values (\pm standard deviations) of CTD values for (A) oysters and (B) mussels at three different sampling points from the Bay of Biscay. Statistically significant differences ($p < 0.05$) between sampling points in (*).

6. Histopathological alterations

Histopathological alterations occurred in both oysters and mussels. In general, oysters displayed low prevalence of histopathological alterations while mussels from all the sampling points showed higher prevalence of alterations (Table 5). Histopathological alterations were particularly frequent in mussels from the Oka Estuary with 60% of the individuals having haemocytic infiltrations in their tissues and 40% of them showing inflammations. Moreover granulocytomes were also present in 20% of the cases, and parasite (mainly *Mytilicola intestinalis*) prevalence in mussels from the Oka Estuary was 20%. Mussels from the Ibaizabal Estuary also showed haemocytic infiltrations and inflammations but no granulocytome was observed, whereas parasite prevalence was higher than in the Oka Estuary 30% (Fig. 7 D). Finally, lowest histopathological alterations were observed in mussels

from the Gironde Estuary, only 10% of individuals from this site presenting haemocytic infiltrations (Fig. 7 F).

Table 5: Histopathological alterations in oysters and mussels from three estuaries in the Bay of Biscay in February 2013.

February 2013	Estuary	GRN(%)	INF(%)	HAE(%)	PAR(%)
Oysters	Ibaizabal	0	0	0	0
	Oka	0	0	10	0
	Gironde	0	0	0	0
Mussels	Ibaizabal	0	20	40	30
	Oka	20	40	60	20
	Gironde	0	10	10	0

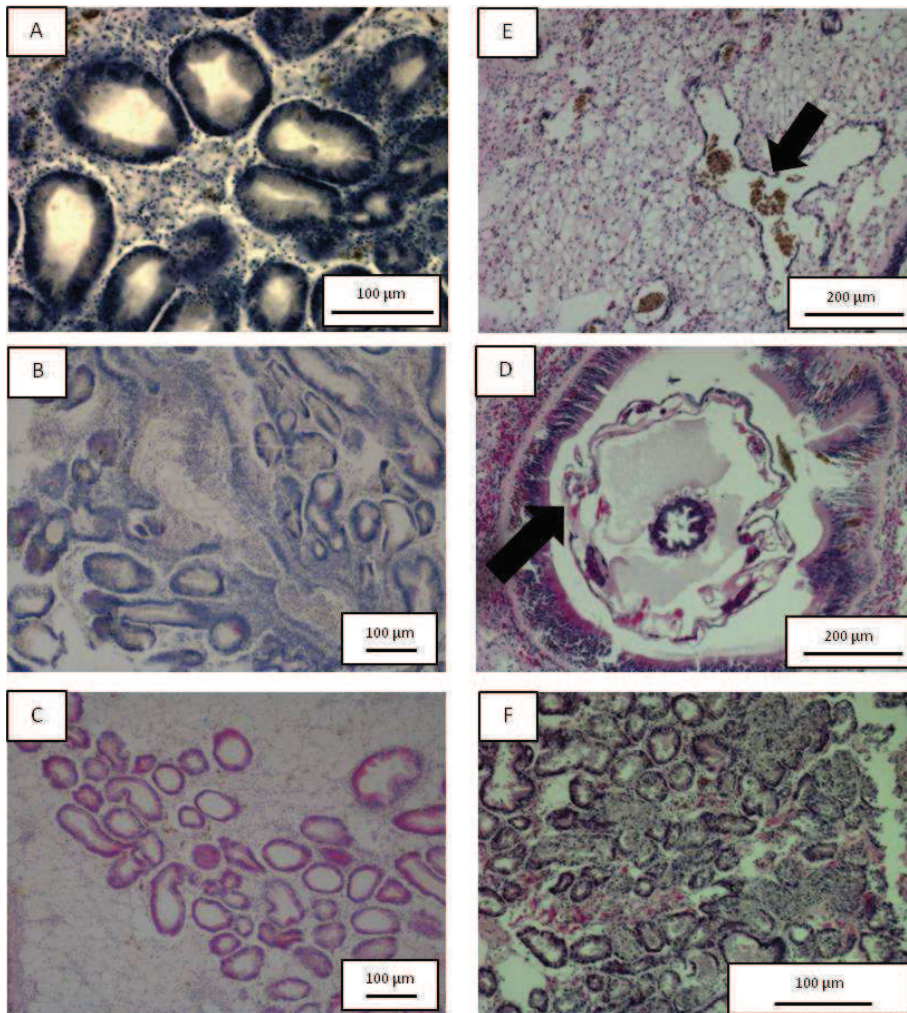


Figure 7: (A) Oysters collected in the Ibaizabal Estuary with low levels of atrophy in digestive gland tubules. (B) Haemocytic infiltration in digestive gland tissue of an oyster from the Oka Estuary. (C) Atrophied digestive gland tubules surrounded by interstitial connective tissue in oyster from the Gironde Estuary. (D) Parasite infection by *Mytilicola intestinalis* in the gut of a mussel from the Ibaizabal Estuary. (E) Empty gonad follicle with Brown cell infiltration in mussel collected in the Oka Estuary. (F) Severe haemocytic infiltration in the digestive gland tissue of a mussel from the Gironde Estuary.

7. Metal distribution and intralysosomal metal accumulation

The distribution of BSD through the tissues of oysters and mussels varied according to the sampling point and specie. Oysters presented higher amounts of BSD in the basal lamina of the digestive gland, particularly in the Gironde site and to a lower extent in the Oka Estuary (Fig. 8 A, B and C). The digestive epithelium of oysters also showed BSD, in particular in bivalves from the Gironde Estuary (Fig. 8 C), and BSD were also present in the haemocytes of the connective tissue that surrounds the digestive gland (Fig. 8 A and B). In mussels, lower amounts of BSD were observed, apart from the intralysosomal accumulation of BSD, BSDs also occurred in the digestive gland interstitial connective tissue. The basal lamina of mussels from the Gironde Estuary was also stained, whereas in the Ibaizabal Estuary mussels, the interstitial connective tissue displayed BSDs between gonads and in one case also within oocytes.

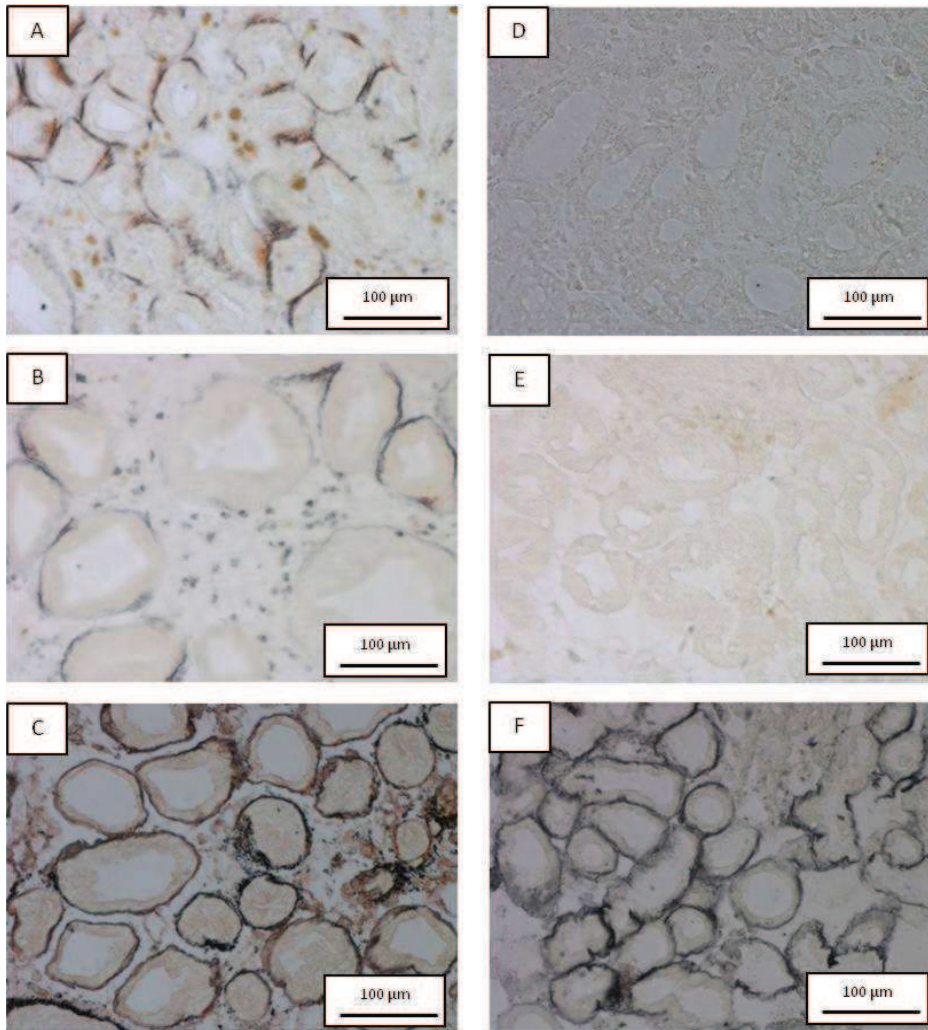


Figure 8: Autometallographical staining of paraffin sections of (A, B and C) oysters and (D, E and F) mussels samples from February 2013 obtained in the (A, D) Ibaizabal, (B, E) Oka and (C, F) Gironde Estuaries. Oysters from the Ibaizabal (A) and Oka (B) Estuaries had slight amounts of BSD in the basal lamina and in the haemocytes. Note the intense staining in oysters from the Gironde Estuary (C). Mussels from the Ibaizabal and Oka Estuaries (D, E) showed very low levels of BSD. Mussels from the Gironde Estuary also had high amounts of BSD in the basal lamina of digestive tubules.

The intralysosomal metal accumulation of BSD in the digestive cells of oysters and mussels followed a similar distribution pattern. In both species, statistically significantly higher amounts of intralysosomal BSDs occurred in Gironde Estuary bivalves. In the Ibaizabal and Oka Estuaries, similar amounts of BSD were present, however higher values for both species were observed in bivalves from the Oka Estuary (Fig. 9).

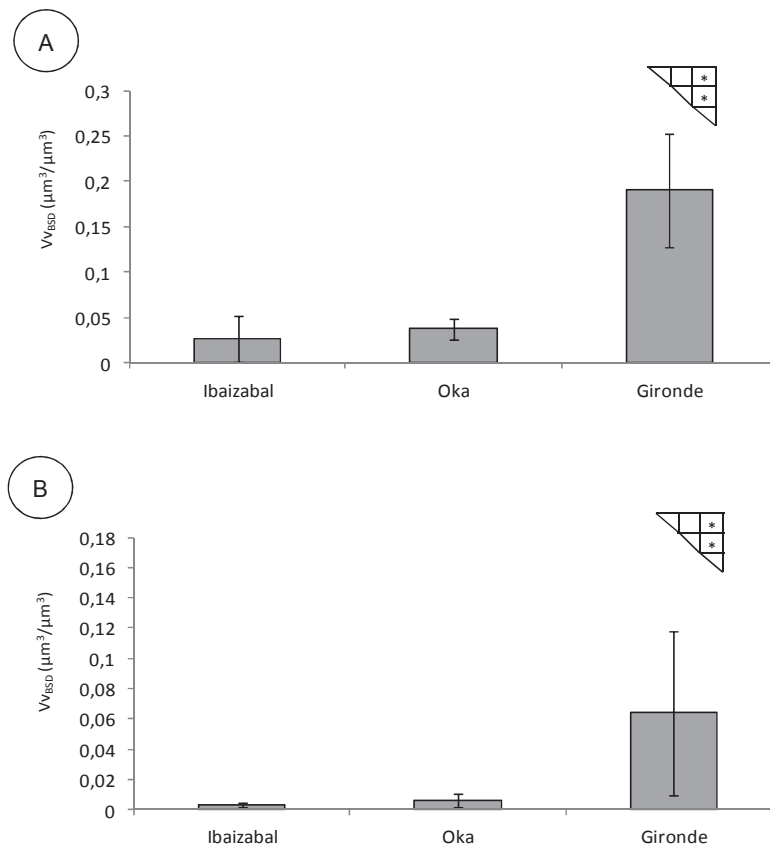


Figure 9: Mean values (\pm standard deviation) of intralysosomal metal accumulation in February 2013 in three different estuaries from the Bay of Biscay for (A) oysters and (B) mussels. Statistically significant differences ($p < 0.05$) between sampling points in (*).

8. Integrative Biological Response (IBR)

The Integrative Biological Response Index (IBR) of oysters varied according to the sampling site (Fig. 10 A). A higher response of oysters from the Gironde Estuary was detected with a marked expression of intralysosomal metal accumulation and CI. Responses in oysters from the Oka and Gironde Estuaries were similar for MLR/MET and CTD ratio, whereas oysters from the Ibaizabal Estuary did not show a noticeable response for biomarkers. The IBR/n (Fig. 10 B) reflected this pattern with higher values for Gironde Estuary oysters followed by oysters from the Oka Estuary and almost no response in Ibaizabal oysters.

A very similar pattern for IBR and IBR/n values was observed for mussels collected in February 2013 (Fig. 10 C and D). Mussels from the Gironde Estuary showed a

marked biomarkers response, in particular for intralysosomal metal accumulation and CI. Meanwhile, mussels from the Oka and Ibaizabal Estuaries responded to MLR/MET and CTD ratio, but in different intensity. Finally, the IBR/n values for mussels from Gironde Estuary were clearly the highest and were followed by those of the Oka and Ibaizabal Estuaries (Fig. 10 D).

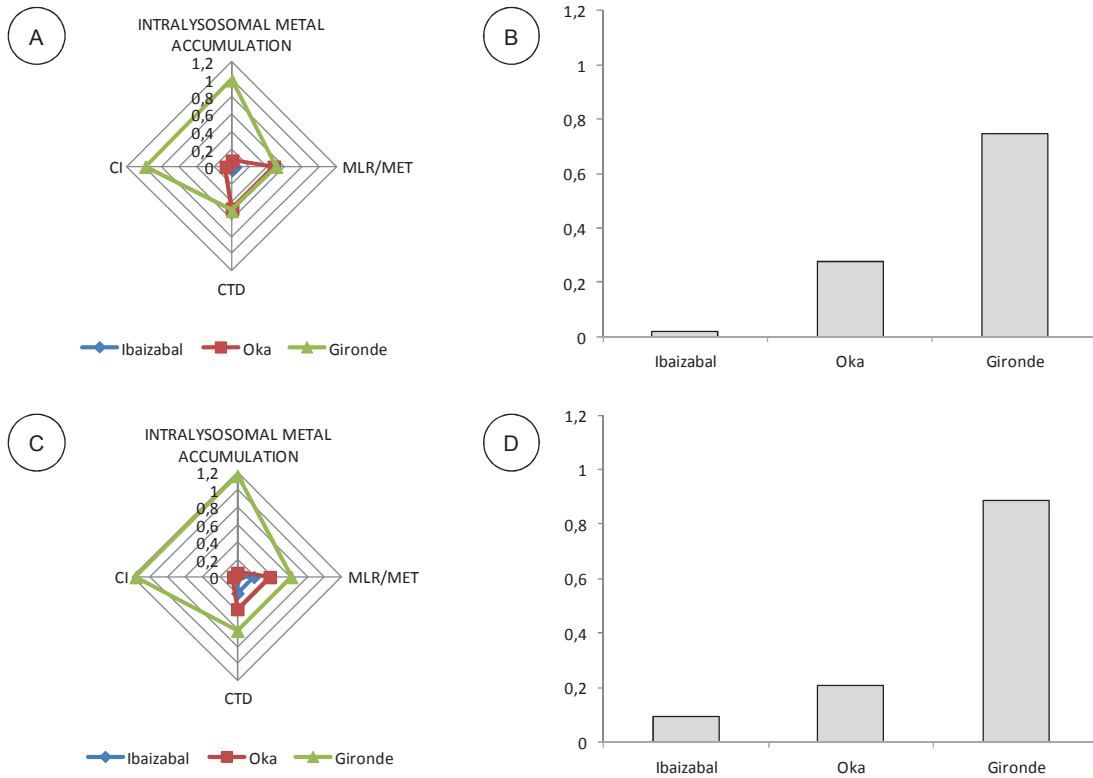


Figure 10: (A) IBR value and (B) IBR/n values for oysters collected in February 2013. (C) IBR value and (D) IBR/n value for mussels collected in February 2013 in three different estuaries from the Bay of Biscay.

DISCUSSION

Wild-living individuals of two different sentinel species (*Crassostrea gigas* and *Mytilus galloprovincialis*) from three representative estuaries of the Bay of Biscay (Ibaizabal, Oka and Gironde) were sampled in February 2013 to assess the health status of the respective aquatic biota using cell and tissue level biomarkers.

The sampling season was selected to avoid possible dilution factors of the metal concentrations in the soft tissues due to the development of gametes or the seasonal related weight variations (Lanceleur et al., 2011b). In this context, the histopathological examination of individuals allowed to determine the gamete developmental stage, being in accordance with the generally described seasonal reproductive cycle for bivalves, which in the studied area consists on: (i) initiation of gametogenesis in late winter or early spring, (ii) maturity and spawning in summer and (iii) gonad reabsorption phase in autumn (Enrriquez-Díaz et al., 2009; Fabioux et al., 2005; Ortiz-Zarragoitia and Cajaraville, 2010). Thus, both oysters and mussels collected in February 2013 at all the studied sites, were in the early stages of gamete development and considered appropriate for chemical analysis (Lanceleur et al., 2011b).

However, although in general the main stages observed were the same in the three estuaries (early development and developing) some differences in the GI index occurred between sites in both species, with an earlier development of gonads observed in individuals from the Oka Estuary. Gonad development in bivalves is known to be governed by two main factors: temperature (affecting speed of gametogenesis) and nutrient availability (affecting the intensity of gametogenesis) (Enrriquez-Díaz et al., 2009; Garmendia et al., 2010). It is conceivable that bivalves from the Gironde Estuary would develop their gonads slightly later due to the lower water temperature (expected from its most northern position). However, the possible

impact of pollutants should not be discarded, since the energy demand required for detoxification can reduce energy investment for reproductive purposes (Rementeria et al., 2016; Séguin et al., 2016) (Further discussed in Appendix I).

At all sites, Cu and Ag concentrations were generally clearly higher in oysters than in mussels. These results are in agreement with previous works (Geffard et al., 2004; Solaun et al., 2013) although it should be noted that for Cu direct comparisons between oysters and mussels are not pertinent since it is known that Cu is a principal component of hemocyanine which is highly present in oysters (Borja et al., 2014). However, Ag not being an essential metal, the different concentrations measured in oysters and mussels suggest different sensibility of the species in terms of Ag accumulation, implying that oysters are (i) more efficient accumulators for Ag and (ii) more suitable for the study of Ag effects in bivalves. In the present work, the difference in metal accumulated in both species was not only observable from total metal concentration in the tissues, but also from the calculated BCF which clearly expressed higher Ag accumulation capacity of oysters compared to mussels. The obtained chemical data for both mussels and oysters from the Ibaizabal and Gironde Estuaries clearly point to a higher metal pressure in the French estuary. In fact, previous studies have shown the highest Ag concentration values in oysters' tissues, mainly in digestive gland, compared to all other Mussel Watch sites along the French coast (Lanceleur et al., 2011b). In contrast, Ag levels in individuals from the Ibaizabal Estuary, were within the regional background values defined for the Basque coast for oysters *Crassostrea gigas* (0.01-16.3 mg.kg⁻¹ for Ag and 17.6-1250 mg.kg⁻¹ for Cu) and mussels *Mytilus galloprovincialis* (0.03-0.12 mg.kg⁻¹ for Ag and 2.07-18.2 mg.kg⁻¹ for Cu) (Solaun et al., 2013). The fact that mussels from the Ibaizabal Estuary had higher Cu concentrations than mussels from the Gironde Estuary is in line with recent work reporting that (i) Cu concentrations in mussels from the nearby Ibaizabal Estuary are among the highest of the Spanish northern

coast (Besada et al., 2014) and (ii) mussels transplanted to Arriluze, i.e. the Ibaizabal Estuary sampling point of the present work located within a leisure marina, experience high Cu accumulation attributed to the use of Cu containing antifouling paints (De los Ríos et al., 2016).

Absolute metal concentrations recorded for oysters from the Gironde Estuary were also in agreement with those of the national Mussel Watch program (RNO/ROCCH) published for the same studied sampling site (Mikolaczyk, 2016). Recent work has attributed a significant decrease in Cu concentrations (49% to 74% from 2001 to 2014 depending on the locality) in juvenile oysters from the Gironde Estuary to lower amounts of copper sulphate used in the composition of vineyards pesticides (Baudrimont et al., 2016).

The Cu/Ag ratios in oysters and mussels, recently proposed as a complementary tool for the interpretation of biomonitoring data (e.g. source discrimination) (Mikolaczyk, 2016), were compared for both sites. This ratio reflects the proportional accumulation of both metals as observed in wild oysters from several sites along the French Atlantic Coast between 2003 and 2014 (Mikolaczyk, 2016). The present results confirm the close relationship between Cu and Ag for the oysters from the Gironde ($R^2=0.8634$), but not for the oysters from the Ibaizabal ($R^2=0.3256$). The average Cu/Ag ratio (51.05) in oysters from the Ibaizabal Estuary being clearly higher than that in the Gironde oysters (39.8) (this study) and in all sites of the French Atlantic coast (Mikolaczyk, 2016) suggests that in the Ibaizabal Estuary the dominance of Cu pressure over that of Ag is greater.

In mussels, the Cu/Ag correlation coefficients were lower than the ones recorded in oysters indicating that the accumulation of both metals is not as proportional as in oysters. However, although a deeper study of the spatial and temporal ranges of this

ratio in mussels is needed, the obtained results seemed to reflect once again the dominant Cu pressure in the Ibaizabal Estuary.

The obtained chemical contents in both oyster and mussel tissues were in good agreement with the intralysosomal metal accumulation observed by autometallography that has been widely employed for metal distribution determination in sentinel organisms (De los Ríos et al., 2016; Raftopoulou and Dimitriadis, 2011; Rodriguez-Iruretagoiena et al., 2016; Soto et al., 2002). Metal quantification by means of BSD quantification clearly discriminated between sampling sites for both bivalve species, with clearly higher contents in individuals from the Gironde Estuary. Furthermore, the BSD quantification also showed higher metal accumulation in oysters, being in good agreement with chemical analyses. Interestingly, differences in the location of metal accumulation between species were observed. In oysters, BSD were preferentially accumulated in the basal lamina of digestive gland tubules (Fig. 8 A, B and C), especially in oysters from the Gironde Estuary. Previous studies on oysters (both wild-living and oysters under experimental conditions) have also reported metal accumulation in this compartment (Amiard-Triquet et al., 1991; Rementeria et al., 2016; Rodriguez-Iruretagoiena et al., 2016). In the case of mussels, BSD typically occur in the lysosomes of digestive cells (Raftopoulou and Dimitriadis, 2011; Soto et al., 2002). In this work, BSD were present in these organelles as expected, but also in the basal lamina of digestive gland tubules in mussels from the Gironde Estuary. A similar distribution pattern was observed in mussels and oysters translocated into the Gironde Estuary (Geffard et al., 2004). This dissimilar distribution suggests a different metal accumulation strategy in oysters and mussels. Indeed, Rementeria et al., 2016 (Chapter IV) suggested that haemocytes could be a relevant site for metal accumulation in oysters and only when their maximum storage capacity exceeded, metals would accumulate in the basal lamina of digestive gland tubules. In contrast, in mussels,

lysosomes of digestive epithelium cells would be preferential storage sites and metals would only appear in the basal lamina of digestive gland tubules under high metal exposure.

Bivalves in general, have a very plastic digestive gland that may undergo several changes even during normal tidal cycles (Langdon and Newel, 1996). These changes can be enhanced due to the presence of different kind of stressors including harvesting or pollutant exposure. In fact, the thinning of digestive gland epithelium is one of the most described alterations that can occur as a consequence of the metal exposure. The accumulation of metals in the digestive cells present in the epithelium and their apocrine extrusion into the lumen has already been described as one of the metal detoxification processes that may occur (Marigómez et al., 2002). Such loss of cells or at least cell fragments, may not only lead to the epithelial thinning but also induce the shrink and reduction of digestive gland tubules resulting in relative augment of connective tissue which is another major alteration of digestive gland (Brooks et al., 2011; Garmendia et al., 2011b; Izagirre et al., 2014; Zaldibar et al., 2007).

The obtained MLR/MET values were close to the MLR/MET values for mussels from the Oka Estuary (Garmendia et al., 2010) and were also similar to the ones obtained for oysters from this estuary in 2011 (Rodríguez-Iruretagoiena et al., 2016). Moreover, the recorded atrophy values in the Ibaizabal Estuary were in concordance with the ones previously described for this estuary in mussels (Garmendia et al., 2011c), suggesting that the results of the present work reflect environmental conditions at pluri-annual timescales.

The CTD ratio showed a similar site distribution pattern for both species with the highest values for the Gironde Estuary. However, the very high CTD values in oysters from the Gironde Estuary, i.e. strong increase in the connective tissue and

reduction of digestive gland tubules, probably reflects digestive cell loss due to high pollutant pressure.

Histopathological alterations have been routinely included in water quality biomonitoring programs in order to assess the effects on aquatic biota caused by pollutant presence (Cuevas et al., 2015). Indeed, different inflammatory responses including granulocytomas in bivalves have been previously linked to the presence of environmental stressors including organic or metallic pollutants (Bignell et al., 2011; Rasmussen, 1986). Moreover increased parasitic infestation has been detected under pollutant exposure (Garmendia et al., 2011b).

In the present study, the alteration prevalence values varied according to the specie and sampling point. In fact, very low alteration levels were detected in oysters from the Oka Estuary with only 10% of individuals presenting haemocytic infiltrations. This value is lower than the ones recorded for this same estuary by Rodríguez-Iruretagoiena et al., (2016) in 2010/2011. The overall low prevalence values obtained for oysters in all sampling points may be indicative of some kind of adaptation of oysters to face chronic metal pollution, whereas a possible season dependence of pathologies should not be discarded (Appendix I). Meanwhile, mussels showed higher prevalence values of lesions than oysters in all sampling points but in particular in the Oka Estuary, for which similar discrimination between species had been previously described (Díez, 1996). In any case, prevalence values in mussels were lower than the ones recorded by Garmendia et al., (2011b) and they in the range of the ones observed for the Oka and Ibaizabal Estuaries by Cuevas et al., (2015). Conversely, it should be mentioned that granulocytomes were present at higher percentages than the maximum values reported by Garmendia et al. (2011b) and Cuevas et al. (2015). Given that this type of alteration has been described also in bivalves under strong metal pollutant exposure (Svärdh and Johannesson, 2002) this point deserves special attention in future studies in the

area (mainly the Oka Estuary). On the other hand, it is noticeable that although relevant histopathological alterations were detected in oysters from Gironde (high levels of CTD), very low levels of other pathologies (parasites, haemocytic infiltrations...) occurred. Some of the lesions studied in this work (granulocytomes or haemocytic infiltrations) may also be linked to the presence of different parasites in molluscan tissues (Bignell et al., 2011). The high Ag and Cu concentrations and the high BSDs levels in bivalves from the Gironde Estuary indicate high metal levels in tissues of those mussels and oysters. Therefore it may hypothesise that due the toxicity of metals the colonization by parasites is limited, which would be consistent with the low levels of immune response in bivalves from the Gironde Estuary.

Condition Index measurements to obtain as general perspective of the physiological status of collected bivalves, have revealed lower levels in oysters from the Ibaizabal Estuary than those from the Oka and Gironde Estuaries, while no CI differences occurred in mussels from the three sites (Fig. 2). This result may suggest that health status of oysters in the Ibaizabal Estuary was affected. However, due to the highly irregular shell shapes of these oysters (compared to those from the Oka and Gironde Estuaries) bias in CI determination cannot be excluded.

The IBR index based on intralysosomal metal accumulation, MLR/MET ratio, CTD ratio and CI suggests that the worst health status occurred in both oysters and mussels coming from the Gironde Estuary, probably due to the chronic metal contamination of this estuary.

This responsiveness pattern was also reflected by the IBR/n values, with clearly higher values for bivalves from the Gironde Estuary. It is noticeable that higher IBR/n values were observed for the Oka Estuary than for the Ibaizabal Estuary (Fig. 10). The Ibaizabal Estuary has commonly been considered a heavily polluted area, in contrast to the Oka Estuary (Solaun et al., 2013). However, recent work has

shown reduction in lysosomal membrane stability, peroxisome proliferation and gamete atresia in mussels transplanted into the Oka Estuary concluding that this estuary is not pristine (De los Ríos et al., 2016). Indeed, the obtained IBR scores for the Ibaizabal and Oka estuaries after 21 days were very similar to those previously reported (De los Ríos et al., 2016).

In conclusion, both sentinel species provided consistent information suggesting the highest metal exposure in the Gironde Estuary compared to the other estuaries. Although biomarkers differed in their response magnitude they showed in general a similar response pattern for both species. In this context, the IBR index appeared as an alternative tool not only to integrate the measured biomarkers but also to allow comparisons between species, since it reduced the differences in response magnitudes between sentinel organisms. However, the ability of oysters to accumulate Ag at higher levels combined with the relatively low amount of pathological alterations (infiltrations, parasites...) that may interfere on tissue level biomarkers (MLR/MET, CTD) make them a very suitable organism for the determination of environmental Ag pressure and toxicity.

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Chapter II

Assessment of the effects of Cu and Ag in oysters *Crassostrea gigas* (Thunberg, 1793) using a battery of cell and tissue level biomarkers

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ABSTRACT

Assessment of the effects of Cu and Ag in oysters *Crassostrea gigas* (Thunberg, 1793) using a battery of cell and tissue level biomarkers.

Oysters are considered sentinel organisms in environmental water quality monitoring programs in which cell and tissue level biomarkers are reliable tools. Copper (Cu) and silver (Ag) are present in relatively high concentrations in several estuaries, potentially affecting environmental and human health. *Crassostrea gigas* oysters were exposed during 28 days to a range of environmentally relevant concentrations of Cu and Ag alone or in mixture. Effects were studied through cell and tissue level biomarkers approach. Results indicated: changes in the Condition Index (CI), altered digestive gland epithelium and presence of histopathological alterations in the gonad and digestive gland of exposed oysters. A time-dependent increase in lipofuscin contents in exposed oysters and an increase in intralysosomal metal accumulation in digestive cells through the experiment were also recorded. The Integrative Biological Response (IBR) Index showed that even at low exposure levels, Ag and Cu can produce alterations on oysters' health status.

RÉSUMÉ

Évaluation des effets de Cu et Ag dans les huîtres *Crassostrea gigas* (Thunberg, 1973) en utilisant une batterie de biomarqueurs aux niveaux cellulaires et tissulaires.

Les huîtres sont considérées comme des organismes sentinelles par les programmes de surveillance de la qualité de l'eau et de l'environnement qui considèrent les biomarqueurs comme des outils fiables de contrôle. Le cuivre (Cu) et l'argent (Ag) sont présents à des concentrations relativement élevées dans plusieurs estuaires, affectant potentiellement l'environnement et la santé humaine. Des huîtres *Crassostrea gigas* ont été exposées pendant 28 jours à une gamme de concentrations de Cu et Ag seuls ou en mélange préalablement observés dans l'environnement. Les effets produits ont été étudiés par l'approche des biomarqueurs cellulaires et tissulaires. Les résultats indiquent: des variations de l'indice Condition (CI), des modifications dans l'épithélium de la glande digestive et la présence d'altérations histopathologiques dans les gonades et la glande digestive d'huîtres exposées. Les contenus de lipofuscine étaient supérieurs dans les huîtres exposées. Une augmentation dans l'accumulation de lipofuscine dans les huîtres exposées a été enregistrée au cours de l'expérience. L'index connu comme Integrative Biological Response (IBR) index, a montré que, même à faibles niveaux d'exposition, Ag et Cu peuvent altérer l'état de santé des huîtres.

LABURPENA

Kobrea eta zilarraren efektuen azterketa *Crassostrea gigas* (Thunberg, 1793) ostretan zelula eta ehun mailako biomarkatzaileen bateria bat erabiliz.

Ostrak ingurumen uren kalitatearen jarraipen programetan espezie behale gisa erabiltzen dira, eta programa hauetan zelula eta ehun mailako biomarkatzaileak tresna baliagarriak direla kontsideratzen da. Kobrea (Cu) eta zilarra (Ag) zenbait estuariotan kontzentrazio altuetan agertzen dira ingurumen eta baita gizakien osasunaren gain eragin kaltegarriak sortuz. *Crassostrea gigas* ostrak 28 egunez ingurumenean gailendu daitekeen Cu eta Ag kontzentraziopean jarri ziren, bakarka zein nahasketan. Efektuak zelula eta ehun mailako biomarkatzaileen bitartez neurtu ziren. Emaitez erakutsi zuten zenbait aldaketa behatu zirela “Egoera-Indizean; ingelesez Condition Index (CI)”, bestetik asaldaturako digestio-epitelioa behatu zen eta baita zenbait alterazio histopatologiko ere behatu ziren gonada zein digestio-guruinean metalenpean jarritako ostretan. Denboraren menpekota zen lipofuszen metaketa zein lisosometako metalen metaketa bat ere neurtu zen esperimentuan zehar. Integrative Biological Response (IBR) indizeak erakutsi zuen bai Ag zein Cu dosi baxuek asaldurak eragin ditzaketela ostren osasun egoeran.

RESUMEN

Evaluación de los efectos del Cu y Ag en ostras *Crassostrea gigas* (Thunberg, 1793) utilizando una batería de biomarcadores celulares y tisulares.

En los programas de seguimiento de la evaluación de la salud del agua, las ostras son ampliamente empleadas como organismos centinela y los biomarcadores celulares y tisulares son herramientas fiables. Algunos estuarios presentan concentraciones de cobre (Cu) y plata (Ag) relativamente altas que potencialmente pueden afectar a la salud del medio e incluso a la salud humana. En el presente estudio, ostras de la especie *Crassostrea gigas* fueron expuestas a concentraciones relevantes desde el punto de vista ambiental de Cu y Ag individualmente y en combinación durante 28 días. Los efectos producidos se estudiaron empleando biomarcadores celulares y tisulares. Los resultados indicaron cambios en el índice de condición, alteraciones en el epitelio digestivo y alteraciones histopatológicas en la gónada y glándula digestiva de las ostras expuestas a los contaminantes. Se observó además, una respuesta dependiente del tiempo en la acumulación de lipofuscinas y un incremento en la acumulación intralisosómica de metales en la glándula digestiva a lo largo del experimento. El índice denominado "Integrative Biological Response (IBR)" demostró que incluso a dosis bajas la Ag y el Cu producen alteraciones en el estado de salud de las ostras.

INTRODUCTION

During the last few decades, aquatic environments over the world have suffered from different anthropogenic pressures, leading to an increasing presence of pollutants in estuaries (David et al., 2012; Millward and Turner, 2001). Although a general decrease in metal pollution levels has been described for several estuaries in Europe, the persistence of metals in estuaries is still high enough to provoke deleterious effects on the biota and some metals even show locally increasing trends in marine biota (Lanceleur et al., 2011a; Solaun et al., 2013).

Silver (Ag) and copper (Cu) are considered amongst the most toxic metals for marine biota (Haberkorn et al., 2014; Money et al., 2011; Ratte, 1999; Santovito et al., 2015; Tappin et al., 2010). Copper is an essential micronutrient for living organisms but at high concentrations induces adverse health effects (Haberkorn et al., 2014; Ringwood et al., 1998; Santovito et al., 2015) and Ag is an especially toxic element, even at low exposures (Ratte, 1999; Tappin et al., 2010). Silver inputs have been closely related with anthropogenic activities since ancient times (metallurgy, jewellery, electronics...) (Lanceleur et al., 2013) and recently, new sources for this metal are described including cloud seeding practices and massive use of personal care products containing Ag nanoparticles (Fabrega et al., 2011; Lanceleur et al., 2011b; Luoma et al., 2008). Copper sources are to some extent similar to those of Ag, however the use of fertilisers, pesticides, algaecides and antifouling paints together with domestic sewage constitute some of the major inputs for this metal (Lee et al., 2010).

Previous research works have indicated the deleterious effects that Ag and Cu, alone or in combination, may exert in invertebrates (Amiard-Triquet et al., 1991; G eret et al., 2002), however in these experiments relatively high concentrations of Ag and Cu were used. More recently, spikes of stable isotopes of Cd have been

presented as an alternative allowing the detection and quantification of metal accumulation in oyster tissues after exposure to relatively low and therefore close-to-real environment concentration levels (Mikolaczyk et al., 2016). Presently, a similar approach is proposed with oyster exposure to Ag and Cu stable isotopes at concentrations close to the ones described in the environment that ranged from values below 0.05 ng/L to over 100 ng/L for Ag (Barriada et al., 2007; Flegel et al., 2007) and values over 5 µg/L for Cu (Ellingsen et al., 2005). Furthermore, since natural environments present several stressors at the same time, the understanding of the joint effects exerted by the combination of a variety of stressors (pollutants) in sentinel organisms is of increasing interest.

Bivalves, such as oysters, have been considered relevant candidates to monitor environmental health status of aquatic ecosystems (David et al., 2012; Kim et al., 2008; Rodriguez-Iruretagoiena et al., 2016), while cell and tissue level biomarkers have been recently proved to be reliable tools in biomonitoring programs carried out in coastal and estuarine areas subjected to metal pollution (Marigómez et al., 2013; Séguin et al., 2016). The application of a battery of cell and tissue level biomarkers and their subsequent integration into a more comprehensive index such as the Integrative Biological Response Index (IBR Index) has been proposed as a powerful tool to carry out an overall assessment of the health status of sentinel organisms and, hence of the ecosystem (Beliaeff and Burgeot, 2002; Broeg and Lehtonen, 2006; Marigómez et al., 2013).

In the present work, a battery of cell and tissue level biomarkers has been applied and integrated into the IBR index for oysters *Crassostrea gigas* exposed to relatively low concentrations of Ag and Cu. The IBR index integrated biomarker responses from tissue to individual level, including histopathological alterations (digestive gland atrophy, tissue integrity in the digestive gland), intralysosomal metal accumulation, metallothionein (MT) concentrations and Condition Index (CI). Additionally, the

applied battery of measured biomarkers also included: neutral lipid and lipofuscin content determination and gamete developmental stage determination.

The objectives of this work were (i) to analyse the responses of a battery of recognised biomarkers at different organisational levels of *Crassostrea gigas* exposed to Ag and Cu at concentrations close to the ones found in the environment, and (ii) to integrate the most relevant responses into the IBR index in order to assess the overall health status of sentinel oysters and to verify its validity to be routinely applied in biomonitoring programs in estuaries.

MATERIALS AND METHODS

1. Experimental design

Oysters *Crassostrea gigas* (7-8 cm long) were purchased in April 2013 from an oyster farm (Ostranor S.L) located in a relatively pristine area (San Vicente de la Barquera, Cantabria, Spain). Recent works carried out in bivalves collected in the vicinity of the farm (De los Ríos et al., 2016) and in oysters directly collected from the farm (unpublished data from Ostranor) reported low Cu levels ($2.07 \pm 0.75 \mu\text{g Cu/g dw}$) while Ag was under the detection limit. Then, oysters were transferred to Plentzia Marine Station (Plentzia, Basque Country, Spain), acclimatised and depurated in naturally filtered sea water ($\text{pH} = 7.92 \pm 0.05$; $T = 15.48 \pm 0.18 \text{ }^\circ\text{C}$; $\text{salinity} = 28.18 \pm 0.08$) with a continuous water and air flow during 4 days and photoperiod was established at 12 h:12 h (light:dark).

The experiment was carried out in conjunction with Mikolaczyk et al. (2016). Briefly, oysters were distributed into 40 L high density polypropylene aquaria containing naturally filtered sea water and were directly exposed to a range of environmentally relevant concentrations of Ag and Cu as mixtures of ^{107}Ag and ^{63}Cu for 28 days. Solutions of stable isotopically-labelled Cu and Ag were prepared as described in Mikolaczyk et al. (2016). Four experimental groups were defined combining high

and low metal concentrations of both isotopes: 500 ng $^{107}\text{Ag}/\text{L}+2000$ ng $^{63}\text{Cu}/\text{L}$ (HAg+HCu), 500 ng $^{107}\text{Ag}/\text{L}+1000$ ng $^{63}\text{Cu}/\text{L}$ (HAg+LCu), 50 ng $^{107}\text{Ag}/\text{L}+2000$ ng $^{63}\text{Cu}/\text{L}$ (LAg+HCu) and 50 ng $^{107}\text{Ag}/\text{L}+1000$ ng $^{63}\text{Cu}/\text{L}$ (LAg+LCu). A parallel control series without Cu and Ag was also carried out. Stable isotopes were chosen for mixture exposures in order to study possible antagonism or synergistic relationships between both metals through highly sensitive isotope detection techniques (Strady et al., 2011), which allowed the detection of metal accumulation kinetics in tissues of individual oysters over the exposure period (Mikolaczyk et al., 2016). In addition to the experimental set up described in Mikolaczyk et al. (2016) single exposures of Ag and Cu were prepared diluting AgNO_3 and CuCl_2 salts, respectively, in distilled water to give two experimental groups: 500 ng Ag/L (HAg) and 2000 ng Cu/L (HCu). Single exposures were selected due to the high toxicity of these ions over a known concentration threshold (Ratte 1999; Money et al., 2011).

Direct exposure to waterborne contaminants was assured by daily feeding the oysters during 4 h in separate clean tanks containing non polluted filtered natural sea water and commercial food (Marin Coraliquid, Sera® Ltd., GmbH Heinsberg, Germany). Meanwhile, exposure tanks were cleaned and sea water was renewed. Then, animals were placed back into their corresponding exposure tanks and metal solutions were spiked; limiting exposure time to 20 h per day. Water volume and food amount for each experimental tank was proportionally adapted through the experiment to the remaining oyster individuals to minimise possible changes in food or pollutant availability for oysters: 80 oysters/30 L (0-7 days), 60 oysters/20 L (7-14 days), 40 oysters/10 L (14-21 days) and 20 oysters/10 L (21-28 days).

2. Sample collection and processing

Fifteen individuals were collected from each experimental group at days 0, 7, 14, 21 and 28. Oysters were opened and shell and flesh weights were individually measured for the calculation of Condition Index (CI). Then, five of the collected

animals were dissected for organotropism analysis of accumulated metal isotopes (Mikolaczyk et al., 2016), while the remaining 10 individuals were dissected for biomarker studies.

For histology, a ~5 mm thick cross-section of the soft body including all main organs and tissues (gills, digestive gland and gonad) was obtained per animal ($n=10$). Sections were rinsed in formalin fixative for 24 h and routinely processed for histological examination.

Small pieces of five digestive glands per experimental group were rapidly frozen in liquid nitrogen for cryosectioning. The remaining digestive glands were also frozen and stored at -80°C until processing for metallothionein content determination.

3. Biological measurements

3.1. Condition Index

Oyster's Condition Index (CI) was calculated according to the formula: $CI = (\text{WW visceral content} / \text{WW shell} \times 100)$ (in grams) (Strady et al., 2011).

3.2. Digestive gland and gonad histology

From digestive gland and gonads, 4 μm thick sections were obtained in a microtome from paraffin embedded samples and stained with haematoxylin-eosin. Afterwards, microscopic slides were analysed under a light microscope (Olympus BX 61).

3.2.1. Gamete developmental stage

Sex and gonad developmental stages were determined as well as Gonad Index (GI Value) for each individual according to the procedure described by Kim et al. (2006) which is based on a subjective scale of the developmental stage of follicles and gametes after examination under the light microscope.

3.2.2. Histopathological alterations

3.2.2.1 Digestive gland atrophy

A planimetric procedure was followed in order to determine changes in the morphology of digestive tubules (Garmendia et al., 2011). Micrographs of digestive gland tubules were obtained with a digital camera attached to an Olympus BX-61 microscope (Olympus, Japan). Five fields of digestive glands were recorded per oyster, and 2 tubule profiles per field were transferred into an image analysis system Visilog 5.4 Noesis to calculate the following parameters and ratios: MET (Mean epithelial thickness, μm), MLR (Mean luminal radius, μm) and MLR/MET ratio.

3.2.2.2 Tissue integrity in digestive gland (CTD ratio)

The integrity of the digestive gland tissue was determined by calculating the connective-to-diverticula (CTD) ratio. For this, 5 fields of digestive gland tubules were obtained per individual with a digital camera attached to an Olympus BX-61 microscope (Olympus, Japan) and processed through Image J program (Image Processing and Analysis in Java, Maryland, USA). CTD ratio is defined as the extent of the interstitial connective tissue relative to the area occupied by digestive diverticula. The following formula was applied to obtain CTD ratio: $\text{CTD ratio} = c / (b + d + l)$. Where: (c) interstitial connective tissue, (b) basophilic cells, (d) digestive cells and (l) diverticular lumen (Brooks et al., 2011).

Histopathological analyses were completed in oyster's whole body cross-sections. Prevalences of the most relevant alterations were obtained: Granulocytomes (GRN), Inflammations (INF), Haemocytic infiltrations (HAE), Haemocytic infiltrations in Gonads (HAE gn) and Parasites (Par).

3.3. Neutral lipid content determination

Frozen 8 μm thickness sections from digestive glands ($n=5$) were obtained in the cryostat at -26°C chamber temperature. Slides were stored at -40°C and stained following Lillie and Ashburn's Oil Red O (ORO) method (Culling, 1974). The volume density ($V_{V_{\text{NL}}}$) of neutral lipids was quantified in respect to connective tissue by using Image J program. $V_{V_{\text{NL}}}$ was calculated as the ratio between neutral lipid volume and connective tissue volume ($V_{V_{\text{NL}}} = V_{\text{NL}}/V_{\text{CT}}$). Where: (V_{NL}) volume of neutral lipids, and (V_{CT}) volume of connective tissue (Marigómez and Baybay-Villacorta, 2003).

3.4. Lipofuscin content determination

Frozen 8 μm thickness sections from digestive glands ($n=5$) were obtained in the cryostat at -26°C chamber temperature, slides were stored at -40°C until processing for histochemical detection of lipofuscins through the Schmorl method (Pearse, 1985). The volume density ($V_{V_{\text{LF}}}$) of lipofuscins was quantified with respect to digestive tissue by using Image J program, as $V_{V_{\text{LF}}} = V_{\text{LF}}/V_{\text{DT}}$, where: (V_{LF}) volume of lipofuscins, and (V_{DT}) volume of digestive tissue (Marigómez et al., 2013).

3.5. Intralysosomal metal accumulation

Histochemical detection of metals was carried out in 4 μm thick sections of paraffin embedded samples ($n=10$ per treatment). After embedding, sections were dewaxed in xylene and hydrated, then the slides were left at 37°C oven at least 24 h for drying. Samples were covered with the commercial silver enhancement kit (Silver enhancing kit for light and electron microscopy, BB International) at room temperature, development was checked under light microscope and stopped after 15-20 min. Slides were then washed with tap water and metals were developed as black silver deposits (BSD). Finally, slides were mounted in Kaiser's glycerine

gelatine and volume density of BSDs ($V_{V_{\text{BSD}}}$) in the lysosomes of digestive gland cells was measured with an image analysis system (Soto et al., 2002).

3.6. Metallothionein concentration

Frozen and stored (-80°C) digestive gland samples ($n=10$ per treatment at each sampling time) were pooled in order to obtain at least 1 g tissue. Several digestive gland pools (3 to 5 depending on the sample size) were obtained per experimental unit and sampling day. Afterwards, metallothionein (MT) content was determined spectrophotometrically according to UNEP/RAMOGÉ (United Nations Environment Programme) method modified from (Viarengo et al., 1997). The supernatant absorbance was evaluated at 412 nm and MT concentration was estimated using reduced glutathione (GSH) as reference standard. Results are expressed as mean MT concentrations (MT $\mu\text{g/wet weight tissue g}$) for each digestive gland pool.

3.7. Integrative Biological Response (IBR) index

Integrative Biological Response (IBR) index was calculated following the procedure described by Beliaeff and Burgeot, 2002. A total of 5 biomarkers were used for this purpose ordered from less biological complexity level to highest: subcellular level (metallothionein content), cellular level (intralysosomal metal accumulation), tissue level (MLR/MET), organ level (CTD index) and individual level (Condition Index). Finally, as the IBR value depends on the number of applied biomarkers, the IBR/ n was obtained dividing IBR by the number of biomarkers applied ($n=5$) (Brooks et al., 2011; Marigómez et al., 2013).

3.8. Statistics

All statistical analyses were carried out with the SPSS v 22.0 (SPSS. Inc., Chicago, Illinois). Analyses of variance (ANOVA) were performed after checking data normality and homogeneity (Kolmogorov Smirnov and Levene's test) for determining

significant differences between experimental groups ($p < 0.05$) followed by Duncan's post hoc test. In those cases where data did not have normal and homogeneous distribution, Kruskal Wallis non parametric method was used (Dunns post hoc test).

RESULTS

Low mortality values were recorded in all experimental groups with values ranging from 13.75% in HAg and 12.5% to 0% in HAg+LCu after 28 days of exposure. Controls presented a mortality of 5% at day 28. Mortality values for isotopically spiked oysters are explained in detail in Mikolaczyk et al. (2016). On the other hand, chemical analyses for oysters from the present experiment indicated that significant Ag and Cu accumulation occurred in tissues (gills, digestive gland, digestive gland+gonads, muscle and mantle) (Mikolaczyk et al., 2016).

1. Condition Index (CI)

No statistically significant differences between experimental groups were found, however a progressive decrease of the CI through the experimentation period occurred indicating a general mass loss of individuals.

2. Gamete developmental stage

A progressive development of the gonad through the experiment from mid to late development occurred. Although no clear pattern of GI values was observed between experimental groups, lowest values were found at 7 and 14 days in oysters from HCu. The percentage of spawned oysters increased from 21 days on, reaching maximum (up to 20 %) at 28 days, especially for the highest exposure levels. Moreover, some oysters presented haemocytic infiltrations in gonads (HAE gn), suggesting a progressive arrest of the gamete development (Fig. 1).

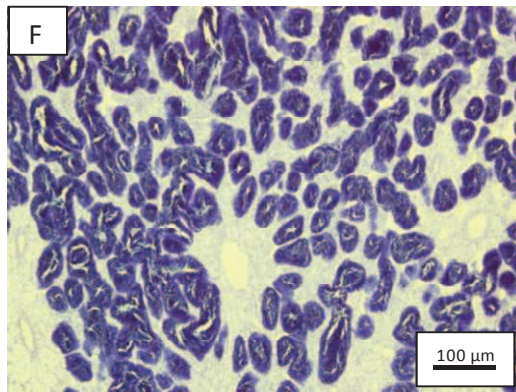
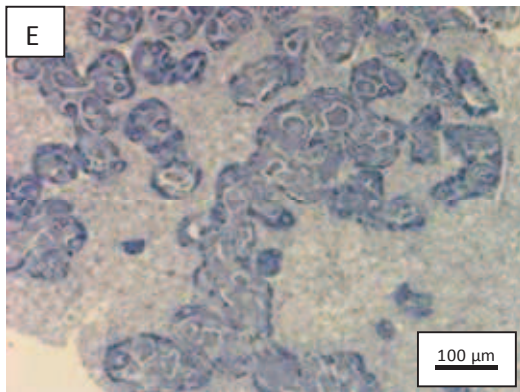
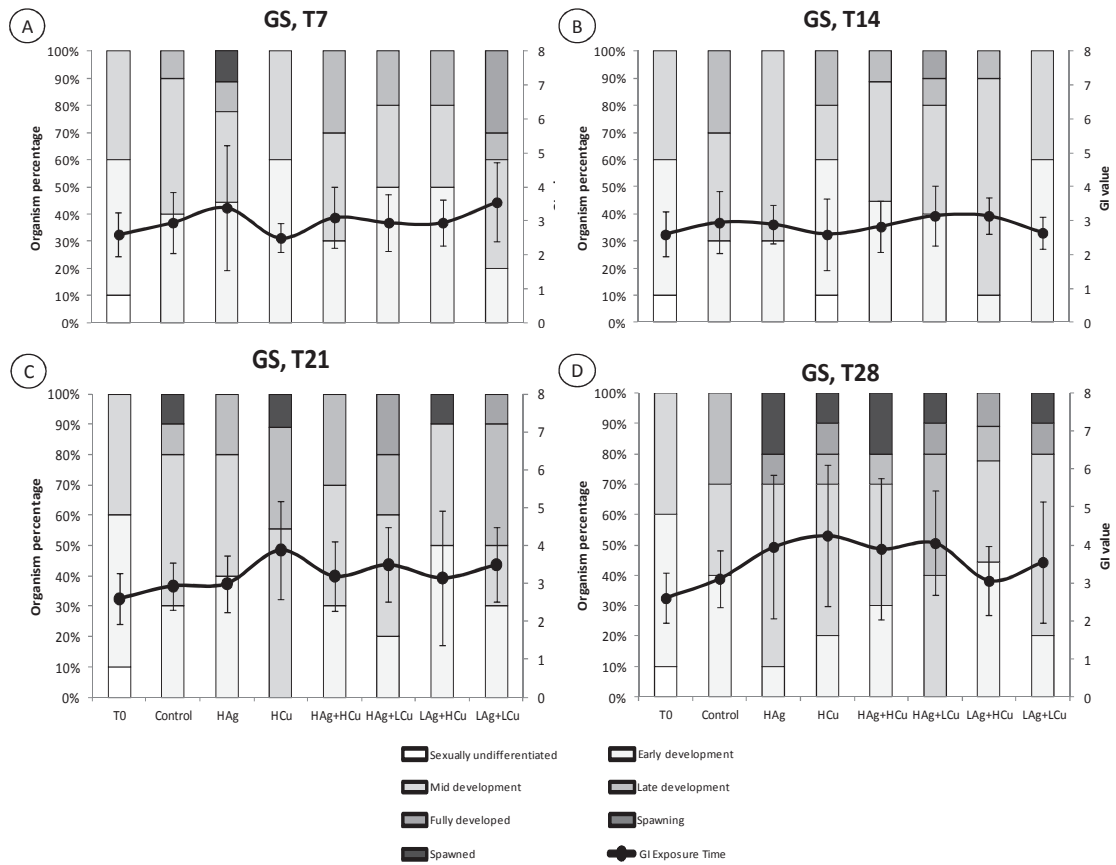


Figure 1: Percentages of gamete developmental stage (stacked bars) and mean values (\pm standard deviations) of the Gonad Index values (represented with a line) for 7 (A), 14 (B), 21 (C) and 28 days (D). Atresic female gonad at day 28 in HAg+LCu exposed oysters (E) and (F) developing male gonad at day 7 in control group, scaled with GI=4.

3. Digestive gland atrophy

Differences for MLR/MET ratio, between experimental groups were not statistically significant after 7 days of exposure, although a slight increase in the atrophy of oysters exposed to HAg+HCu appeared. After 28 days, oysters exposed to LAg+LCu and HAg exhibited significantly higher values of atrophy than controls (Fig. 2).

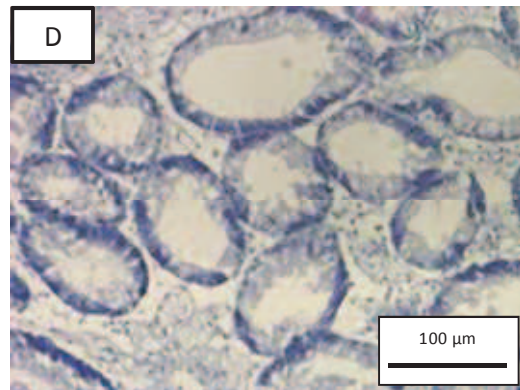
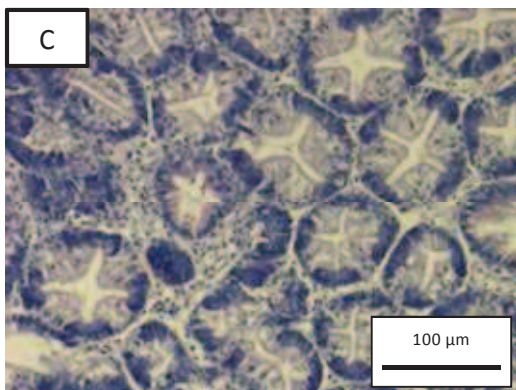
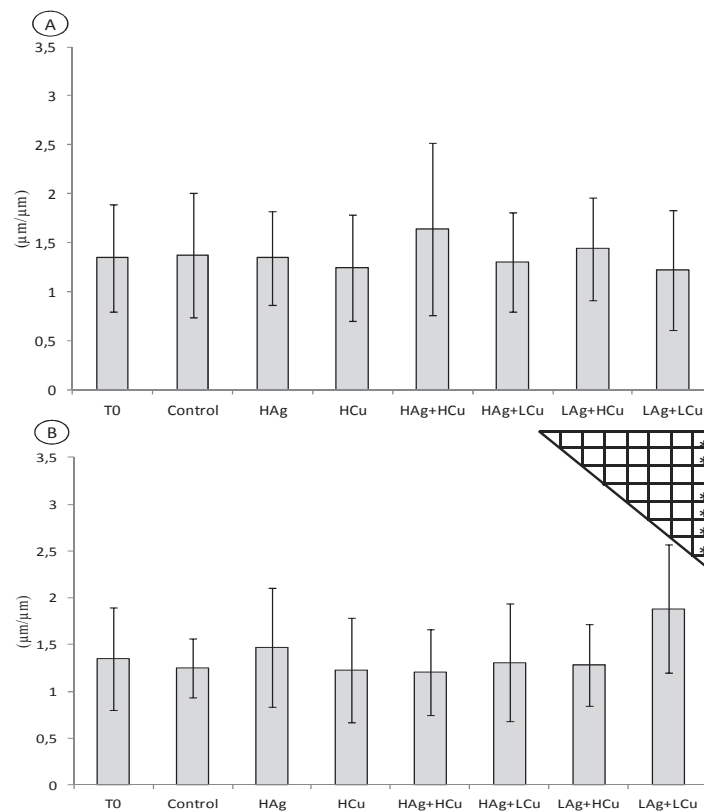


Figure 2: Mean values (\pm standard deviations) of digestive gland atrophy (MLR/MET) at 7 days (A) and 28 days (B). (C) normal digestive tubules from control group at 28 days and (D) atrophied tubules from LAg+LCu exposure group in 28 days. Significant differences between groups * ($p < 0.05$).

4. Tissue integrity in digestive gland (CTD ratio)

No significant differences occurred between groups at 7 days, although CTD values increased in oysters exposed to HAg. After 28 days of exposure, significant differences for CTD index were observed, with the highest values occurring in oysters exposed to HAg and LAg+LCu. Clearly lower values occurred in oysters from: Control group, LAg+HCu and HCu (Fig. 3). On the other hand, a decrease in CTD ratio was observed for the Control group.

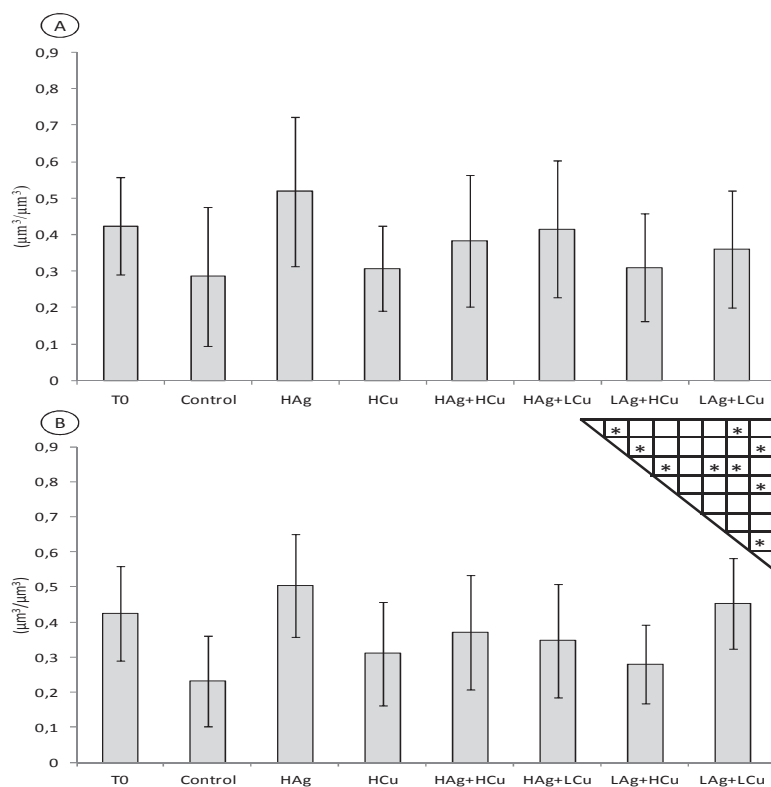


Figure 3: Mean values (\pm standard deviations) of tissue integrity in the digestive gland (CTD) at 7 (A) and 28 days (B). Significant differences between groups * ($p < 0.05$).

Regarding other types of pathologies, a gradual increase of HAE in gonads through time was detected in oysters exposed to HAg+HCu. These oysters showed an increase from 14 (10%) to 28 day (20%). Similarly, oysters exposed to HAg+LCu also had higher values through the time from 0% at day 14 to 20% after 28 days. Parasite prevalence also followed an increasing tendency through time in oysters

exposed to HAg+HCu changing from 10% at 21 days to 20% at 28 days (Fig. 4 A and B).

5. Neutral lipid content determination

No significant differences occurred between experimental groups at any sampling time. Neutral lipids were mainly detected in the connective tissue of oysters and to a lesser extent in female gametes and in digestive gland ducts (Fig. 4 C and D).

6. Lipofuscin content determination

Significant differences in lipofuscin contents between experimental groups were found at day 7 and 21. At the first sampling day, higher lipofuscin contents were found in oysters exposed to HAg+LCu and Control oysters. From day 7 to day 14, a decrease in lipofuscin contents occurred in the majority of the experimental groups while lipofuscins in Control oysters remained stable. At day 21, statistically significant higher values were found in Control group oysters. After 28 days, lipofuscin contents increased in all the treatments, mainly after exposure to high Ag concentrations alone or in mixture (Fig. 4 E and F).

7. Metal distribution

Metals were detected as black silver deposits (BSD) in the basal lamina of Control and T0 oysters and to a lower extent in the lysosomes of the digestive cells and in the haemocytes of the connective tissue which surrounded the digestive tubules (Fig. 4G). Exposure to metals increased intralysosomal metal accumulation after only 14 days of exposure (Fig. 4 H). The BSD present in the gills were very scarce and mainly localised in haemocytes and in the apical part of the frontal cells (results not shown). Only BSD present in the lysosomes of digestive gland cells were quantified to obtain intralysosomal metal accumulation as volume density of BSD (V_{BSD}).

Significant differences in intralysosomal metal accumulation between experimental groups were obtained for all sampling times (Fig. 5), the lowest V_{BSD} values being recorded in oysters at 0 days. After 7 days, V_{BSD} were significantly higher in oysters exposed to HAg+HCu with a great variability that was reduced after 14 days (Fig. 5 A-B). After 21 days, oysters exposed to LAg+HCu and HAg+HCu exhibited higher amounts of V_{BSD} (Fig. 5 C). After 28 days of exposure, HAg and HAg+HCu had the highest amounts of V_{BSD} .

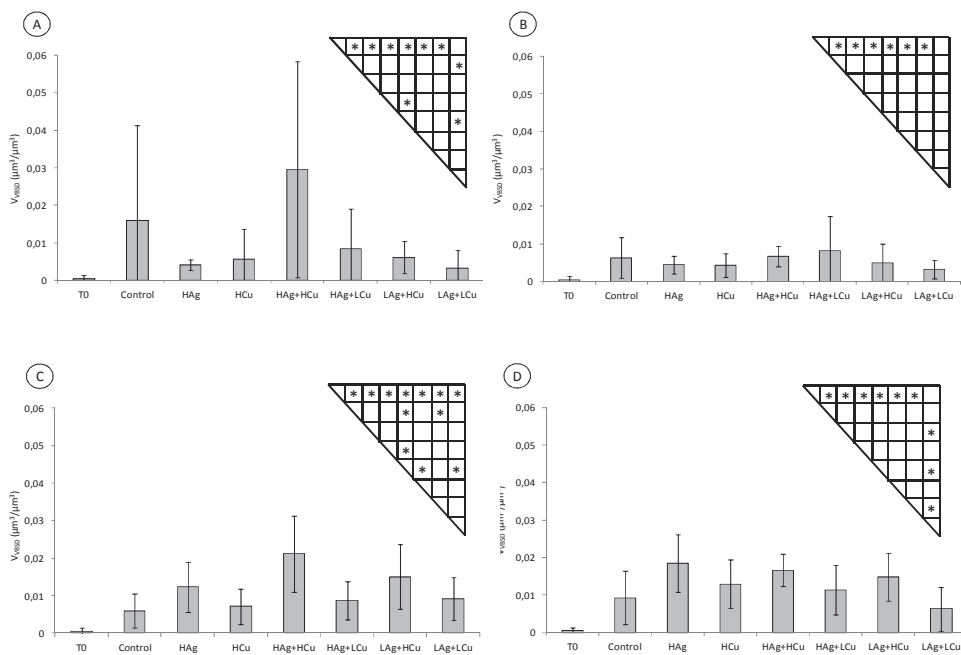


Figure 5: Mean values (\pm standard deviations) of intralysosomal metal accumulation (V_{BSD} , volume density of BSD, $\mu\text{m}^3/\mu\text{m}^3$) in oysters exposed to Ag and Cu concentrations: (A) 7 days, (B) 14 days, (C) 21 days and (D) 28 days. Statistically significant differences between groups * ($p < 0.05$).

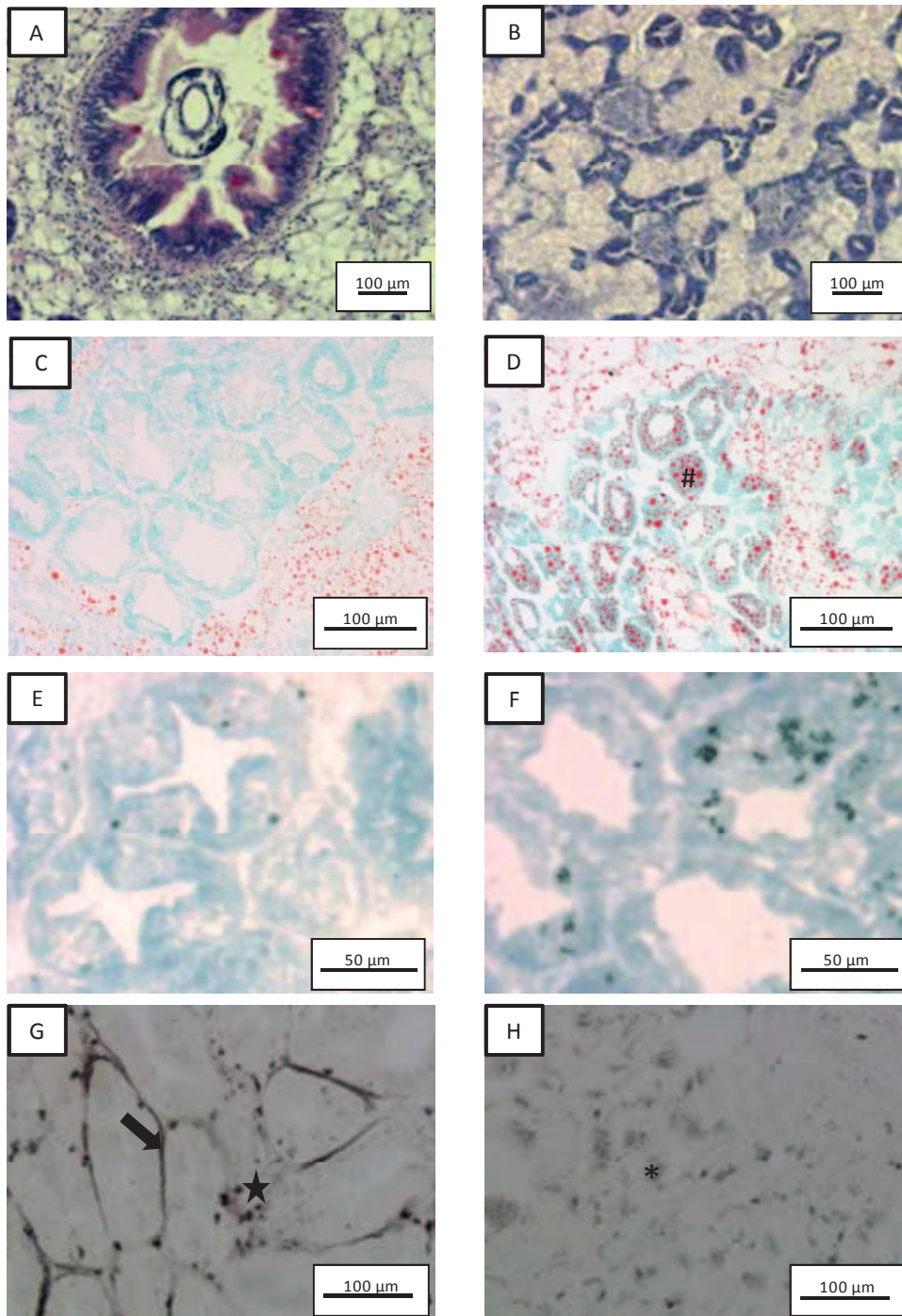


Figure 4: Histopathological alterations in oysters. (A) Parasite infection by *Mytilicola intestinalis* in the stomach of oysters exposed to HAg+HCu (21 days); (B) Haemocytic infiltration in the gonads of a female oyster exposed to HAg+LCu (28 days). Accumulation of neutral lipids (C-D), lipofuscins (E-F) and metal ions (G-H) in the digestive gland of oysters. (C) Neutral lipids in the digestive gland connective tissue of oysters LAg+LCu exposure group at 28 days and in (D) female gonads (#) of HCu at 7d. (E and F) Lipofuscin accumulation in the digestive gland tubules of LAg+HCu at 7 days and in HAg at 28 days. (G) Autometallographed Black Silver Deposits in the basal lamina (arrows) and haemocytes of connective tissue (stars) of farmed oysters at t0 and, (H) in the lysosomes (*) of oysters exposed to HAg+HCu 14 days.

8. Metallothionein (MT) content determination

An increase in MT levels was rapidly detected after the beginning of the experiment (7 days), although statistically significant differences with Control group only occurred after 28 days of exposure to the highest concentrations of Cu and Ag. (Fig. 6)

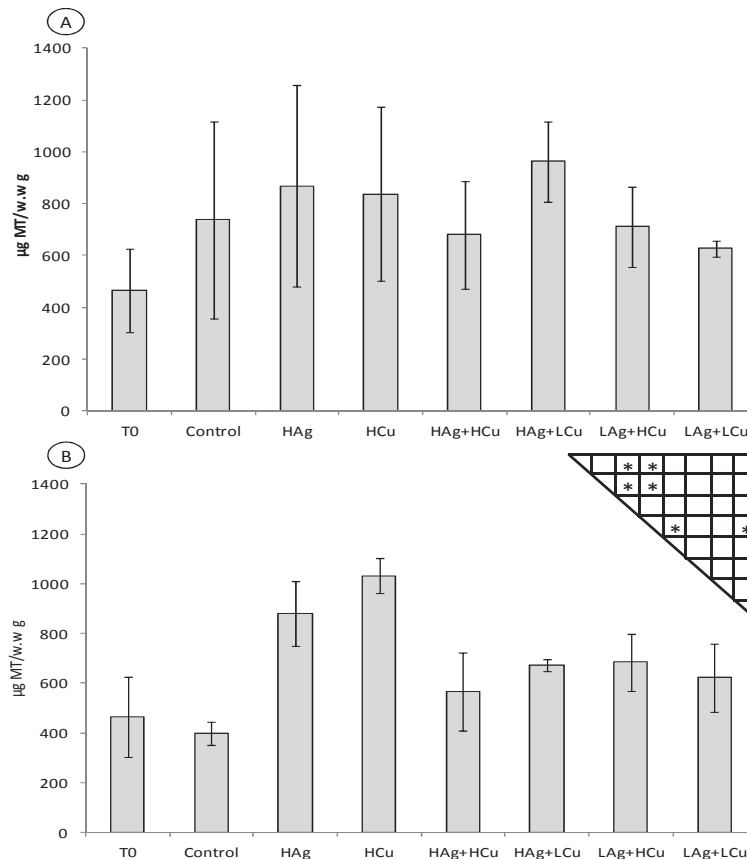


Figure 6: Mean values (± standard deviations) of MT content (µg MT/w.w g) in digestive gland of oysters at (A) 7 and (B) 28 days. Statistically significant differences between groups * ($p < 0.05$).

9. Integrative Biological Response (IBR) index

The IBR index showed different responses for the experimental groups at 7 days depending on the Ag exposure concentrations. The highest value was recorded in oysters exposed to HAg+HCu mainly due to a high intralysosomal metal accumulation and, to a lower extent, to digestive tubule atrophy and increased MT levels. Oysters exposed to HAg+LCu presented medium IBR values due to changes

in CI, while the response produced in oysters exposed to HCu was very low (Fig. 7A-B).

After 14 days, the highest IBR values were recorded in (i) oysters exposed to HAg+LCu due to tubule atrophy and changes in CTD, and in (ii) oysters exposed to HAg+HCu due to intralysosomal metal accumulation and high MT levels.

After 21 days, oysters exposed to HAg+HCu followed the same pattern of response as at 14 days (increased Vv_{BSD} and MTs) while oysters exposed to HAg+LCu showed a decrease in overall response. Oysters exposed to LAg+LCu and LAg+HCu exhibited moderate-to-high IBR/n values due to changes in tissue level biomarkers (MLR/MET, CTD; Fig. 7E-F).

After 28 days, the responses of oysters exposed to LAg+LCu were related to tubule atrophy (increased MLR/MET values) and CTD, whereas oysters exposed to HAg showed increased MT values. A global response involving biomarkers at tissue and organism level was recorded in oysters exposed to LAg+LCu resulting in the highest values for IBR/n index (Fig. 7, G-H).

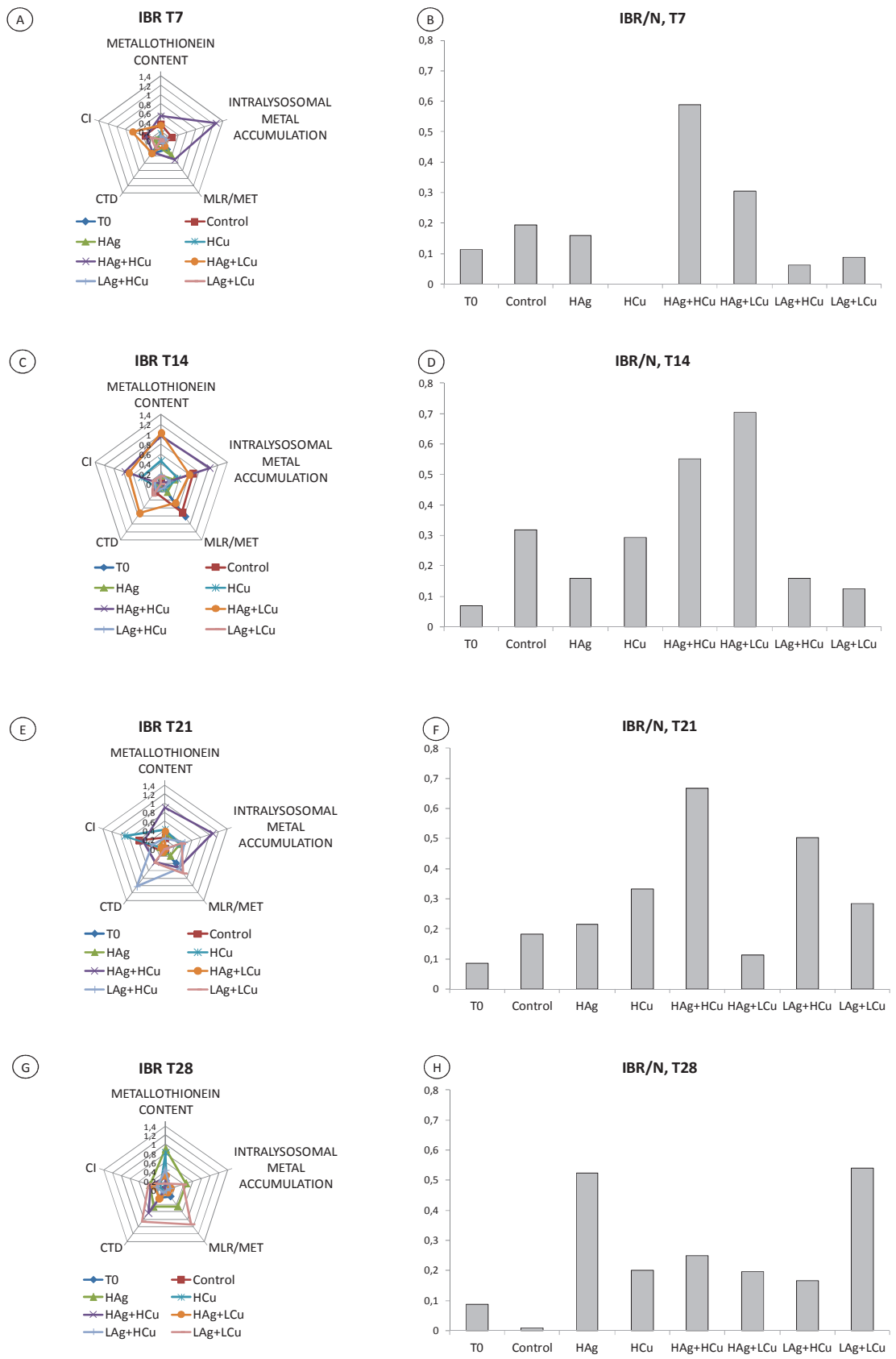


Figure 7: Integrative Biological Response Index (IBR) star plots after (A) 7, (C) 14, (E) 21 and (G) 28 days of exposure. IBR/n values per sampling day where (B) 7, (D) 14, (F) 21 and (H) 28 days of exposure.

DISCUSSION

Exposure of uncontaminated, farmed oysters to levels of dissolved Ag and Cu isotopes (direct exposure) similar to those described in some coastal areas (Elligsen et al., 2005; Flegal et al., 2007), separately or in mixtures, in controlled experiments over 28 days has induced a variety of biomarker responses from cell/tissue to organism levels. The nature and the degree of the responses varied over time and between exposure conditions.

Induction of MT levels can occur at very short times to cope with the presence of high levels of metals (Ringwood et al., 1998; Santovito et al., 2015). Likewise, in the present study a general increase in MT concentration was observed at the beginning (7 days) of the exposure to Ag and Cu. This response is a well known protective mechanism in digestive cells that includes sequestration by MTs and engulfment in digestive cell lysosomes followed by excretion (exocytosis) of residual bodies bearing metals to the gut lumen and subsequently to faeces (Amiard et al., 2006; Marigómez et al., 2002). Accordingly, together with MT induction, intralysosomal metal accumulation was observed at short exposure times. According to our results, at longer periods of exposure (14 and 21 days) the endolysosomal system and the newly synthesised MTs were able to cope with metal exposure, until day 28 when a second increase of MT levels was also recorded as described in clams (*Venerupis philippinarum*) (Santovito et al., 2015). In fact, it has been reported that chronic exposure to pollutants may produce high MT levels for long periods without toxicity onset. For instance, Yu et al. (2013) found that oysters inhabiting a strongly metal polluted estuary in China presented higher amounts of Cu bound to MT-like proteins when Cu burden in organisms was low, suggesting that MTs are more involved in Cu regulation (homeostatic control) than in storage.

In summary, the quick increase (<7 days) in MT concentrations and intralysosomal metal accumulation, may indicate the rapid onset of detoxification/accumulation processes, that were maintained up to 28 days. However, at these longer exposure periods a general decline of the health status of the oysters occurred (e.g. increasing digestive tubule atrophy, see below). The impairment of the excretory function has been considered as a general stress response to pollutants (Marigómez et al., 2002) that can lead to the accumulation of metals in other tissue compartments of the digestive tract, such as the basal lamina of digestive tubules and wandering haemocytes in the connective tissue. Alike, Amiard-Triquet et al. (1991) reported strong accumulation of Ag in haemocytes and in the basal lamina of digestive tubules of oysters exposed to higher Ag levels than in the present work. On the other hand, the presence of BSDs in the gills of oysters followed the same distribution previously described in autochthonous oysters from an estuary exhibiting low pollution levels (Rodríguez-Iruretagoiena et al., 2016). Moreover, lipofuscin accumulation over the experiment duration was attributed to accumulation of cell debris in lysosomes after metal exposure, which has already been described in bivalves under stress conditions (Marigómez et al., 2013) and in winkles exposed to Cd (Zaldibar et al., 2007). Furthermore, lipofuscin accumulation has also been linked to the production of reactive oxygen species (ROS) in bivalves, which ends up in an increase of lipofuscin contents due to lipid peroxidation (Moschino and Da Ros., 2016).

The accumulation of neutral lipids in the lysosomes of digestive cells is also considered as an indicator of non-specific stress under pollutant exposure (Marigómez et al., 2013). However, in the present work, higher amounts of neutral lipids were detected in oyster's connective tissue, which is known to act as an energy reservoir (Thompson et al., 1996). Relatively constant values of neutral lipids and unaltered morphology and functionality of the digestive gland of control oysters

indicate that food supply was enough for maintenance all along the experimental period. The generally low amount of neutral lipids in digestive cells of oysters in the present experiment was probably related with the seasonal cycle and the transference of lipids to the gonad during spawning periods (Cancio et al., 1999; Dridi et al., 2007). However, it cannot be excluded that exposure to Cu and Ag caused a certain imbalance in the energy budget reflected by a general consumption of neutral lipids from the connective tissue. Moreover, Guerlet et al. (2006, 2007) reported a depletion of neutral lipids reserves in freshwater molluscs, and a similar phenomenon was described by Séguin et al. (2016) in oysters translocated into a metal polluted site, suggesting that energy reserves were relocated for survival mechanisms.

Stress conditions are also known to provoke changes in the digestive gland of bivalves such as atrophy of the digestive epithelium (Izagirre et al., 2014; Zaldibar et al., 2007) or the shrink and reduction in size of digestive gland tubules, with an increase in the relative proportion of the interstitial connective tissue (Brooks et al., 2011; Garmendia et al., 2011). In fact, the ratio between digestive and connective tissues (CTD), was previously applied in mussels as a successful biomarker of tissue integrity (Brooks et al., 2012, 2011; Garmendia et al., 2011). This is the first time CTD ratio being applied to oysters, showing that this biomarker is sensitive enough to detect a general stress effect in oysters after 28 days of exposure to HAg and to the combination of Ag and Cu at close-to-real exposure levels. Furthermore, increased CTD values coincided with the increased atrophy of digestive tubules. Other histopathological alterations such as HAE also appeared, mainly in oysters exposed to Ag for 21 days, and have been attributed to stress conditions appearing as a result of exposure to Cu and Ag among other factors (Bayne et al., 1985; Haberkorn et al., 2014; Lowe and Moore, 1979; Séguin et al., 2016). Interestingly, a large HAE occurred in gonads, especially after 21 days at high concentrations of Cu

and Ag. Upon this time, gamete development was in concordance with the seasonal reproductive cycle of *Crassostrea gigas* (Fabioux et al., 2005) while after this period, oysters exposed to the highest dose of Cu and Ag (alone or in combination) exhibited arrested gonad development together with strong HAE. This general picture seems to reflect a natural reabsorption process of unspawned gametes in order to clean and prepare gonads for new reproduction cycles (Fabioux et al., 2005; Ortiz-Zarragoitia and Cajaraville, 2010). Nevertheless, one cannot exclude that the increased energy demand for detoxification processes can require the cessation of energy invest for growth and reproduction, causing an arrest of gamete development and the subsequent release of immature gametes (Ratte, 1999). Furthermore, the presence of organic and metallic pollutants can induce the spawning of gametes (Amiard et al., 2004; Bignell et al., 2011; Marigómez et al., 2013), which may have occurred under the present exposure conditions.

The Index of Biological Response (IBR) has been successfully applied to other sentinel species (Brooks et al., 2011; Cravo et al., 2012; Garmendia et al., 2011; Marigómez et al., 2013). IBR has been used as a method to integrate the responses of the different biomarkers in oysters, in order to discriminate biological responses to metal exposure (Ag and Cu) over time. In the present work, IBR produced satisfactory discrimination between exposure groups and controls suggesting different health status. The overall response pattern suggested a possible synergy between high Ag concentrations and Cu, hence, causing a decrease in oysters' health status. Assuming competition for the regulation of both metals, Cu presents higher affinity for MTs than Ag (Amiard et al., 2006), probably due to its nature as an essential element being therefore preferentially regulated, even in the presence of Ag. However, the organotropism of both metals in the same experimental set up as used in the present work suggested a synergistic relationship between both

elements, in which high (but environmentally realistic) concentrations of Cu enhance Ag bioaccumulation (Mikolaczyk et al., 2016).

Furthermore, a plateau in Ag accumulation after 2 weeks in oysters exposed to HAg in combination with both concentrations of LCu and HCu occurred, presenting these groups Ag accumulation values of 70% and 50% higher than controls after 14 days of exposure. The use of stable metal isotopic spikes allowed tracing this metal accumulation at relatively low exposure levels since the beginning of the experiment (Mikolaczyk et al., 2016) this fact could explain the higher response of IBR and IBR/n indices during the first weeks of exposure and their following decrease at longer exposure periods.

In summary, our results suggest that the presence of high concentrations of Ag and Cu produced the quick activation of detoxification processes in oysters at low levels of biological organisation (i.e. increased levels of MTs and enhanced intralysosomal metal accumulation) resulting in effects at tissue, organ and organism levels (histopathological alterations, reserve consumption, reproductive impairment by gonad reabsorption or enhanced spawning). Accordingly, it has become evident that the exposure to levels of Cu and Ag alone that can be found in the environment, and especially in combination, resulted in a diminished health status (easily quantified through IBR) in oysters following a cascade of effects through time, even if metal accumulation values in oyster tissues (Mikolaczyk et al., 2016) were lower than values observed in natural environments such as the Gironde Estuary (Lanceleur et al., 2011b). The synergistic processes that occur between Cu and Ag accumulation in oysters (Mikolaczyk et al., 2016) and the integrative conclusions obtained with the aid of IBR in the present study indicate that the monitoring of Ag and Cu is needed since deleterious effects can occur even if they appear at very low concentrations.

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Chapter III

Influence of salinity in Ag and Cu toxicity in oysters (*Crassostrea gigas*) through the integrative biomarker approach

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ABSTRACT

Influence of salinity in Ag and Cu toxicity in oysters (*Crassostrea gigas*) through the integrative biomarker approach.

Human activities have altered estuarine environments leading to increased presence of different pollutants including metals. Although the implementation of new environmental policies has caused a considerable decrease in trace metal concentrations in estuaries from the Bay of Biscay, some elements such as copper (Cu) and silver (Ag) are still present in relatively high concentrations. Oysters have been widely used in environmental biomonitoring programs as sentinel organisms. However, the influence of natural variables such as salinity which rapidly changes in estuarine environments is of relevant interest, since it may influence dissolved metal concentrations and speciation, i.e key factors to bioavailability. Presently, oysters *Crassostrea gigas* were exposed to sublethal, environmentally relevant concentrations of Cu (2000 ng Cu/L) and Ag (500 ng Ag/L) during 14 days in (S=18) seawater. A battery of cell and tissue level (exposure) biomarkers at different levels of biological complexity was applied and integrated into the Integrative Biological Response (IBR) index including: metallothionein contents, intralysosomal metal accumulation, digestive gland atrophy and digestive gland tissue integrity. Condition Index (CI) was incorporated into the IBR index as a complementary parameter that reflects the general physiological condition of oysters (organism level). Results indicated an increase in intralysosomal metal accumulation after 7 and 14 days of exposure to Ag together with an increase in the digestive epithelium atrophy and lipofuscin content after 7 days of exposure to Ag. The responses detected with the aid of biomarkers integrated in the IBR index showed a higher toxicity in oysters exposed to Ag-inducing the clear onset of detoxification processes which also occurred, to a lower extent, in Cu-exposed oysters.

RÉSUMÉ

Étude de l'influence de la salinité sur la toxicité d'Ag et Cu chez l'huître (*Crassostrea gigas*) à travers l'approche d'intégration des biomarqueurs.

Les activités humaines ont modifié les milieux estuariens conduisant à une présence accrue de différents polluants, notamment les métaux. La mise en œuvre de nouvelles politiques environnementales, a permis une diminution considérable des concentrations en métaux traces dans les estuaires du Golfe de Gascogne. Cependant, certains métaux tels que le cuivre (Cu) et l'argent (Ag) sont toujours présents à des concentrations relativement élevées. Les huîtres ont été largement utilisées comme des organismes sentinelles dans les programmes de biosurveillance de l'environnement. Néanmoins, des variables naturelles comme la salinité (qui change rapidement dans les estuaires) ont une forte influence sur les concentrations dissous des métaux et leur spéciation, i.e. des facteurs clés de la biodisponibilité. Dans le présent travail, des huîtres *Crassostrea gigas* ont été exposées à des concentrations sublétales de Cu (2000 ng Cu/L) et Ag (500 ng Ag/L) dans une eau de salinité 18 pendant 14 jours. Une batterie de biomarqueurs cellulaires et tissulaires (dits biomarqueurs d'exposition) à différents niveaux de complexité biologique ont été étudiés et intégrés dans le « Integrative Biological Response » (IBR) index, soit: le contenu en métallothionéines, l'accumulation intralysosomale métallique, l'atrophie de la glande digestive et l'intégrité du tissu de la glande digestive. L'Indice de Condition (Condition Index, CI) a été incorporé dans l'IBR index comme paramètre complémentaire qui reflète l'état physiologique global des huîtres (niveau de l'organisme). Les résultats ont montré une augmentation de l'accumulation intralysosomale de métaux après 7 et 14 jours d'exposition à l'Ag avec une augmentation de l'atrophie de l'épithélium digestif et des lipofuscines après 7 jours d'exposition à l'Ag. Les réponses biologiques détectées grâce à l'intégration des biomarqueurs par l'IBR index ont montré une toxicité plus élevée dans les huîtres exposées à l'Ag, indiquant le début des processus de détoxication. Ces processus ont aussi commencé, à moindre mesure, dans les huîtres exposées au Cu.

LABURPENA

Gazitasunaren eragina zilar eta kobrearen toxikotasunean *Crassostrea gigas* ostretan: biomarkatzaile bidezko hurbilketa integratua.

Gizakien jarduerak direla eta, estuarioak bezalako ekosistemetan kutsatzaile desberdinen presentzia areagotua, metalak barne, behatu da hauek asaldatuz. Ingurumen-politika berriak martxan jarri diren arren eta Bizkaiko Golkoko estuarioetan metal kontzentrazioak behera egin duten arren, zenbait elementu, kobrea (Cu) eta zilarra (Ag) bezala oraindik ere kontzentrazio esangarrietan agertzen dira. Ostrak modu orokorrean erabili dira organismo behale gisa ingurumen biojarraipen programetan. Hala ere, aldagai naturalen eraginak, gazitasuna adibidez, zeina modu azkarrean aldatzen den estuariotan, uretan disolbatutako metalen kontzentrazioan eragina izan dezake eta baita euren espeziazioan ere, hauek bioeskuragarritasunean berebiziko garrantzia izanik. Ikerlan honetan *Crassostrea gigas* ostrak, Cu (2000 ng Cu/L) eta Ag (500 ng Ag/L) kontzentrazio azpihilgarrien pean jarri dira 14 egunez eta 18-ko gazitasunpean. Konplexutasun biologiko desberdineko zelula eta ehun mailako biomarkatzaileak aplikatu dira eta "Integrative Biological Response (IBR)" delako indizean integratu dira hurrengo biomarkatzaileak barneratuz: metalotioneinen kopurua, lisosomen barneko metalen metaketa, digestio-guruineko atrofia eta digestio-guruineko ehunaren osotasuna. Egoera-indizea (Condition Index, CI) parametro osagarri gisa barneratu zen IBR indizean ostren egoera fisiologiko orokorra islatzen duelako (organismo maila). Esperimentuko 7 eta 14. egunetan lisosomen barneko metalen metaketan handipena behatu zen Ag-pean jarritako ostretan, gainera digestio-epitelioaren atrofia eta lipofuszinaren handipena behatu zen Ag-pean jarritako ostretan 7. egunean. Biomarkatzaileen emaitzak IBR indizean integratu zirenean toxikotasun altuagoa behatu zen Ag-pean jarritako ostretan, baina baita ere detoxifikazio prozesuen abiarazpena behatu zen, prozesu hauek mugatuagoak izanik Cu-ren kasuan.

RESUMEN

Influencia de la salinidad en la toxicidad de Ag y Cu en ostras (*Crassostrea gigas*) utilizando una aproximación integradora de biomarcadores.

Las actividades humanas han alterado los medios estuarinos dando como resultado un aumento en la presencia de diversos contaminantes incluyendo los metales. A pesar de que con la implantación de nuevas políticas medioambientales se ha observado un descenso considerable en la concentración de metales en los estuarios del Golfo de Bizkaia, algunos elementos como el cobre (Cu) y la plata (Ag) siguen presentes en concentraciones relativamente altas. Las ostras han sido ampliamente empleadas como organismos centinela en programas de bioseguimiento. Sin embargo, la influencia de variables naturales como la salinidad, que varía de manera rápida en los estuarios, puede ser importante ya que puede influir tanto en la concentración de metales disueltos así como en su especiación, los cuales son factores clave en su biodisponibilidad. En el presente estudio se han expuesto ostras *Crassostrea gigas* a concentraciones subletales de Cu (2000 ng Cu/L) y Ag (500 Ag ng/L) durante 14 días a una salinidad de 18. Se ha integrado una batería de biomarcadores incluyendo la concentración de metalotioneinas, la acumulación intralisosómica de metales, la atrofia de la glándula digestiva y la integridad del tejido de la glándula digestiva en el denominado índice "Integrative Biological Response (IBR)". El índice de condición (Condition Index, CI) fue incorporado al IBR como un parámetro complementario que refleja la condición fisiológica general de las ostras (nivel de organismo). Los resultados indicaron un aumento de la acumulación intralisosómica de metales después de 7 y 14 días de exposición a Ag así como un aumento de la atrofia del epitelio digestivo y lipofuscinas después de 7 días de exposición a Ag. Las respuestas detectadas mediante el uso integrado de los biomarcadores en el índice IBR indicaron mayor toxicidad de la Ag y la puesta en marcha de los mecanismos de detoxificación por parte de las ostras que también se observó en aquellas expuestas a Cu aunque en menor medida.

INTRODUCTION

Aquatic environments, and in particular estuarine systems have been impacted by human pressures leading to pollutant presence in their waters (David et al., 2012; Millward and Turner, 2001; Spencer et al., 2007). In this context, some of the main estuaries in the Bay of Biscay including the Gironde or Ibaizabal estuaries have been polluted for many decades by metals derived from various sources. In both cases, concentrations of some metals are still relatively high and can affect the general health status of the of the aquatic biota inhabiting mentioned estuaries (Baudrimont et al., 2016; Cuevas et al., 2016; David et al., 2012; Lanceleur et al., 2011a; Solaun et al., 2013). Silver (Ag) and copper (Cu) are both present in the Gironde and Ibaizabal estuaries (Lanceleur et al., 2011a; Solaun et al., 2013) and are amongst the most toxic metals for marine biota (Haberkorn et al., 2014; Money et al., 2011; Ratte, 1999; Santovito et al., 2015; Tappin et al., 2010). While Ag is a model toxic element even at low exposure levels (Luoma et al., 1995; Ratte, 1999; Tappin et al., 2010), Cu is an essential micronutrient for living organisms that becomes toxic at higher concentrations (Debelius et al., 2009; Foster et al., 2011; Gamain et al., 2016; Raftopoulou and Dimitriadis, 2011). Accumulation and toxicity of these two elements in invertebrates including oysters has been considered relevant by previous studies (Amiard-Triquet et al., 1991; G ret et al., 2002).

Environmental factors such as salinity are known to affect both metal partitioning between the dissolved and the particulate phases, speciation and organisms. It has been demonstrated that salinity is a key physiological factor that modifies the bioavailability and toxicity of metals by altering their speciation in the environment (Lanceleur et al., 2011b; Money et al., 2011). For instance, an increase in metal accumulation has been described in soft tissues of bivalves when salinity decreases (Amiard-Triquet et al., 1991; Mouneyrac et al., 1998; Ratte, 1999; Renault, 2015; Roesijadi, 1996). The influence of salinity on toxicity/accumulation processes has

been previously reported for different invertebrate species (Amiard-Triquet et al., 1991; Gamain et al., 2016; Leung et al., 2002; Mouneyrac et al., 1998; Renault, 2015) being these effects specially interesting not only at low salinity levels but in environments showing periodic/cyclic changes (gradients) in salinity, such as estuaries. In estuarine waters, maximum dissolved Ag concentrations usually occur in brackish waters, at 15-20 salinity range (Tappin et al., 2010) because salinity enhances Ag desorption from the particulate phase by chloride complexation (Lanceleur et al., 2011b). This process causes the formation of electrically neutral Ag-chlorocomplexes (AgCl^0) which are lipophilic molecules of low polarity, able to cross biological membranes (Barriada et al., 2007; Tappin et al., 2010). However, the most toxic form of Ag is the free ion Ag^+ (Ratte, 1999). Conversely, Cu speciation is mainly governed by organic complexation, where the ionic forms of Cu, which are considered as the most toxic ones (Cu^{2+} , Cu^+ and $\text{Cu}(\text{OH})^+$) bind to dissolved organic matter reducing their bioavailability (Money et al., 2011; Roesijadi, 1996). At higher salinity, sodium (Na) concentration increases and directly competes with Cu^{2+} , Cu^+ and $\text{Cu}(\text{OH})^+$ for binding sites thus affecting Cu bioavailability and toxicity (Lee et al., 2010). Recently, biomonitoring programs carried out in coastal and estuarine areas subjected to metal pollution have proven the strength of cell and tissue level biomarkers (Marigómez et al., 2013b; Séguin et al., 2016; Zorita et al., 2006). In order to integrate all the measured biomarkers, an inclusive index has already been successfully applied in biomonitoring programs (Cravo et al., 2012; Marigómez et al., 2013a; Serafim et al., 2012): the Integrative Biological Response Index (IBR Index) has been used as a powerful tool for the health status assessment of sentinel organisms and their surrounding ecosystems (Beliaeff and Burgeot, 2002; Broeg and Lehtonen, 2006; Marigómez et al., 2013b). However, the responsiveness of biomarkers measured in sentinel organisms can be altered by changes in salinity and should be taken into account when assessing environmental health status of aquatic ecosystems. Oysters, that inhabit estuaries, have been

widely employed in monitoring programs (Kim et al., 2008; Liu and Wang, 2016; Morley, 2010; Raposo et al., 2009) but can suffer salinity-related physiological and behavioural changes in feeding activity, growth and respiration rates, oxygen consumption and excretion rates and eventually produce histopathological alterations (Knowles et al., 2014). Therefore, the main objectives of the work were (i) to ascertain the effects exerted by environmentally relevant Cu and Ag concentrations at relatively low salinities in oysters *Crassostrea gigas*, and (ii) to integrate biomarker responses at different levels of biological complexity using a comprehensive index (IBR). The fulfilment of these two objectives will help to obtain a more precise environmental health assessment using oysters in coastal sites with changing salinities. A battery of biomarkers has been applied at subcellular (metallothionein –MT- concentration), cell (intralysosomal metal accumulation, neutral lipid and lipofuscin contents) and tissue levels (histopathological alterations, digestive gland atrophy, digestive gland integrity), together with complementary parameters quantified at individual level (gamete developmental stage, Condition Index -CI- and mortality rate). A selection of five biomarkers were integrated into the IBR index (MT concentration, intralysosomal metal accumulation, digestive gland atrophy, tissue integrity and CI) in order to assess the health status of oysters exposed to environmentally relevant concentrations of Cu and Ag in brackish waters.

MATERIALS AND METHODS

1. Experimental design

Oysters *Crassostrea gigas* (7 - 8 cm length) from an oyster farm (Ostranor S.L) located in an estuary with low levels of pollution (San Vicente de la Barquera, Cantabria, Spain; De los Ríos et al., 2016) were transferred to the Plentzia Marine Station (Plentzia, Basque Country, Spain) in April 2013. Prior to the exposure

experiment, the oysters were acclimatised and depurated in naturally filtered sea water (pH=7.9; T=15.5 °C; S=28.2) with a continuous water and air flow during 4 days and photoperiod was established at 12 h:12 h (light:dark).

Oysters were distributed into 20 L high density polypropylene aquaria containing a mixture of naturally filtered sea water and distilled water in order to control salinity values ($V_{\text{Total water}}=1/3V_{\text{distilled water}}+2/3V_{\text{sea water}}$) (final S=18.8) and mimic the values found in the mid-salinity range inside estuaries. Three experimental groups were defined: Control, 500 ng Ag/L and 2000 ng Cu/L. These concentrations did not produce significant mortality in oysters as previously demonstrated in natural sea water (Mikolaczyk et al., 2016; Rementería et al., 2016). For metal addition, AgNO₃ and CuCl₂ salts were dissolved in distilled water before spiking the water in the tanks of each corresponding experimental group to obtain the above mentioned nominal concentrations.

Direct exposure to waterborne contaminants was assured by avoiding contact of food with spiked water in the tanks. For this, daily feeding with commercial food (Marin Coraliquid, Sera® Ltd., GmbH Heinsberg, Germany) during 4 h in separate clean tanks was carried out. These tanks contained the same water mixture explained before, but without metal spikes. Meanwhile, exposure tanks were cleaned and water was renewed and spiked. After feeding, animals were placed back into their corresponding exposure tanks; limiting exposure time to 20 h per day. Water volume and food amount for each experimental tank were adapted to the number of organisms throughout the experiment: 30 oysters/21 L (0-7 days), 15 oysters/10.5 L (7-14 days).

2. Sample collection and processing

Oysters were collected from each experimental group at days 0, 7 and 14. Oysters were opened and shell and flesh weights were individually measured for the

calculation of Condition Index (CI, see below). Then, 10 individuals were dissected for biomarker studies. For histology, a ~5 mm thick cross-section of the soft body including all main organs and tissues (gills, digestive gland and gonad) was obtained per animal ($n=10$). Sections were rinsed in formalin fixative for 24 h and routinely processed for histological examination. Small pieces of five digestive glands per experimental group were rapidly frozen in liquid nitrogen for cryosectioning. The remaining digestive glands of each experimental group were also frozen and stored at -80° C until processing for metallothionein content determination.

3. Biological measurements

3.1. Mortality

Mortality of oysters was checked daily. An individual was considered dead when after physical stimulation valves gaped and failed to close (Veldhuizen-Tsoerkan et al., 1991).

3.2. Condition Index

Oyster's Condition Index (CI) was calculated according to the formula: $CI = (\text{WW visceral content} / \text{WW shell} \times 100)$ (in grams) (Strady et al., 2011).

3.3. Digestive gland and gonad histology

Sections of 4 μm thickness were obtained in the microtome from paraffin embedded samples and were stained with haematoxylin-eosin. Afterwards, microscopic slides were analysed under a light microscope (Olympus BX 51) (Olympus, Japan).

3.3.1. Gamete developmental stage

Sex and gonad developmental stages as well as Gonad Index (GI Value) were determined for each individual based on a subjective scale of the developmental

stage of follicles and gametes after examination under the light microscope according to the procedure described by Kim et al. (2006).

3.3.2. Histopathological alterations

3.3.2.1 Digestive gland atrophy

A planimetric procedure was followed in order to determine changes in the morphology of digestive tubules (Garmendia et al., 2011). Micrographs of digestive gland tubules were obtained with a digital camera attached to an Olympus BX-61 microscope (Olympus, Japan). Five fields of digestive glands were recorded per oyster, and 2 tubule profiles per field were transferred into an image analysis system Visilog 5.4 Noesis to calculate the following parameters and ratios: MET (Mean epithelial thickness, μm), MLR (Mean luminal radius, μm) and MLR/MET ratio.

3.3.2.2 Tissue integrity in digestive gland (CTD ratio)

The integrity of the digestive gland tissue was determined by calculating the connective-to-diverticula (CTD) ratio. For this, 5 fields of digestive gland tubules were obtained per individual with a digital camera attached to an Olympus BX-61 microscope (Olympus, Japan) and processed through Image J program (Image Processing and Analysis in Java, Maryland, USA). CTD ratio is defined as the extent of the interstitial connective tissue relative to the area occupied by digestive diverticula. The following formula was applied to obtain CTD ratio: $\text{CTD ratio} = c / (b + d + l)$. Where: (c) interstitial connective tissue, (b) basophilic cells, (d) digestive cells and (l) diverticular lumen (Brooks et al., 2011).

Histopathological analyses were completed in oyster's whole body cross-sections. Prevalences of the most relevant alterations were obtained: Granulocytomes (GRN), Inflammations (INF), Haemocytic infiltrations (HAE), Haemocytic infiltrations in Gonads (HAE gn) and Parasites (Par).

3.4. Neutral lipid content determination

Frozen 8 μm thickness sections from digestive glands ($n=5$) were obtained in the cryostat at -26°C chamber temperature. Slides were stored at -40°C and stained following Lillie and Ashburn's Oil Red O (ORO) method (Culling, 1974). The volume density ($V_{V_{\text{NL}}}$) of neutral lipids was quantified in respect to connective tissue by using Image J program. $V_{V_{\text{NL}}}$ was calculated as the ratio between neutral lipid volume and connective tissue volume ($V_{V_{\text{NL}}} = V_{\text{NL}}/V_{\text{CT}}$). Where: (V_{NL}) volume of neutral lipids, and (V_{CT}) volume of connective tissue (Marigómez and Baybay-Villacorta, 2003).

3.5. Lipofuscin content determination

Lipofuscins in the digestive gland ($n=5$) were histochemically detected using the Schmorl method (Pearse, 1985). The volume density ($V_{V_{\text{LF}}}$) of lipofuscins was quantified with respect to digestive tissue by using Image J program, as $V_{V_{\text{LF}}} = V_{\text{LF}}/V_{\text{DT}}$, where: (V_{LF}) volume of lipofuscins, and (V_{DT}) volume of digestive tissue (Marigómez et al., 2013b).

3.6. Intralysosomal metal accumulation

Histochemical detection of metals was carried out in 4 μm thick sections of paraffin embedded samples ($n=10$ per treatment). After embedding, sections were dewaxed in xylene and hydrated, then, the slides were left at 37°C oven at least 24 h for drying. Samples were covered with the commercial silver enhancement kit (Silver enhancing kit for light and electron microscopy, BB International) at room temperature, development was checked under light microscope and stopped after 15-20 min. Slides were washed with tap water and metals were developed as black silver deposits (BSD). Finally, slides were mounted in Kaiser's glycerine gelatine and volume density of BSDs ($V_{V_{\text{BSD}}}$) in the lysosomes of digestive gland cells was measured with an image analysis system (Soto et al., 2002).

3.7. Metallothionein concentration

Frozen and stored (-80°C) digestive gland samples ($n=10$ per treatment at each sampling time) were pooled in order to obtain at least 1 g tissue. Several digestive gland pools (3 to 5 depending on the sample size) were obtained per experimental unit and sampling day. Afterwards, metallothionein (MT) content was determined spectrophotometrically according to UNEP/RAMOGÉ (United Nations Environment Programme) method modified from Viarengo et al. (1997). The supernatant absorbance was evaluated at 412 nm and MT concentration was estimated using reduced glutathione (GSH) as a reference standard. Results are expressed as MT concentrations (MT $\mu\text{g/wet weight tissue g}$).

3.8. Integrative Biological Response (IBR) index

Integrative Biological Response (IBR) index was calculated according to Devin et al., (2014). This procedure is based on the method described by Beliaeff and Burgeot, (2002). A total of 5 biomarkers were used for this purpose ordered from less biological complexity level to highest: subcellular level (metallothionein content), cellular level (intralysosomal metal accumulation), tissue level (MLR/MET), organ level (CTD index) and individual level (Condition Index). Finally, as the IBR value depends on the number of applied biomarkers, the IBR/ n was obtained dividing IBR by the number of biomarkers applied ($n=5$) (Brooks et al., 2011; Marigómez et al., 2013a, 2013b).

3.9. Statistics

All statistical analyses were carried out with the SPSS v 22.0 (SPSS. Inc., Chicago, Illinois). Analyses of variance (ANOVA) were performed after checking data normality and homogeneity (Kolmogorov Smirnov and Levene's test) for determining significant differences between experimental groups ($p < 0.05$) followed by Duncan's

post hoc test. In those cases, where data did not have normal and homogeneous distribution, Kruskal Wallis non parametric method was used (Dunns post hoc test).

RESULTS

1. Mortality

Very low mortality rates were recorded in all experimental groups with values ranging from 3.33% in Control and 500 ng Ag/L exposure groups after 14 days to 6.66% in 2000 ng Cu/L (Table 1).

Table 1: Percentage (%) of accumulated mortality through the experiment.

%	Control	500 ng Ag/L	2000 ng Cu/L
T0	0	0	0
T7	0	3.33	3.33
T14	3.33	3.33	6.66

2. Gamete developmental stage

During the experimental period, a progressive development of the gonads from early-mid to late development occurred especially in the Control group. Although no clear pattern of GI values was observed between experimental groups, lowest values were found at day 7 for Ag-exposed oysters (Fig. 1). After 14 days of exposure, GI values in Ag and Cu-exposed oysters showed a slight increase due to the presence of some oysters in late development phase. This increase was more marked in Control group oysters that reached the maximum percentage of oysters having spawned (up to 20 %) at 14 days (Fig. 1 B).

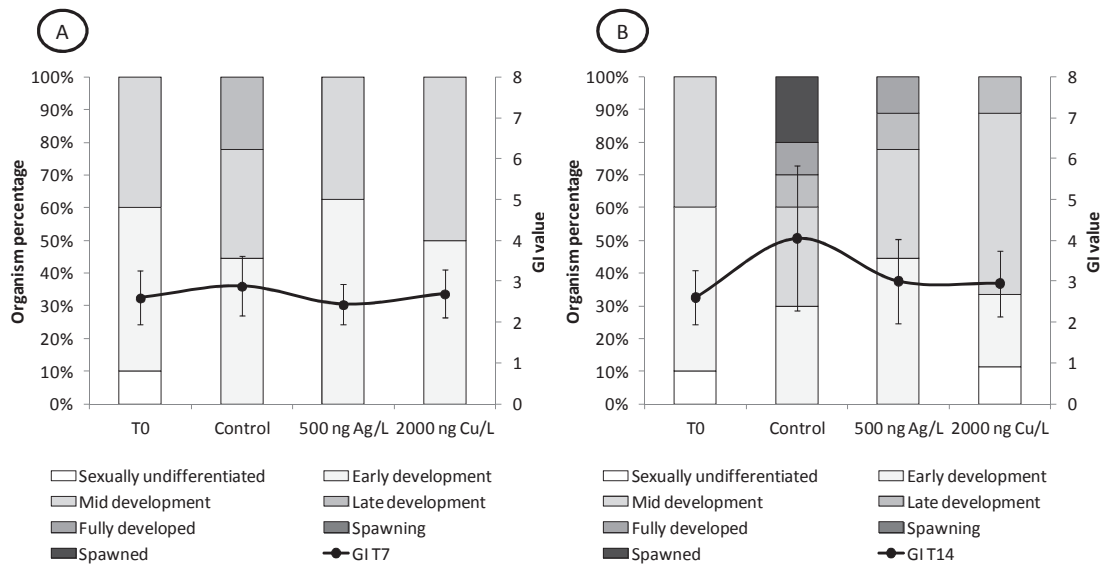


Figure 1: Percentage of organisms in the different gamete developmental stage (stacked bars) and mean values (\pm standard deviations) of the Gonad Index values (line) after 7 (A) and 14 days (B) of experiment.

3. Condition Index (CI)

Condition Index values through the experiment remained rather similar to the values found at day 0, although statistically significant differences between experimental groups were found at day 7, when Control and oysters exposed to 2000 ng Cu/L presented significantly higher values than at 0 day (Fig. 2 A). No statistically significant differences between experimental groups were observed after 14 days of exposure.

4. Tissue integrity in digestive gland (CTD ratio) and other pathologies

No significant differences in the relative abundance of connective tissue were observed after 7 days of exposure to Ag and Cu. After 14 days a significant increase was found between day 0 and Control group, and between day 0 and Ag-exposed group. At this time, Ag-exposed oysters showed increased amounts of connective tissue with respect to digestive tubules, being statistically higher than in Cu-exposed oysters (Fig. 2 B).

Regarding other types of pathologies, very low prevalences were detected. After 7 days only 10% of the Cu-exposed oysters displayed haemocytic infiltrations in their gonads while at day 14, parasites (*Mytilicola intestinalis*) were present in 10% of Ag-exposed oysters (Fig. 3 A).

5. Digestive gland atrophy

No significant differences between exposure groups were detected for MLR/MET ratio at any sampling time (Fig. 2 C).

6. Neutral lipid content determination

Neutral lipids were mainly detected in the connective tissue surrounding gonads and in the interstitial connective tissue from the digestive gland (Fig 3. C and D). Neutral lipids were present at a lesser extent in female gametes and digestive gland ducts. No significant differences occurred between experimental groups at any sampling time (data not shown).

7. Lipofuscin content determination

Lipofuscins were mainly located in the digestive cells lysosomes of the digestive gland tubules of oysters (Fig. 3 E and F). No significant differences were observed between treatments at any sampling time (Fig. 2 D).

8. Intralysosomal metal accumulation

Metals were detected as black silver deposits (BSD) in the basal lamina of the digestive epithelium of Control and day 0 oysters and to a lower extent in the digestive cell lysosomes and in the haemocytes of the connective tissue which surrounded the digestive tubules (Fig. 3 G). Exposure to metals increased intralysosomal metal accumulation after only 14 days of exposure (Fig. 3 H). The BSD present in the gills were very scarce and mainly localised in haemocytes and in the apical part of the frontal cells (results not shown). Significant differences in

intralysosomal metal accumulation ($V_{V_{\text{BSD}}}$) were obtained between experimental groups after 7 and 14 d of exposure (Fig. 2 E). The lowest $V_{V_{\text{BSD}}}$ values were recorded in oysters at day 0. After 7 days, $V_{V_{\text{BSD}}}$ were significantly higher in oysters from all exposure groups including Controls. High values of $V_{V_{\text{BSD}}}$ were detected in oysters exposed to Ag (7 and 14 d). After 14 days, oysters exposed to Cu and Ag exhibited statistically higher $V_{V_{\text{BSD}}}$ than Controls and oysters from day 0.

9. Metallothionein (MT) content

No significant differences in MT concentrations occurred at any sampling time (Fig. 2 F).

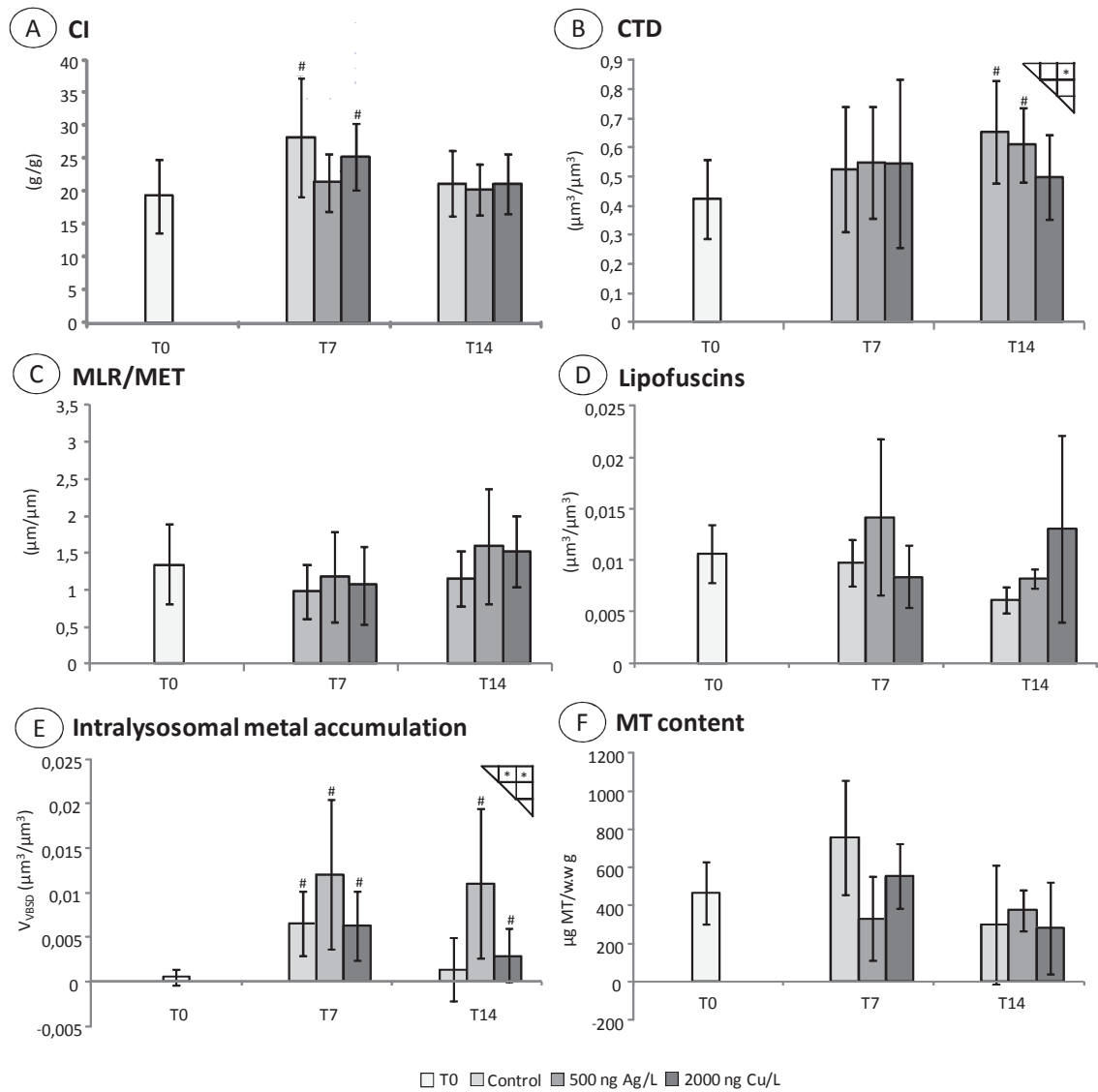


Figure 2: Mean values (\pm standard deviations) of Condition Index (A). Tissue integrity in the digestive gland (CTD) (B). Digestive gland atrophy measured as MLR/MET (C). Lipofuscins contents in digestive cells (D) Intralysosomal metal accumulation (V_{vBSD}) (E) and Metallothionein contents (F) in oysters of different treatments after 7 and 14 days of exposure. Significant differences between groups and T0 is indicated by a #; significant differences between experimental groups in the same day is indicated by an * ($p < 0.05$).

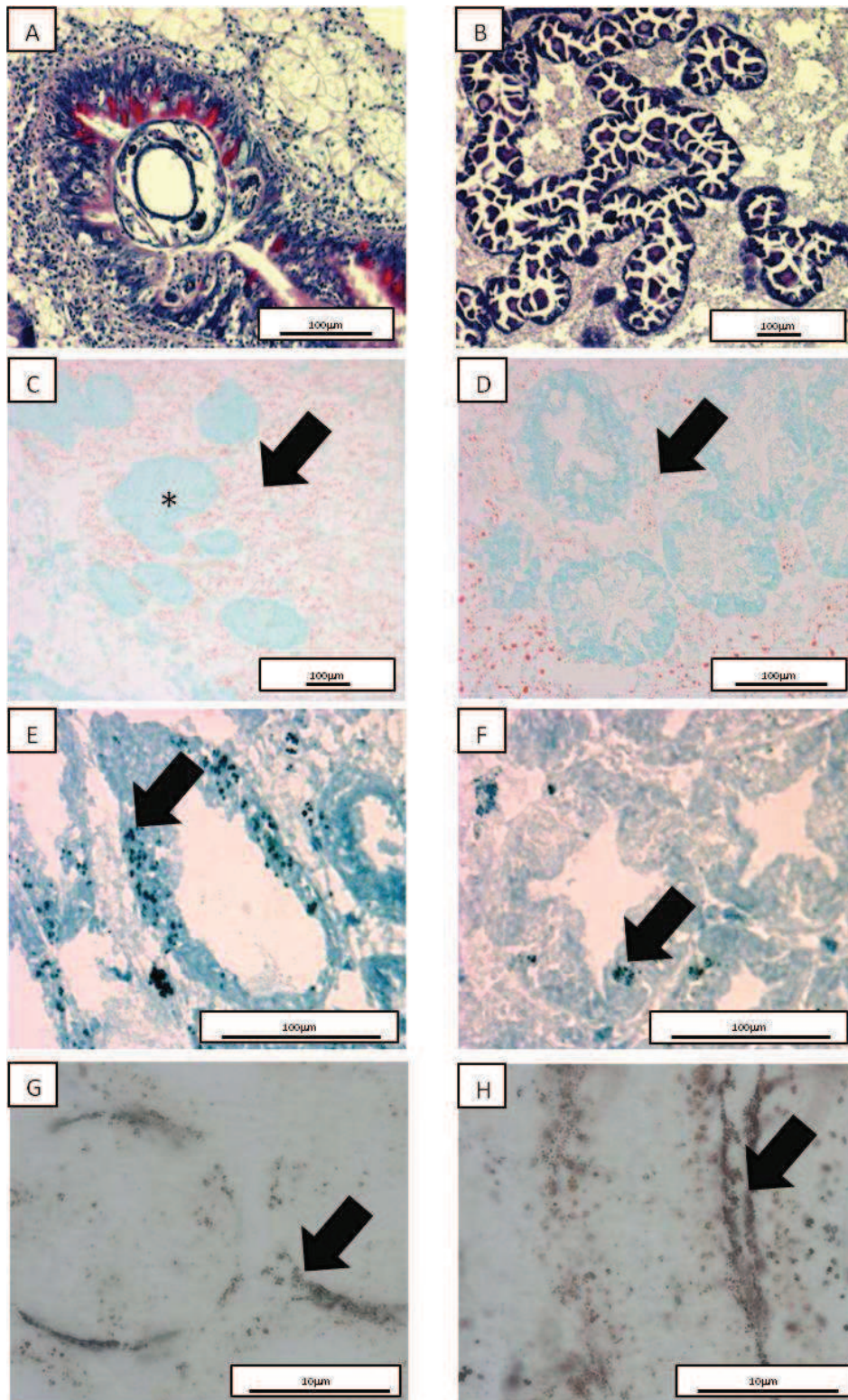


Figure 3: (A) Parasite infection by *Mytilicola intestinalis* in the stomach of oysters exposed 14 d to 500 ng Ag /L (Haematoxylin eosin –H/E- staining); (B) Abnormal development of a female gonad in a Cu-exposed oyster (7 days) (H/E). (C) Accumulation of neutral lipids in gonad connective tissue (arrow, Oil Red O –ORO-) of a Ag-exposed male oyster (*) and (D) in the digestive gland connective tissue in Cu-exposed oyster (14 days, ORO). (E) Lipofuscin accumulation (arrow) in digestive cells of oyster exposed to Ag (7 days, Schmorl staining) and (F) Control oyster nearly devoid of lipofuscins (14 days). (G) Metal detection in the basal lamina (arrow) and in the connective tissue of control oysters (14 days), (H) intralysosomal metal accumulation (arrow) in digestive cells of Ag-exposed oyster (14 days).

10. Integrative Biological Response (IBR) index

The IBR index showed different responses for the experimental groups after 7 days of exposure. The highest values were recorded in Ag-exposed oysters, which clearly indicated an increased intralysosomal metal accumulation, enlarged digestive tubule lumen (high MLR/MET) and a higher CTD ratio (Fig. 4 A). Regarding IBR/n index, Ag-exposed oysters had the highest values followed by Cu-exposed oysters (Fig. 4 B).

After 14 days of exposure, the highest IBR values were recorded again in Ag-exposed oysters, mainly due to higher values of the following biomarkers: intralysosomal metal accumulation, MLR/MET and CTD ratio (Fig. 4 C). The highest IBR/n values were found for oysters exposed to Ag and the lowest for Controls. Oysters collected at day 0 and exposed to Cu exhibited intermediate values (Fig. 4 D).

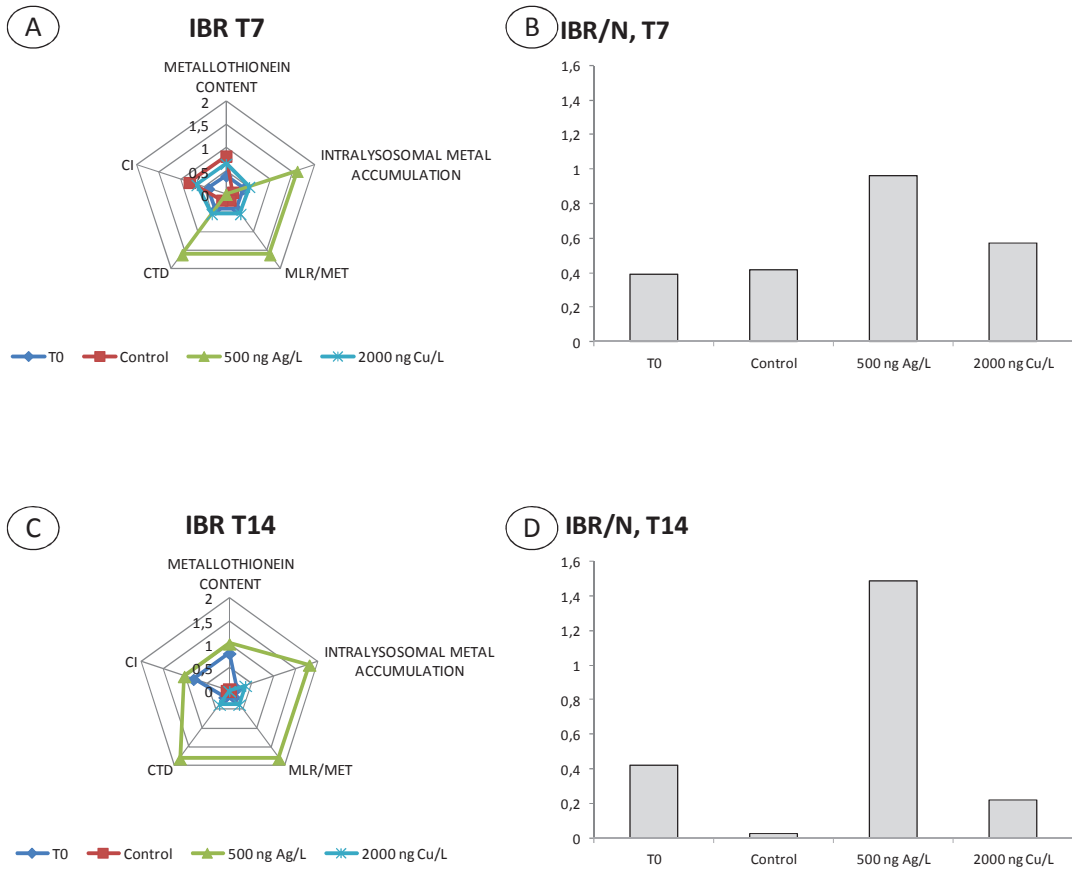


Figure 4: Integrative Biological Response Index (IBR) star plots (A) 7 and (C) 14 days. IBR/n values per sampling day, (B) 7 and (D) 14 days.

DISCUSSION

In the present study, oysters *Crassostrea gigas* collected in a farm were exposed during 14 days to environmental concentrations of Ag (500 ng/L) and Cu (2000 ng/L) in brackish waters (S=18). Previously, a similar experiment was carried out in coastal sea water salinity conditions (S=28), indicating that similar exposure to the same pollutants diminished the health status of oysters (Rementeria et al., 2016). In this context, salinity fluctuations are known to influence both Ag and Cu speciation and hence their bioaccumulation and toxicity. In particular, Ag has been described to be more bioavailable to biota and thus more toxic in lower salinities than at sea water salinity (Amiard-Triquet et al., 1991; Ratte, 1999; Roesijadi, 1996). Regarding Cu, a recent study has described an enhancement of the toxicity caused by this element in oyster *Crassostrea gigas* larvae at lower salinities (S=18-21) (Gamain et al., 2016). Therefore, salinity fluctuation could increase uncertainty in biomarker interpretation that needs of a deeper understanding.

Metallothionein induction is regarded as a metal exposure biomarker and the role of this protein as a protective mechanism to face metal exposure is quite well known, especially in digestive cells: metallothioneins are in charge of metal sequestration and their subsequent engulfment in digestive cell lysosomes to be then excreted by exocytosis to the gut lumen and finally to faeces (Amiard et al., 2006; Marigómez et al., 2002). Previous authors have reported an influence of salinity on MT synthesis, since increased MT contents were observed under experimental conditions in dogwhelks (*Nucella lapillus*) kept in brackish waters (S=22) (Leung et al., 2002). In the present experiment, no significant induction of MT was detected suggesting that the basal or physiological MT amounts present in oysters were enough to cope with metal exposure confirming previous results in oysters exposed to the same concentrations, where MT induction was only observed after 28 days of exposure (Rementeria et al., 2016). On the other hand, salinity did not seem to affect the

response pattern in MT induction. Moreover, Liu et al., (2016) also found after transplanting oysters (*Crassostrea hongkongensis* and *Crassostrea angulata*) into a Cu-, Zn- and Ni-polluted estuary (with salinity values ranging from 10 to 20), that MT contents increased after 5 days but then decreased and remained constant until the end of exposure (65 days). Therefore, at least MT induction in oysters does not seem to be strongly influenced by salinity changes.

The results of the present study suggest that the metallothionein concentrations already present in oysters, were sufficient to initiate the detoxification processes, sequestering metals and incorporating them into lysosomes. Accordingly, a significant increase in intralysosomal metal accumulation was observed in all exposure groups. However, the lower intralysosomal metal accumulation displayed by BSDs in Cu-exposed individuals may indicate that this element was better regulated than Ag.

Previous research works have attributed increased lipofuscin accumulation in lysosomes to the presence of oxidised cell debris accumulation in lysosomes under stress conditions, including exposure to metals (Marigómez et al., 2013b; Rementería et al., 2016; Zaldibar et al., 2007). However, in this work, lipofuscin accumulation was not so clear, probably due to the increase of apocrine secretion of the digestive cells, which contained residual bodies charged with metals. This extrusion mechanism was mainly observed in Ag-exposed oysters and led to an increased atrophy of the digestive epithelium (see below). After 14 days of exposure, some increase in lipofuscin content was observed in the Cu-exposed oysters coinciding with increased digestive epithelium atrophy. This observation may indicate the accumulation of oxidised cell debris after Cu exposure, yet after a longer exposure period compared to Ag. Although accumulation of neutral lipids in the digestive cell lysosomes is regarded as an indicator of non specific stress (Marigómez et al., 2013b), in the present study, neutral lipids were mainly detected

in the connective tissue as previously reported by Rementeria et al., (2016). This tissue is characterised by the presence of vesicular cells that store glycogen for its use in energy demanding processes, such as (i) reproduction (Berthelin-Heude et al., 2000; Jouaux et al., 2013; Thompson et al., 1996) when neutral lipids can be transferred to gonads in order to prepare for spawning periods (Cancio et al., 1999; Dridi et al., 2007) or (ii) defence against stress including pollutant presence (Guerlet et al., 2006; Séguin et al., 2016). In this work, salinity or pollutant exposure did not induce such effect, yet a translocation of neutral lipids to gonads was observed in the Control group which can be attributed to the preparation for the spawning period. In fact, the gonad development was in concordance with the seasonal reproductive cycle of oysters *Crassostrea gigas* (Fabioux et al., 2005). However, assuming longer exposure periods, it is conceivable that an arrest of the gamete development would occur as a consequence of the increased energy demand for detoxification processes (Ratte, 1999). This process was described in oysters exposed to Ag and Cu in seawater but only after more than 21 days of exposure (Rementeria et al., 2016). Accordingly, the lower salinity levels employed in the present work do not seem to impact gamete development.

Stress conditions are known to cause changes in the structure of digestive gland in bivalves, usually reflected by: the atrophy of digestive epithelium (Izagirre et al., 2014; Zaldibar et al., 2007) or the shrink and reduction in size of digestive gland tubules followed by an increase of the interstitial connective tissue (Brooks et al., 2011; Garmendia et al., 2011). Recently, the ratio between connective tissue and digestive gland tubules (CTD) was successfully proposed as general stress biomarker in oysters exposed to Ag and Cu, individually or in combination (Rementeria et al., 2016).

In the present experiment, oysters exposed to both metals individually showed an increasing tendency for digestive gland atrophy values, implying the risk of

impairment of digestive gland functions due to cell loss/death, as observed in winkles *Littorina littorea* exposed to high cadmium concentrations (1.25 mg Cd/L) for 20 days (Zaldibar et al., 2007). In concordance, the CTD ratio decreased with increasing atrophy values due to the previously mentioned cell death in the digestive epithelium, while connective tissue amounts in all exposure groups remained similar.

Based on these results, the response of the selected tissue level biomarkers did not seem to be influenced by lower salinity conditions. However, recent studies have shown that low salinity conditions can induce morphological alterations in stomach, intestines, digestive gland tubules and kidney of oysters and also cause the presence of haemocytic infiltrations in all these organs (Knowles et al., 2014). Such histopathological alterations are routinely used in biomonitoring programs because of their potential to reflect bivalve's health status and possible interactions with environmental stressors (Cuevas et al., 2015). In the present experiment, very low percentages of lesions occurred similar to the ones recorded in oysters exposed to Cu and Ag in coastal sea water (Rementeria et al., 2016), suggesting that, again, reduced salinities did not modify the biomarker response to pollutant exposure.

In order to obtain a general view of the health status of animals, the IBR index was used. A clear response pattern of biomarkers was obtained suggesting that the worst health status occurred in Ag-exposed oysters compared to the rest of the experimental groups. These results suggest that under the experimental conditions, Cu has been better regulated by oysters than Ag, probably because this element is an essential micronutrient and the exposure concentration was too low for producing significant deleterious effects at short exposure times. Interestingly, Ag exposure perfectly reflected a cascade effect starting from low biological levels at day 7 (MT induction) to higher biological levels at 14 days (CTD decrease) of exposure.

It is noteworthy that biomarker responsiveness varied according to salinity: in oysters exposed to the same concentrations of Ag in coastal seawater, clear responsiveness to MTs contents and intralysosomal metal accumulation did not occur before day 28 (Rementeria et al., 2016), whereas in brackish waters this responses appeared after only 7 days. This result suggests that at lower salinity, oysters seem to respond faster to environmental pressure, yet with very minor changes in response intensity. Therefore, even if decreased salinities did not induct clearly increased responses on biomarkers separately, the IBR index was useful to detect an earlier initiation of detoxification processes in brackish waters.

CONCLUSIONS

The present study applied a battery of biomarkers to oysters exposed to Ag and Cu in brackish water (S=18), at environmentally relevant concentrations and compared the responses to a similar experiment in coastal seawater (S=28) (Rementeria et al., 2016) at similar exposure levels. The application of the IBR index clearly pointed to higher Ag toxicity compared to Cu for the experimental conditions, where individual biomarkers at different biological levels did not always clearly respond. Reduced salinity conditions did not affect biomarker response as shown by stable or slightly decreasing IBR/n index in the Control group through exposure time.

Finally, the IBR index allowed detecting that the onset of detoxification processes occurs earlier in brackish water than in coastal seawater. This observation is in line with the general assumption of a higher toxicity of Ag at lower salinity levels. Further research should focus on the role of salinity-dependent speciation of dissolved Ag and the uptake kinetics of this metal in different oyster tissues.

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Chapter IV

Assessment of the effects exerted by copper and silver in oysters *Crassostrea gigas* (Thunberg, 1793) after dietary exposure

This chapter has been presented in:

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ABSTRACT

Assessment of the effects exerted by copper and silver in oysters *Crassostrea gigas* (Thunberg, 1793) after dietary exposure.

Oysters have been widely used as sentinel organisms in environmental health status programs because of their sedentary way of life and ability to accumulate pollutants with little metabolic transformation. Metal exposure in bivalves can occur from both, direct and trophic pathway. When the uptake follows the direct pathway, possible synergistic effects may exist in the accumulation of Ag and Cu, both highly toxic elements in aquatic systems, but less is known on the toxicity of Ag and Cu uptake via the trophic pathway. Oysters *Crassostrea gigas* have been exposed for 21 days to isotopically labelled ^{63}Cu and ^{107}Ag alone and in mixture through food pathway. Animals were fed for 20 h with previously contaminated microalgae *Isochrysis galbana* exposed for 24 h to: 500 ng $^{107}\text{Ag}/\text{L}$; 8600 ng $^{63}\text{Cu}/\text{L}$ and a mixture of 500 ng $^{107}\text{Ag}/\text{L}+8600$ ng $^{63}\text{Cu}/\text{L}$. Results indicate that algae successfully accumulated metal isotopes, whereas metal accumulation in oysters was not evident from chemical analyses. However, oysters exposed to the combination of ^{63}Cu and ^{107}Ag showed a decrease in neutral lipids and an increase in lipofuscin after 14 days of exposure as well as increased metallothionein levels after 7 and 14 days. The Integrative Biological Response index also indicated a higher toxicity exerted by ^{107}Ag and $^{107}\text{Ag}+^{63}\text{Cu}$ after short exposure time. Toxic effects caused by single exposure to ^{107}Ag and ^{63}Cu increased through the experiment. Overall, the presence of Cu in addition to Ag exposure seems to have an additive/synergistic effect on the Ag toxicity, at least after 14 days exposure.

RÉSUMÉ

Évaluation des effets exercés par le cuivre et l'argent dans les huîtres *Crassostrea gigas* (Thunberg, 1793) après de l'exposition par la voie alimentaire.

Les huîtres ont été largement utilisées comme organismes sentinelles dans les programmes de biomonitorisation en raison de leur mode de vie sédentaire et leur capacité à accumuler des polluants dans leurs tissus avec une transformation métabolique faible. L'accumulation de métaux dans les bivalves peut se produire par différentes voies. Un possible effet de synergie entre le cuivre et l'accumulation d'argent a récemment été décrit chez les huîtres, mais on connaît moins sur la toxicité de l'Ag et du Cu par la voie trophique. Dans cette expérience, les huîtres *Crassostrea gigas* ont été exposées par voie alimentaire aux isotopes ^{63}Cu et ^{107}Ag seuls et en mélange, pendant 21 jours. Les animaux ont été nourris pendant 20 h aux microalgues *Isochrysis galbana* précédemment contaminées. Ces microalgues ont été exposées pendant 24 h à : 500 ng ^{107}Ag / L; 8600 ng ^{63}Cu / L et un mélange de 500 ng ^{107}Ag / L+8600 ng ^{63}Cu / L. Les résultats indiquent que les algues ont accumulés avec succès les isotopes métalliques mais l'accumulation des métaux dans les huîtres n'était pas évidente. D'autre part, les effets significatifs exercés par la combinaison de ^{63}Cu et ^{107}Ag ont été révélés chez les huîtres par une diminution de lipides neutres et une augmentation de lipofuscines après 14 jours d'exposition, les niveaux de métallothionéines sont supérieurs après 7 et 14 jours d'exposition. L'index Integrative Biological Response (IBR) a également indiqué une toxicité plus élevée de l'isotope ^{107}Ag et du mélange isotopique $^{107}\text{Ag}+^{63}\text{Cu}$ après un court temps d'exposition alors que la présence de Cu avec Ag semble avoir un effet additif/synergique sur la toxicité de l'Ag, du moins après 14 jours d'exposition.

LABURPENA

Zilarra eta kobreaken eraginaren azterketa *Crassostrea gigas* (Thunberg, 1793) ostretan dieta bidezko esposizioa erabiliz.

Beraien bizitza modu sedentarioa eta aldaketa metaboliko urriekin kutsatzaileak metatzeko ahalmena direla eta ostrak modu zabalean erabili dira ingurumen osasunaren ebaluazio programetan organismo behale gisa. Metalen metaketa ostretan modu desberdinetatik gertatu daiteke batik bat zuzeneko esposizioz ala dieta bidezko esposizioz. Duela gutxi, kobre eta Ag-aren metaketan elkarrekintza sinergistikoak behatu dira ostretan zuzenean kutsatzaile hauen pean jartzean esposatuak izatean. Ikerlan honetan, isotopikoki markatutako ^{63}Cu eta ^{107}Ag pean jarri dira ostrak 21 egunez bakarka eta nahasketan dietaren bitartez. Horretarako animaliak egunero 20 orduz alde aurretik 24 orduz $500\text{ ng }^{107}\text{Ag/L}$; $8600\text{ ng }^{63}\text{Cu/L}$ eta $500\text{ ng }^{107}\text{Ag/L}+8600\text{ ng }^{63}\text{Cu/L}$ nahasketara esposatutako *sochrysis galbana* mikroalgekin elikatu ziren. Emaitzek erakutsi zuten algek metala metatzen zuten bitartean, ostretan metaketa hau ez zela horren argia izan. Beste alde batetik ^{63}Cu eta ^{107}Ag nahasketak ostretan lipido neutroen gutxipena eta lipofuszenen handipena eragin zuen 14 egunetara eta baita metalotioneinen mailaren handipen bat ere eragin zuen 7. eta 14. egunetan. "Integrative Biological Response" indizeari dagokionez, ^{107}Ag eta $^{107}\text{Ag}+^{63}\text{Cu}$ toxikotasun altua erakutsi zuten esposizio-denbora laburretan eta bakarkako esposizioek (^{107}Ag eta ^{63}Cu) eragindako toxikotasuna denboran zehar emendatu zen. Orotara, kobreaken presentziak Ag-arekin batera, Ag-ren toxikotasunean elkarrekintza aditibo/sinergiko bat duela ematen du behintzat 14 egunetako esposizioa eta gero.

RESUMEN

Evaluación de los efectos producidos por el cobre y la plata en ostras *Crassostrea gigas* (Thunberg, 1793) expuestas mediante la dieta.

Las ostras han sido ampliamente empleadas como organismos centinela en la evaluación de la salud de las aguas debido a su forma de vida sedentaria y su capacidad de acumular contaminantes con poca transformación metabólica. La acumulación de metales en bivalvos puede ocurrir siguiendo la ruta directa o mediante la dieta. Mediante la exposición directa se han observado posibles efectos sinérgicos en la acumulación de Ag y Cu en ostras, ambos elementos muy tóxicos. En el presente estudio se han expuesto ostras *Crassostrea gigas* a isótopos marcados de ^{63}Cu y ^{107}Ag solos o en combinación durante 21 días en la dieta. Los animales se alimentaron durante 20 horas con algas *Isochrysis galbana* previamente contaminadas durante 24 horas con: 500 ng $^{107}\text{Ag/L}$; 8600 ng $^{63}\text{Cu/L}$ y a la mezcla de 500 ng $^{107}\text{Ag/L}+8600$ ng $^{63}\text{Cu/L}$. Los resultados indicaron que las algas acumulaban los metales pero la acumulación de metales en las ostras no fue tan evidente. Por otra parte, la combinación de ^{63}Cu y ^{107}Ag ejerció un efecto negativo en las ostras con un descenso en los lípidos neutros y un incremento de lipofuscinas después de 14 días de exposición además de un incremento en los niveles de metalotioneínas después de 7 y 14 días de exposición. El índice “Integrative Biological Response” también evidenció la mayor toxicidad producida por ^{107}Ag y la mezcla $^{107}\text{Ag}+^{63}\text{Cu}$ después de un periodo breve de exposición y el efecto tóxico causado por la exposición de ^{107}Ag y ^{63}Cu aumentó junto con el tiempo de exposición. En general, la presencia del Cu junto con la exposición a Ag parece producir un efecto aditivo/sinérgico en la toxicidad de la Ag al menos a partir de 14 días de exposición.

INTRODUCTION

Environmental sentinel species (including oysters *Crassostrea gigas*) are widely used in environmental water health status monitoring programs, since these animals are able to provide unique information about changes occurred in the environment. In this context, oysters are considered valid sentinel species, because they are sedentary and filter-feeding organisms that can accumulate pollutants with little metabolic transformation (Moschino and Da Ros, 2016). Both chemical measurements of pollutants and batteries of biomarkers have been successfully applied in bivalves' tissues in order to assess health status of estuarine and coastal areas in biomonitoring programs all over the world (Cuevas et al., 2015; Séguin et al., 2016; Xie et al., 2016). For example, the French Mussel-Watch Program (RNO/ROCCH) has collected oysters for more than 30 years with the aim of assessing coastal water quality levels (Lanceleur et al., 2011).

Among the many different pollutants present in estuaries and coastal areas, silver (Ag) and copper (Cu) are metals of particular interest because they are amongst the most toxic elements for aquatic biota (Haberkorn et al., 2014; Money et al., 2011; Ratte, 1999; Santovito et al., 2015; Tappin et al., 2010). Copper plays an essential role in several biological/biochemical processes but is highly toxic when tolerable exposure levels are exceeded (Haberkorn et al., 2014; Ringwood et al., 1998; Santovito et al., 2015). On the other hand, Ag is a non-essential metal that has been described as toxic even at very low concentration levels (Ratte, 1999; Tappin et al., 2010, Rementeria et al., 2016a). A variety of Cu and Ag sources to the aquatic environment exist; Cu may originate from fertilisers, pesticides, algacides, antifouling paints and sewage waters (Lee et al., 2010), while the classical Ag sources have mainly been metallurgy, jewellery, photography, electronics... (Lanceleur et al., 2013). Nowadays, increasing concern is being focused on Ag due

to its use in cloud-seeding devices and personal care products, generally in form of nanoparticles (Fabrega et al., 2011; Lanceleur et al., 2011; Luoma et al., 2008).

In the aquatic environment, one of the first biological groups affected by metal pollution are microalgae since they are primary producers at the base of the aquatic food chain (Debelius et al., 2009; Satoh et al., 2005). Metal uptake by microalgae is known to be dominated by two major processes: adsorption onto the cellular surface and the absorption of metals. Usually, the external adsorption of metals is followed by subsequent absorption of metals (uptake into the interior of the cell) (Moreno-Garrido et al., 2001). The absorption implies that metals pass through biological membranes in an active or a passive way. Specific membrane transports (ion/voltage-gated channels), are in charge of the active transport of metal ions and also of polar metal-organic complexes (Bielmyer-Fraser et al., 2014). On the other hand, in the passive absorption, metals directly cross the biological membranes (de Souza Machado et al., 2016). Once metals are inside the algae, they bind to the metabolic sites usually occupied by essential ions causing toxic effects (Bielmyer-Fraser et al., 2014). Moreover, when algae are ingested by organisms of higher levels within the trophic chain, metals can be transferred to animals such as bivalves (Fisher and Wang, 1998; Trenfield et al., 2015).

Pollutant accumulation pathways and effects in marine bivalves have been long studied (Abbe and Sanders, 1990; Ettajani et al., 1992; Hédouin et al., 2010; Wildgust et al., 2000). In particular for metals, two main uptake pathways have been classically distinguished: (i) the direct pathway which implies metal uptake from water through filtration and (ii) the particulate pathway; due to the ingestion of contaminated food (also known as trophic, food or dietary pathway) and/or sediments (de Souza Machado et al., 2016; Fisher and Wang, 1998; Hédouin et al., 2010). During the last decades, the food pathway route has gained importance, and it is considered by several authors as the main route for metal accumulation in

marine invertebrates (Fisher and Wang, 1998; Hédouin et al., 2010; Lee et al., 2015; Pan and Wang, 2009). Conversely, other authors have identified the direct pathway uptake as major route for metal pollutant accumulation (Abbe and Sanders, 1990; Ettajani et al., 1992; Strady et al., 2011b). Therefore, the importance of each metal accumulation pathways is still under discussion and suggests the presence of several factors (tested animal, type of nutrition, uptake kinetics, exposure levels, pH, salinity...) influencing the balance between metal accumulation pathways.

The use of stable isotopes presents a real alternative tool to trace metal accumulation kinetics and pathways in oysters tissues at close to real exposure levels (Mikolaczyk et al., 2016; Strady et al., 2011b). In fact, this tool has proven suitable for tracing simultaneously different metal accumulation pathways in oysters *Crassostrea gigas* through the use of cadmium (Cd) stable isotopes, demonstrating that during 28 days of exposure to Cd at close-to-real exposure levels, Cd was preferably accumulated by direct pathway than trophic pathway (Strady et al., 2011b).

The combination of isotopic tracing with measurements of cell and tissue level biomarkers and their subsequent integration, demonstrated to be useful for the assessment of oyster's response to Ag and Cu exposure at relatively low concentrations (Mikolaczyk et al., 2016; Rementeria et al., 2016a). In fact, these authors suggested a synergy on metal accumulation resulting in an enhanced Ag accumulation in the presence of Cu together with a diminished health status of oysters (Rementeria et al., 2016a).

In the present study, the integration of a battery of biomarkers into the Integrated Biological Response (IBR) index in oysters exposed to stable isotopes (^{63}Cu and ^{107}Ag) via trophic pathway aimed at assessing the effects of metal exposure at different biological levels.

MATERIALS and METHODS

1. Experimental set-up

1.1 Oyster obtaining

In January 2015, oysters *Crassostrea gigas* (7-8 cm long) were bought from an oyster farm (Ostranor S.L) situated in a unpolluted estuary (San Vicente de la Barquera, Cantabria, Spain) (De los Ríos et al., 2016). Oysters were immediately transferred to the Plentzia Marine Station (PiE UPV/EHU, Plentzia, Basque Country, Spain) and kept during 7 days in natural filtered sea water for acclimatisation (same as experimental conditions described below) with a continuous air flow. The photoperiod was established at 12:12 h (light:dark) and the room temperature was kept constant (T=17 °C).

After this period, oysters were introduced into 45 L high density polypropylene experimental tanks each one containing 30 individuals with 30 L of natural filtered sea water (S=31.1). Four experimental groups were set (each one with two replicates, thus, in total 8 tanks): Control, ^{107}Ag , ^{63}Cu and $^{107}\text{Ag}+^{63}\text{Cu}$. Solutions of stable isotopically-labelled Cu and Ag were prepared as described by (Mikolaczyk et al., 2016).

1.2 Algae culture

A strain of *Isochrysis galbana* T. Iso clone was grown in previously cleaned 30 L volume methacrylate reactors with natural filtered sea water (S=31.1). Algae were maintained under constant white light exposure (two lamps of 36 W per reactor), room temperature (T=17 °C) and filtered air flow (0.2 µm filters) (Fig. 1). Culture alga density was daily checked on a Neubauer Counting Chamber and when concentration reached 8×10^6 cell/mL it was again diluted and enriched with F/2 Algae Food Part A and B medium (Based on Guillard's formulation, Proline water conditioners).

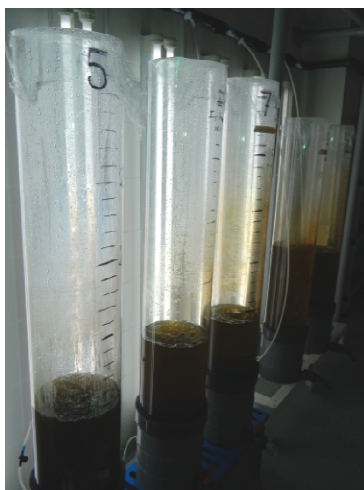


Figure 1: Methacrylate reactors containing *Isochrysis galbana* algae cultures.

1.3 Algae exposure to contaminants

Every experimental day, algae from reactors at exponential growth phase were transferred into previously cleaned (with HCl, H₂O₂ and seawater) glass bottles and diluted with filtrated natural sea water until reaching algae density of 3×10^6 cell/mL in 1 L volume, concentrations were checked on a Neubauer Counting Chamber. Four exposure groups were defined (including two replicates per group): Control, 500 ng ¹⁰⁷Ag/L, 8600 ng ⁶³Cu/L and 500 ng ¹⁰⁷Ag/L+8600 ng ⁶³Cu/L. Exposure time to metal isotopes was limited to 24 h, as previously conducted screening experiments showed that this period was long enough for algae to accumulate pollutants without causing a significant increase in mortality. Pollutant exposition was carried out under constant filtered air, cool light and room temperature (T=17°C) (Fig. 2).

This microalga (*Isochrysis galbana*) specie was selected because it does not present a cell wall and therefore an easier accumulation of metals into cytoplasm is expected as well as more efficient digestion by oysters, indeed this specie is widely employed in aquaculture (Wildgust et al., 2000). Moreover, recent studies have successfully used these algae as Control food (Medhioub et al., 2012).

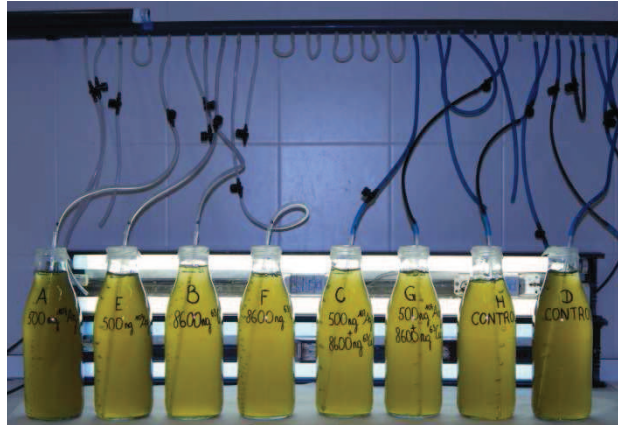


Figure 2: Algae exposure to pollutants in separated glass bottles with constant light and aereation.

1.4 Algae centrifugation

Before feeding oysters, spiked algae were separated from the culture water through centrifugation (4000 rpm during 6 minutes), then resuspended in natural non spiked filtered sea water. The centrifuged algae volume was adjusted through the experiment according to the remaining number of oysters and water volume per tank (Fig. 3).

1.5 Oyster feeding

Oysters were daily fed during 20 h with clean (Control) or previously polluted algae *Isocryshis galbana*, and after this period sea water was renewed and animals were kept for 4 h in clean water in order to mimic previous experiments via direct exposure to ^{107}Ag and ^{63}Cu (Mikolaczyk et al., 2016; Rementeria et al., 2016a). Everyday oysters were fed with approximately 2000 algae cell/mL per individual. Then, exposure tanks were carefully cleaned and sea water was renewed. Water volume and food amount for each aquaria were adapted through the experiment.

Oysters were kept under a 12:12 h photoperiod, constant room temperature ($T=17^{\circ}\text{C}$) and with stable water physic-chemical parameters ($T=14.5^{\circ}\text{C}$, $\text{pH}=8$ and $\text{S}=31.1$) (Fig. 3).

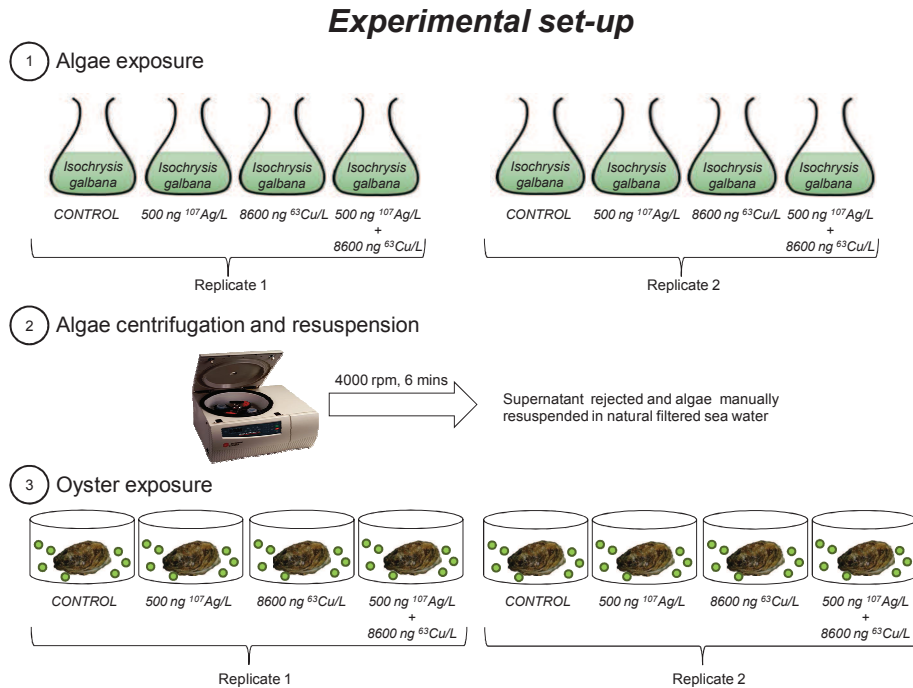


Figure 3: Experimental set-up scheme for the present experiment.

2. Sample collection and processing

Animal dissections were carried out at days 0, 7, 14 and 21. Each time 8 individuals per experimental unit replicate were sampled. Oysters were opened and shell and flesh weights were individually measured for the calculation of the Condition Index (CI). Three of the collected animals were dissected for organotropism analysis of accumulated metal isotopes (Mikolaczyk, 2016) while the remaining five individuals were dissected for biomarker studies. For histology, a ~5 mm thick cross-section of the oyster's soft body including all main organs and tissues (gill, digestive gland and gonad) was obtained per animal ($n=5$ per replicate; $n=10$ per treatment). Sections were rinsed in formalin for fixation during 24 h and routinely processed for histological examination.

Small pieces of digestive glands from five individuals per aquaria were rapidly frozen in liquid nitrogen for cryosectioning. The remaining digestive gland material was also frozen and stored at -80° C until processing for metallothionein content determination.

Spiked water and algae samples were also collected at 0, 7, 14 and 21 days 10 minutes and 24 h after pollutant spiking in order to analyse accumulation of metal isotopes in algae *Isochrysis galbana* (Mikolaczyk, 2016).

3. Chemical measurement

Prior to sample obtaining all the labware used for chemical analyses was thoroughly cleaned by soaking in HNO₃ 10% for 72 h rinsing with deionised water followed by MilliQ® water, then dried under a laminar flow hood and stored in double-sealed plastic bags. Algae samples were collected as bulk by centrifugation, whereas oysters were carefully dissected using Teflon scissors and the following organs were isolated: gills, adductor muscle, digestive gland, mantle and gonads. Obtained algae pellets and each oyster organ were introduced into previously weighed and cleaned polyethylene tubes. Afterwards, samples were deep-frozen (-80°C), freeze-dried, weighed, ground in an agate mortar and homogenised. From each sample of algae and oyster organ 200 mg were introduced into clean polyethylene tubes for acid digestion with 1.4 mL HNO₃ (14 M, PlasmaPur) and 2 mL HCl (12 M, PlasmaPur) following the procedure described by Daskalakis, (1996). Briefly, closed tubes were digested for 3 h in a heating block at 90°C (DigiPREP MS; SCP SCIENCE). Once digestates cooled, they were diluted with Milli-Q® water and analysed for Cu and Ag isotope ratios and concentrations by Inductively Coupled Plasma Mass Spectrometry (ICP-MS; Thermo, X Series II) with external calibration (made with commercially available standard solutions PLASMACAL, SCP Science). At each analytical session accuracy and precision were controlled by parallel analysis of international referenced certified materials (TORT 2, NIST Oyster Tissue 1566b and IAEA 407), and were respectively >90% and 5% (r.s.d.).

4. Biological measurements

4.1 Mortality

Oyster mortality was daily checked. Oysters were considered as dead individuals when after physical stimulation valves gaped and failed to close (Veldhuizen-Tsoerkan et al., 1991).

4.2 Condition Index

The Condition Index (CI) per individual was calculated according to the formula: $CI = (WW \text{ visceral content} / WW \text{ shell} \times 100)$ (Strady et al., 2011a).

4.3 Digestive gland and gonad histology

From digestive gland, gonads and gills, 4 μm thick sections were obtained in a microtome from paraffin embedded samples and stained with haematoxylin-eosin. Afterwards, microscopic slides were analysed under a light microscope (Olympus BX 61).

4.3.1 Gamete developmental stage

Sex and gonad developmental stages were determined as well as Gonad Index (GI Value) for each individual according to the procedure described by Kim et al. (2006) which is based on a subjective scale of the developmental stage of follicles and gametes after examination under the light microscope.

4.3.2 Histopathological alterations

Histopathological analyses through oysters whole body transections were completed paying special attention to most relevant alterations including Granulocytomes (GRN), Inflammations (INF), Haemocytic infiltrations (HAE), Haemocytic infiltrations in Gonads (HAE gn) and Parasite presence (Par). Prevalence values for each alteration were obtained.

4.3.3 Digestive gland atrophy

A semi-quantitative procedure based on Kim et al., (2006) was followed in order to determine the thinning of digestive tubule walls. Individuals were observed under an Olympus BX-61 microscope (Olympus, Japan) light microscope and a score based on digestive gland atrophy degree was assigned to each one. Values ranged from 0 (normal tubule wall thickness) to 4 (wall extremely thin, nearly all tubules affected).

4.3.4 Tissue integrity in digestive gland (CTD ratio)

The integrity of the digestive gland tissue was determined by calculating the connective-to-diverticula (CTD) ratio. For this, 5 fields of digestive gland tubules were obtained per individual with a digital camera attached to an Olympus BX-61 microscope (Olympus, Japan) and processed through Image J program (Image Processing and Analysis in Java, Maryland, USA). CTD ratio is defined as the extent of the interstitial connective tissue relative to the area occupied by digestive diverticula. The following formula was applied to obtain CTD ratio: $CTD\ ratio = c / (b + d + l)$. Where: (c) interstitial connective tissue, (b) basophilic cells, (d) digestive cells and (l) diverticular lumen (Brooks et al., 2011).

4.4 Neutral lipid content determination

Frozen 8 μ m thick sections from digestive glands ($n=5$) were obtained in the cryostat at -26°C chamber temperature. Slides were stored at -40°C and stained following Lillie and Ashburn's Oil Red O (ORO) method (Culling, 1974). The volume density ($V_{V_{NL}}$) of neutral lipids was quantified with respect to connective tissue by using Image J program. $V_{V_{NL}}$ was calculated as the ratio between neutral lipid volume and connective tissue volume ($V_{V_{NL}} = V_{NL} / V_{CT}$), where: (V_{NL}) volume of neutral lipids, and (V_{CT}) volume of connective tissue (Marigómez and Baybay-Villacorta, 2003).

4.5 Lipofuscin content determination

Frozen 8 μm thick sections from digestive glands ($n=5$) were obtained in the cryostat at -26°C chamber temperature. Slides were stored at -40°C until histochemical demonstration of lipofuscins through the Schmorl method (Pearse, 1985). The volume density ($V_{V_{LF}}$) of lipofuscins was quantified with respect to digestive tissue by using Image J program, as $V_{V_{LF}} = V_{LF}/V_{DT}$, where: (V_{LF}) volume of lipofuscins, and (V_{DT}) volume of digestive tissue (Marigómez et al., 2013b).

4.6 Intralysosomal metal accumulation

Histochemical detection of metals was carried out in 4 μm thick sections of paraffin embedded samples ($n=10$ per treatment). After embedding, sections were dewaxed in xylene and hydrated, then, the slides were dried in an oven at 37°C for at least 24 h. Samples were covered with the commercial silver enhancement kit (Silver enhancing kit for light and electron microscopy, BB International) at room temperature, development was checked under light microscope and stopped after 15-20 min. Slides were then washed with tap water and metals were developed as black silver deposits (BSD). Finally, slides were mounted in Kaiser's glycerine gelatine and volume density of BSDs ($V_{V_{BSD}}$) in the lysosomes of digestive gland cells was measured with an image analysis system (Soto et al., 2002).

4.7 Metallothionein concentration

Frozen and stored (-80°C) digestive gland samples ($n=10$ per treatment at each sampling time) were pooled in order to obtain at least 1 g of tissue. Afterwards, metallothionein (MT) content was determined spectrophotometrically according to the modified UNEP/RAMOGÉ (United Nations Environment Programme) method from Viarengo et al., (1997). The supernatant absorbance was evaluated at 412 nm and MT concentration was estimated using reduced glutathione (GSH) as reference

standard. Results are expressed as MT concentrations (MT $\mu\text{g/g}$ of wet weight tissue).

4.8 Integrative Biological Response (IBR index)

Integrative Biological Response (IBR) index was calculated following the procedure described by Devin et al., (2014) which is a modification of the method established by Beliaeff and Burgeot, (2002).

A total of 5 biomarkers were used for IBR calculation ordered according to their biological complexity (from low to high): subcellular level (metallothionein content), cellular level (lipofuscin accumulation), tissue level (Atrophy), organ level (CTD index) and individual level (Condition Index). Finally, as the IBR value depends on the number of applied biomarkers, the IBR/n was obtained dividing IBR by the number of biomarkers applied ($n=5$) (Brooks et al., 2011; Marigómez et al., 2013a, 2013b).

4.9 Statistics

All statistical analyses were carried out with the SPSS v 22.0 (SPSS. Inc., Chicago, Illinois). Analyses of variance (ANOVA) were performed after checking data normality and homogeneity (Kolmogorov Smirnov and Levene's test) for determining significant differences between experimental groups ($p < 0.05$) followed by Duncan's post hoc test. In those cases, where data did not have normal and homogeneous distribution, Kruskal Wallis non parametric method was used (Dunns post hoc test).

RESULTS

1. Metal accumulation in algae and oysters

Results for algae metal accumulation showed that after 24 h of metal exposure, Ag-exposed algae accumulated Ag with significant differences between groups (Table 1). No statistically significant differences between groups were observed for Cu accumulation, however, average Cu concentrations tended to be higher (~50%) in experimental groups with Cu exposure (Table 1). Furthermore, a higher incorporation of Ag occurred as shown by the incorporation ratios for algae after 24 h of exposure: $98.1 \pm 0.16\%$ (^{107}Ag) and $63.1 \pm 8.30\%$ (^{63}Cu).

Table 1: Mean values for total metal concentration (mg / kg) of Ag and Cu in *I. galbana* after 24 h of exposure in different exposure groups. Significant differences groups in super scripts (a,b) ($p < 0.05$).

Accumulation	Control	^{107}Ag	^{63}Cu	$^{107}\text{Ag}+^{63}\text{Cu}$
Ag TOT (mg / kg)	0.022 ± 0.013^a	0.777 ± 0.372^b	0.022 ± 0.008^a	0.578 ± 0.305^b
Cu TOT (mg / kg)	19.28 ± 11.9	21.9 ± 11.3	32.3 ± 12.4	31.3 ± 13.8

Despite the high sensitiveness of the isotope spike method in kinetic accumulation experiments (Mikolaczyk et al., 2016), no statistically significant change in the isotopic ratio was detected in oysters exposed to spiked algae through the experimental period. Thus, oysters did not measurably accumulate metals in their tissues from metal spiked algae (Table 2 and 3).

Table 2 and 3: Change of isotopic ratios (2) $^{107/109}\text{Ag}$ and (3) $^{63/65}\text{Cu}$ in oysters through the experiment at the start and at the end of the 21 days exposure.

$^{107/109}\text{Ag}$	Control	^{107}Ag	^{63}Cu	$^{107}\text{Ag}+^{63}\text{Cu}$
T0	1.07 ± 0.04	1.07 ± 0.04	1.07 ± 0.04	1.07 ± 0.04
T21	1.09 ± 0.04	1.22 ± 0.04	1.12 ± 0.07	1.12 ± 0.05

$^{63/65}\text{Cu}$	Control	^{107}Ag	^{63}Cu	$^{107}\text{Ag}+^{63}\text{Cu}$
T0	2.04 ± 0.02	2.04 ± 0.02	2.04 ± 0.02	2.04 ± 0.02
T21	2.05 ± 0.02	2.03 ± 0.02	2.05 ± 0.02	2.08 ± 0.04

2. Mortality

Mortality values were recorded in all experimental groups ranging from 6.66% in $^{107}\text{Ag}+^{63}\text{Cu}$ exposure group at day 7 to 26.66% at day 21 in ^{107}Ag exposed oysters (Table 4). Values were represented as percentage sums, thus meaning that final values for day 21 show the percentage of oysters that had died since the beginning of the experiment. In general, values were relatively high even in Control groups, but the highest values were observed in Ag-exposed oysters.

Table 4: Percentage of accumulated mortality through the experiment.

%	Control	^{107}Ag	^{63}Cu	$^{107}\text{Ag}+^{63}\text{Cu}$
T0	0	0	0	0
T7	8.33	11.66	8.33	6.66
T14	16.66	18.33	13.33	10
T21	21.66	26.66	18.33	16.66

3. Condition Index (CI)

No statistically significant differences between exposure groups were found after 7 and 14 days exposure. However, at day 21 CI values increased in all experimental groups, moreover, all experimental groups except ^{107}Ag exposure group had significantly higher values than at day 0 (Fig. 4).

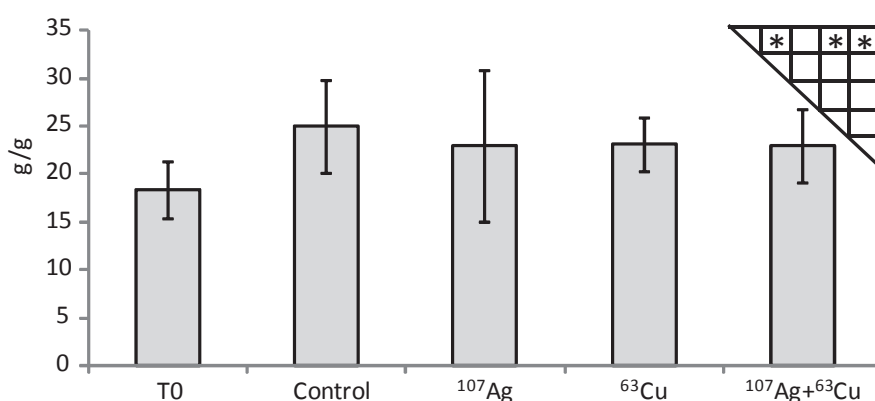


Figure 4: Mean values (\pm standard deviations) of Condition Index (CI) for day 21. CI values significantly increased for all experimental except ^{107}Ag group after 21 days of exposure. Significant differences between groups * ($p < 0.05$).

4. Gamete developmental stage

Oysters were found in a sexually undifferentiated or in an early gamete developmental stage in all the experimental groups and throughout the whole experimental period. However, few individuals at day 7 in the ^{107}Ag -exposed group and at day 14 in the Control group were in spawned phase (Fig. 5). In general, no differences were observed between experimental groups (Fig. 12 A).

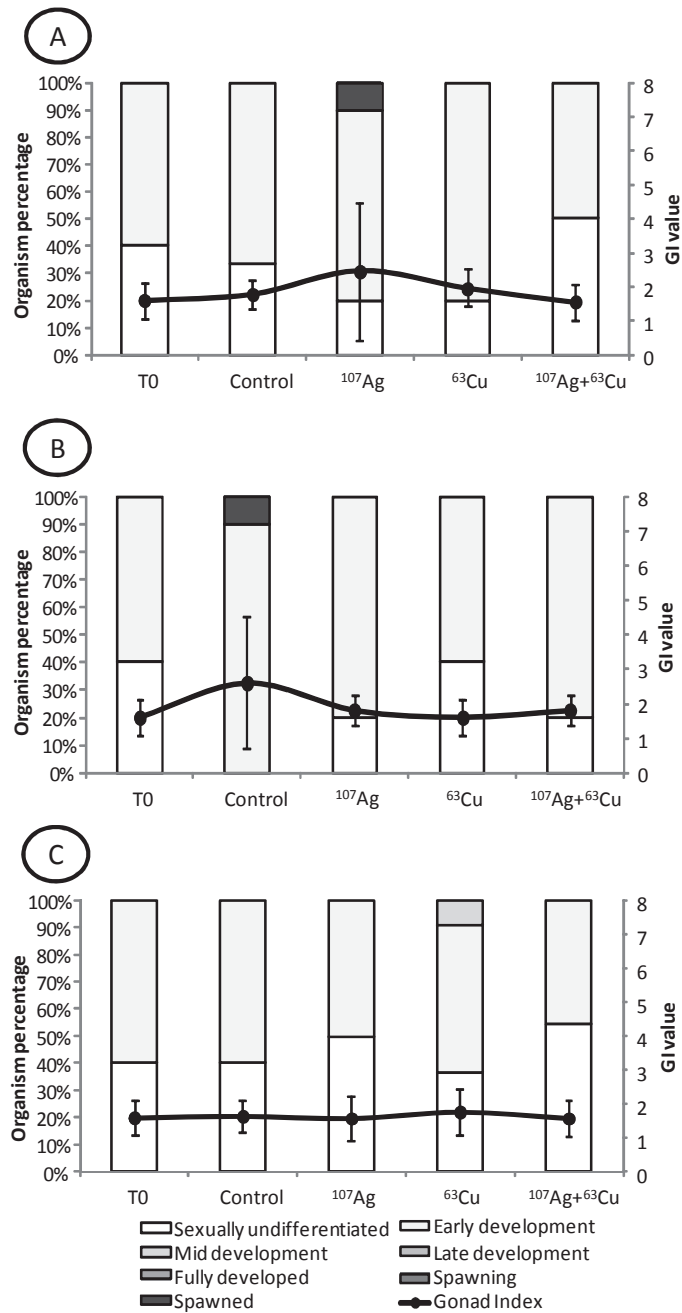


Figure 5: Percentage of gamete developmental stage (stacked bars) and mean values (\pm standard deviations). Gonad Index (represented with a line) for day 0 and after 7 (A), 14 days (B) and 21 days (C) of exposure.

5. Digestive gland atrophy

The digestive gland atrophy results did not show statistically significant differences between treatments at any sampling time. However, somewhat higher atrophy values were recorded in oysters exposed to the combination of both metals at day 7 and 14. After 21 days, individuals exposed to ^{107}Ag alone tended to have the highest atrophy values (Fig. 6).

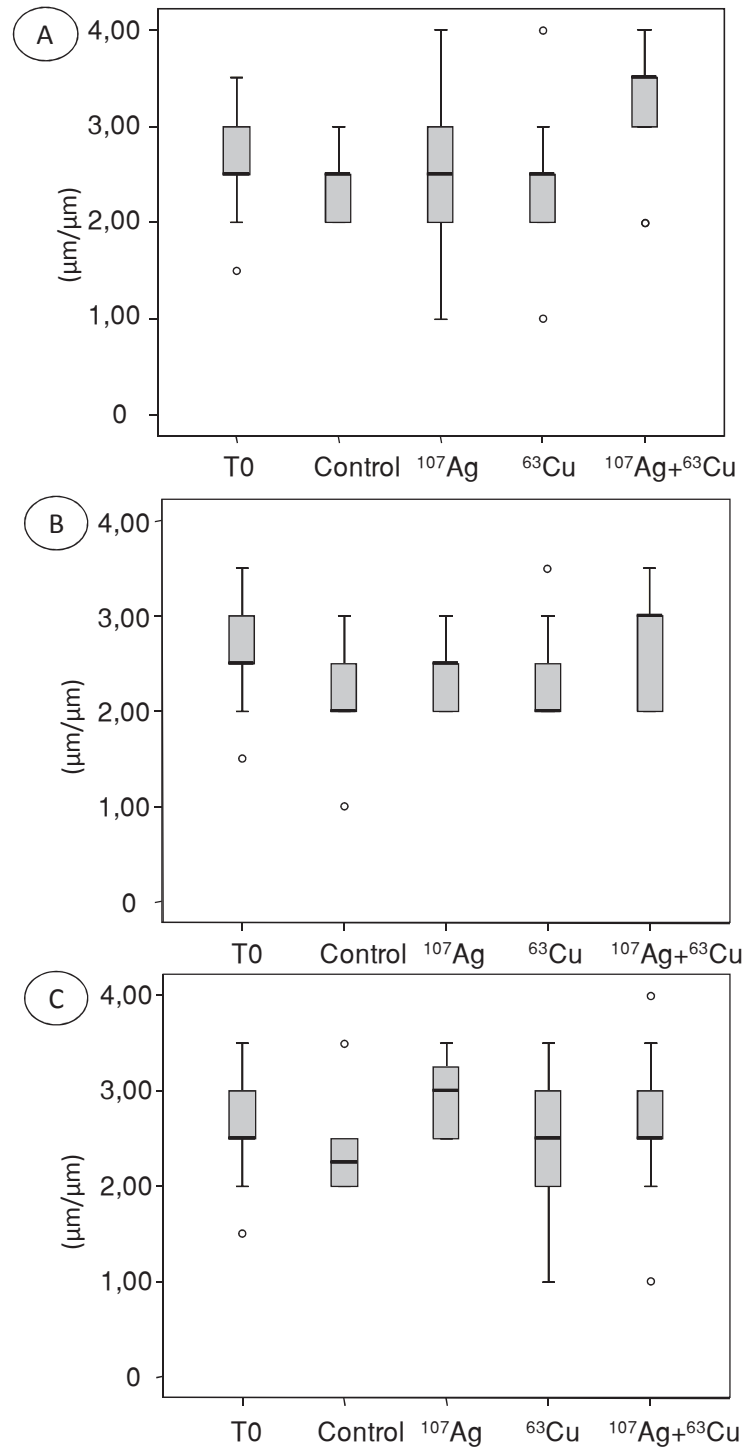


Figure 6: Box-plot of the atrophy values for day 7 (A), day 14 (B) and day 21 (C). Box plots represent the 25th, 50th and 75th percentiles, while Whiskers represent the standard deviations. Circles indicate outliers.

6. Tissue integrity in digestive gland (CTD ratio)

Statistically significant differences between experimental groups were detected at all sampling times. On day 7 higher values occurred in oysters exposed to ¹⁰⁷Ag and

^{63}Cu in combination, followed by Control group oysters. Indeed, these two groups were statistically significant different from day 0 oysters (Fig. 7). After 14 and 21 days exposure, all experimental groups including Control oysters had statistically significantly higher values of CTD ratio than day 0 samples.

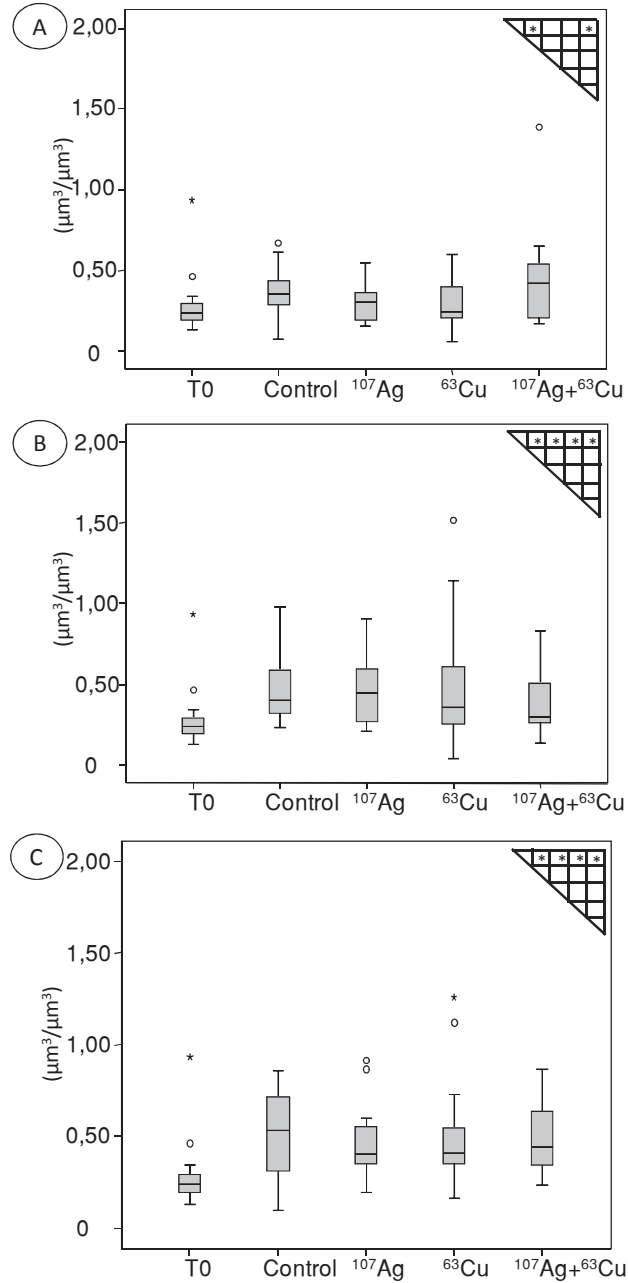


Figure 7: Box-plot of the CTD ratio values for day 7 (A), day 14 (B) and day 21 (C). Box plots represents the 25th, 50th and 75th percentiles, while Whiskers represents the standard deviations. Circles indicate outliers. Values significantly increased over the time. Statistically significant differences between groups * ($p < 0.05$).

Regarding other types of pathologies, prevalence of injuries was generally low. The detected pathologies were mainly related with the presence of haemocytic infiltrations and inflammation. Parasites were only present at day 7 in 10% of the individuals exposed to either Cu or Ag alone (Fig. 12 B).

7. Neutral lipid content determination

Neutral lipids were mainly detected in oysters' connective tissue surrounding digestive gland tubules (Fig. 12 D). Measurements of Vv_{NL} indicated statistically significant differences between experimental groups only after 14 days of exposure. At 7 days of exposure all the groups presented similar neutral lipid amounts, while after 14 days, Control groups had the highest Neutral lipid contents and minimum values occurred in oysters exposed to Ag and Cu in combination (Fig. 8). After 21 days of exposure Vv_{NL} values still were highest in the Control group followed by oysters exposed to Cu only.

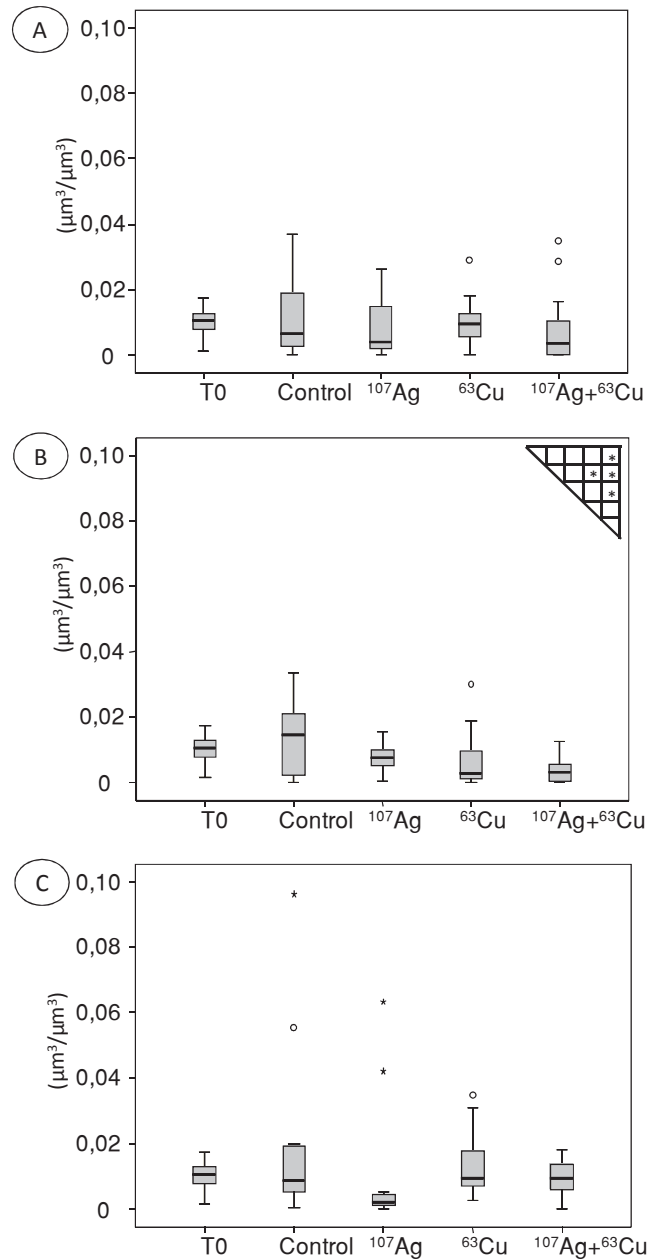


Figure 8: Box- plot of the neutral lipid content values for day 7 (A), day 14 (B) and day 21 (C). Box plot represents the 25th, 50th and 75th percentiles, while Whiskers represents the standard deviations. Circles indicate outliers. Statistically significant differences between groups * ($p < 0.05$).

8. Lipofuscin content determination

Lipofuscin contents were detected in the lysosomes of digestive gland cells. Their amount visibly increased over the experimental period (Fig. 12 E-F). Differences in lipofuscin contents were observed for all sampling times. At day 7, oysters exposed to $^{107}\text{Ag}+^{63}\text{Cu}$ had significantly lower values compared with the other groups.

However, at day 14 this exposure group had the highest lipofuscin content values followed by Ag-exposed oysters. Finally at day 21, all treatments showed an increase in lipofuscin contents being statistically different from day 0 values. Highest values for this exposure time were recorded in the Control group followed by the Ag-exposed group (Fig. 9).

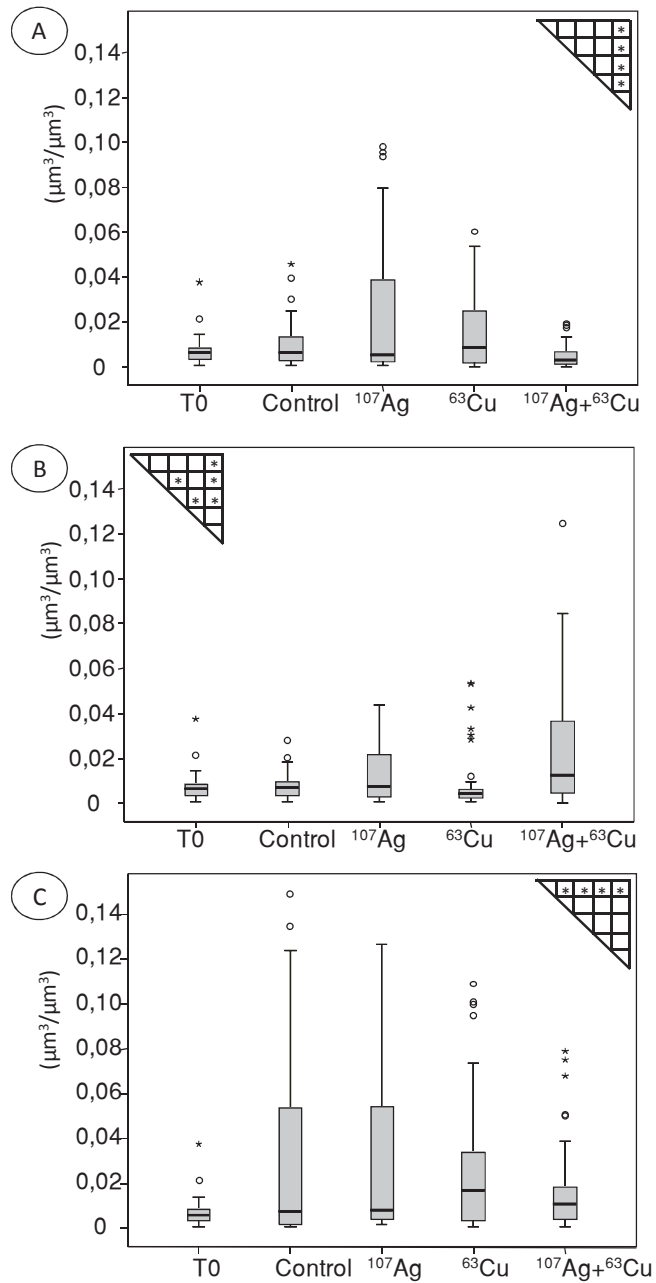


Figure 9: Box-plot of lipofuscin content values for day 7 (A), day 14 (B) and day 21 (C). Box plot represents the 25th, 50th and 75th percentiles, while Whiskers represents the standard deviations. Circles indicate outliers. Statistically significant differences between groups * ($p < 0.05$).

9. Intralysosomal metal accumulation

Differences in the BSD distribution between tissues and cells were observed according to the nature of the exposure metal. Thus, Cu-exposed oysters had BSD in the digestive gland epithelium (Fig. 10 B and D) whereas Ag-exposed oysters showed BSD in haemocytes (Fig. 11 C and D).

Regarding the quantification of intralysosomal metals, significant differences in metal distribution occurred after 14 and 21 days between exposure groups (Fig. 10). Although at day 7 no significant differences were present, Vv_{BSD} contents tended to be higher in Ag-exposed individuals. At day 14, Control and Cu-exposed oysters showed higher Vv_{BSD} contents, while lower values occurred for oysters exposed to the combination of both metals. Finally at day 21, Control values had lowest Vv_{BSD} contents. On the contrary, highest values were found for the $^{107}\text{Ag}+^{63}\text{Cu}$ exposure groups (Fig. 10).

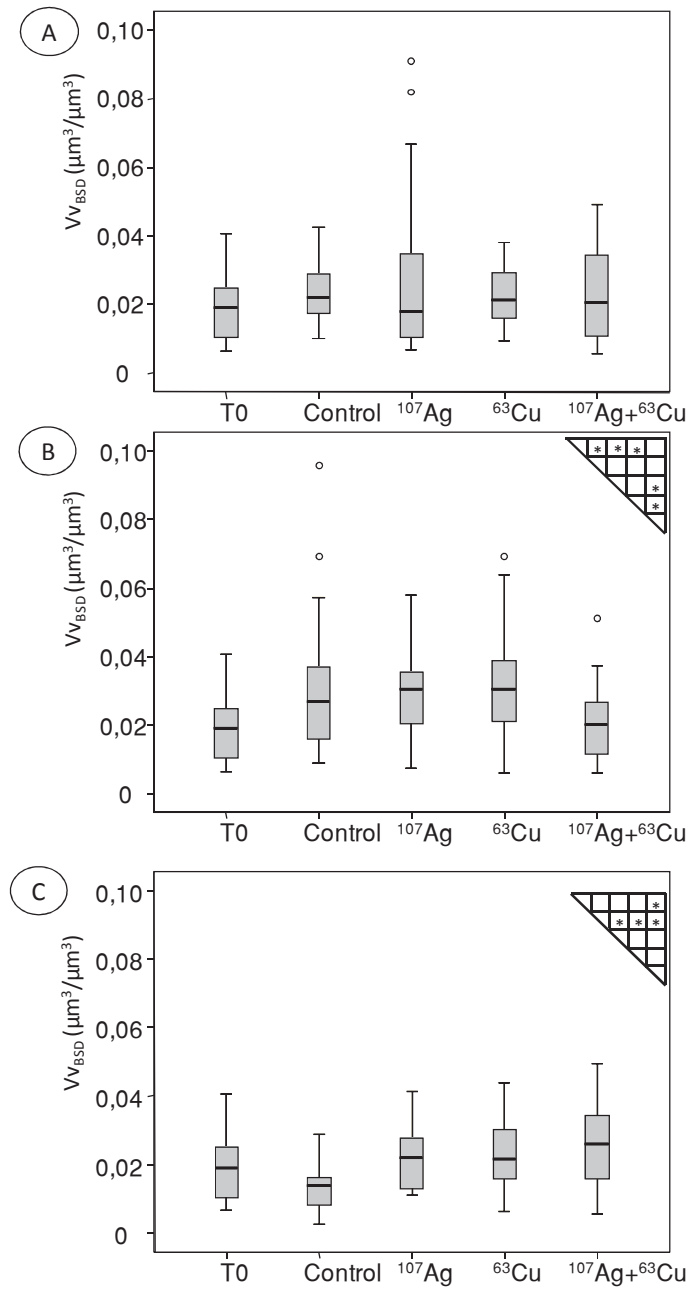


Figure 10: Box-plot of Intralysosomal metal accumulation in oysters at day 7 (A), day 14 (B) and day 21 (C). Box plot represents the 25th, 50th and 75th percentiles, while Whiskers represents the standard deviations. Circles indicate outliers. Statistically significant differences between groups * ($p < 0.05$).

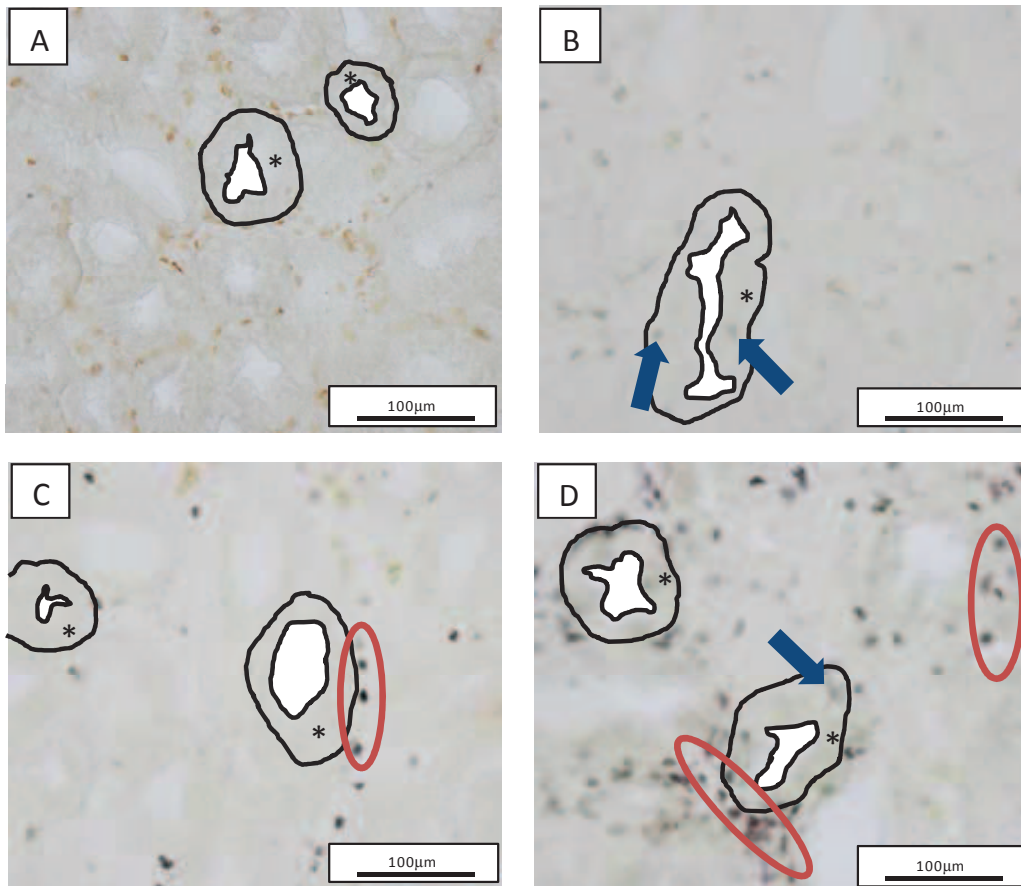


Figure 11: Metal accumulation pattern after autometallography. (A) Low levels of metals at T0 in digestive gland (digestive tubules profiles emphasised, digestive epithelium in *). (B) ^{63}Cu exposure on day 14 with metal accumulation in digestive epithelium (blue arrows). (C) ^{107}Ag exposed oyster on day 14: haemocytes display a metal signal (within red circles). (D) $^{107}\text{Ag}+^{63}\text{Cu}$ at 14 day, digestive epithelium (blue arrow) and haemocytes show metal signal (red circle).

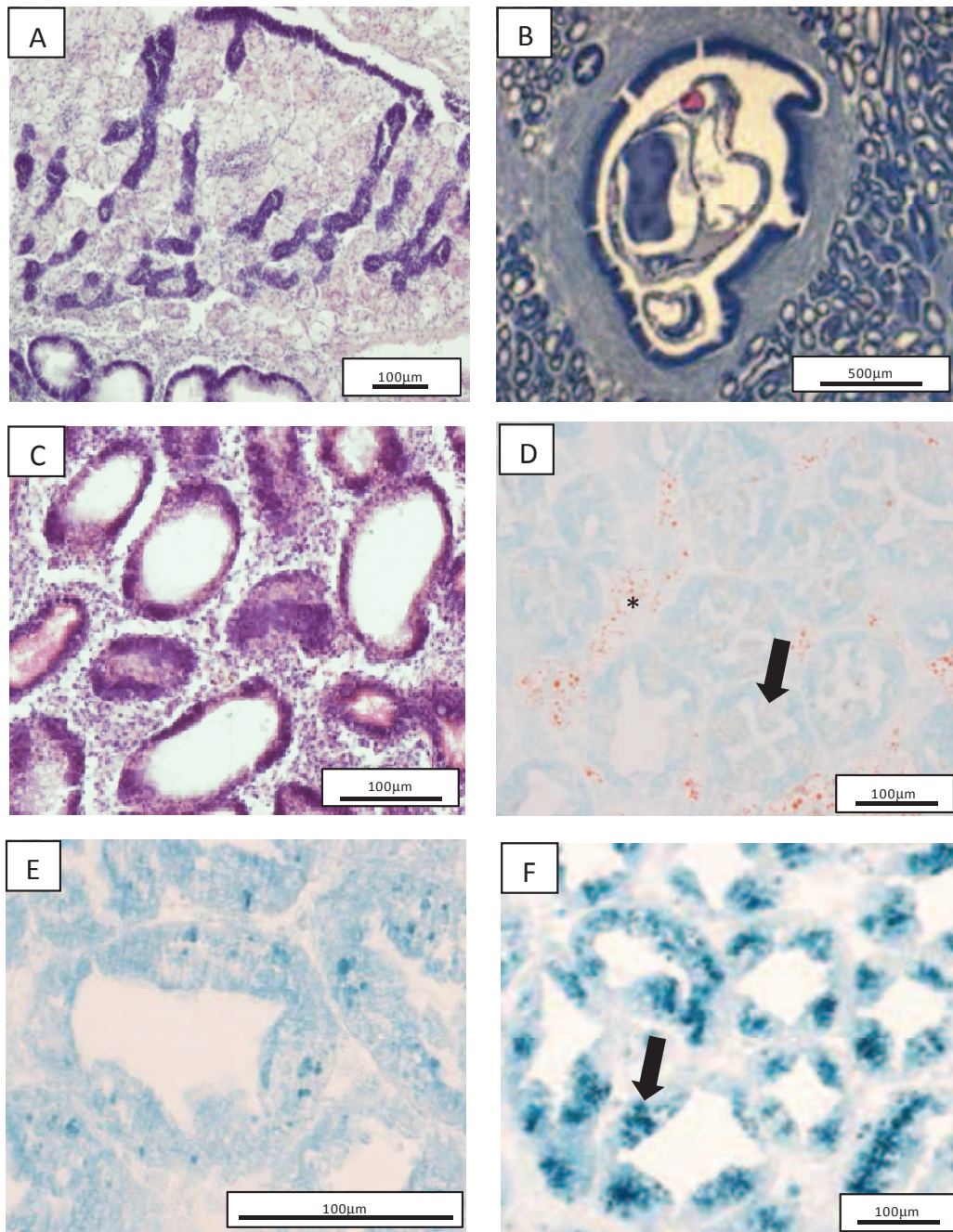


Figure 12: (A) Gonad in early developmental stage at day 14 in $^{107}\text{Ag}+^{63}\text{Cu}$ exposed oyster. (B) Parasite presence in the intestine of a ^{63}Cu exposed oyster at day 7. (C) Atrophied digestive gland tubules surrounded by increase interstitial connective tissue in $^{107}\text{Ag}+^{63}\text{Cu}$ exposed oyster at day 7. (D) Neutral lipid contents in connective tissue (*) and alga debris in digestive gland cells (arrow) in Control oyster at day 14. (E) No lipofuscin accumulation in digestive cells of oysters at day 0. (F) Relevant lipofuscin accumulation in ^{107}Ag exposed oysters at day 14.

10. Metallothionein (MT) content determination

No statistically significant differences between exposure groups were detected at any sampling day. Nevertheless, at day 7 a noticeable increase in MT contents with respect to the rest of the groups occurred in $^{107}\text{Ag}+^{63}\text{Cu}$ exposure group (Fig. 13). On the other hand, MT contents for ^{63}Cu exposed oysters decreased with respect to day 0 values. At day 14 also Control group individuals had reduced MT contents, while ^{107}Ag and $^{107}\text{Ag}+^{63}\text{Cu}$ groups had both the highest values. Finally, at day 21, metallothionein levels between treatments were similar although the previously described distribution pattern observed on day 14 was still present. Thus, ^{107}Ag and $^{107}\text{Ag}+^{63}\text{Cu}$ exposed animals had the highest MT contents, whereas Cu-only exposed group individuals maintained low MT levels (Fig. 13).

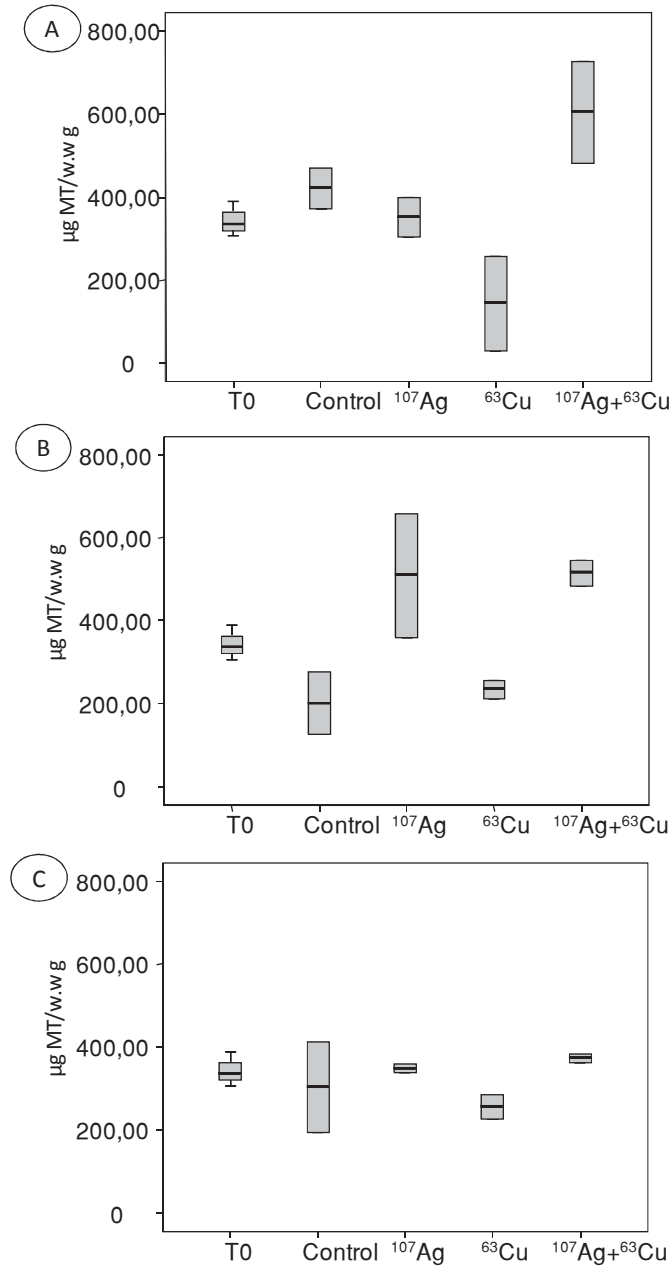


Figure 13: Box-plot of MT content ($\mu\text{g MT/w.w.g}$) in digestive gland of oysters after (A) 7, (B) 14 and (C) 21 days. Box plot represents the 25th, 50th and 75th percentiles, while Whiskers represents the standard deviations. Circles indicate outliers. Statistically significant differences between groups * ($p < 0.05$).

11. Integrative Biological Response (IBR) index

The IBR index and IBR/n values showed different distribution patterns along the experimental period, but values in general depended on the metals oysters were exposed to (Fig. 14). On day 7, $^{107}\text{Ag}+^{63}\text{Cu}$ exposed oysters showed a marked response for the five biomarkers but in particular for metallothionein contents and CI. Meanwhile, the rest of the experimental groups did not show noticeable response (Fig. 14 A). Accordingly, the IBR/n values were markedly higher for $^{107}\text{Ag}+^{63}\text{Cu}$ oysters, whereas the rest of the groups showed low IBR/n values, in particular ^{63}Cu -exposed oysters (Fig. 14 B).

On day 14, $^{107}\text{Ag}+^{63}\text{Cu}$ -exposed oysters still had the highest response for biomarkers, but at this sampling day similar response intensity as day 7 for this group was obtained for the five integrated biomarkers (Fig. 14 C). Moreover, only Ag-exposed individuals maintained their response, whereas individuals from single Cu exposure increased their response intensity for Lipofuscin contents, MLR/MET and CTD ratio (Fig. 14 C). For the IBR/n values, $^{107}\text{Ag}+^{63}\text{Cu}$ exposure group oysters still had the highest values followed by ^{107}Ag and ^{63}Cu (Fig. 14 D).

At day 21, Ag-exposed oysters showed the strongest integrated biomarkers response, with almost same intensity for the five biomarkers. Furthermore, the biomarker response of this group increased throughout the experimental period. Similarly, ^{63}Cu group also showed steady increase in the biomarker response although being more moderated. In addition, this group kept the response pattern for biomarkers observed at day 14. In the $^{107}\text{Ag}+^{63}\text{Cu}$ exposure group a marked response for MT contents and CI occurred. Finally, on day 21 Control oysters showed similar response intensity for all the measured biomarkers (Fig. 14 E). Regarding IBR/n values, ^{107}Ag and $^{107}\text{Ag}+^{63}\text{Cu}$ groups in general displayed higher values while the Control and ^{63}Cu groups showed lower values (Fig. 14 F).

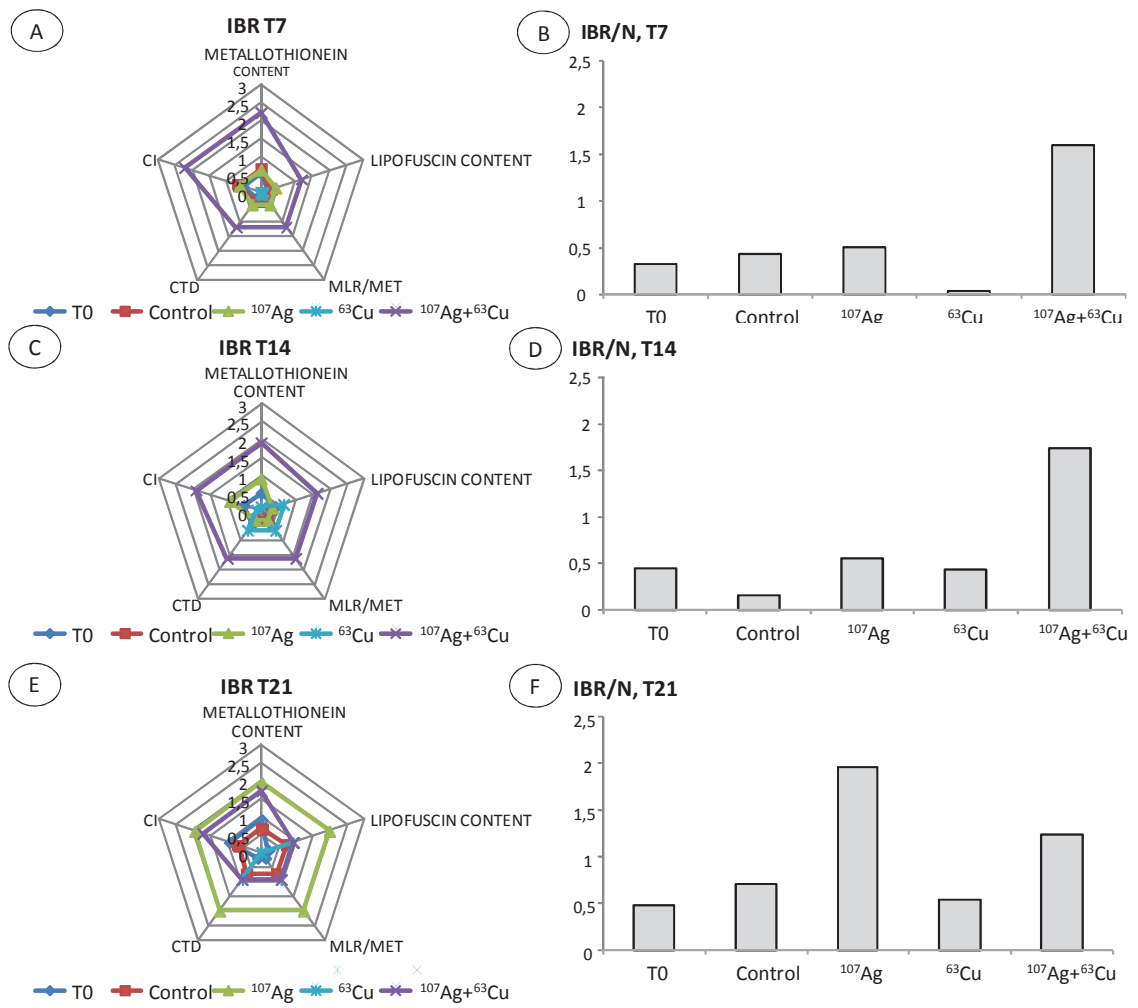


Figure 14: Integrative Biological Response Index (IBR) star plots for: (A) day 7, (B) day 14 and (C) day 21. IBR/n values for: (B) day 7, (D) day 14 and (F) day 21.

DISCUSSION

In the present experiment, oysters *Crassostrea gigas* were exposed to ^{107}Ag and ^{63}Cu alone or in combination through dietary exposure pathway, and a battery of cell and tissue level biomarkers was used to assess the effects exerted in oysters together with metal accumulation patterns in both oysters and algae.

In the past decades, it has been highlighted the relevance of the food pathway as an important route for metal accumulation in bivalves, but some authors have stated out the importance of the direct exposure route and therefore the fact is still under discussion (Strady et al., 2011b; Lee et al., 2015).

Chemical measurements carried out in algae confirmed metal accumulation in algae indicating a suitable exposure of metals to algae. However, the expected isotopic signal reflecting trophic metal accumulation in oysters due to the presence of metals in the cytosol of ingested algae (Fisher and Wang, 1998) was not measurable in oysters of the present experiment. Previous studies conducted by Abbe and Sanders, (1990) concluded after exposing oysters *Crassostrea virginica* to Ag through direct and food pathways (using *I. galbana* previously exposed to Ag among other algae species), that this element was not bioavailable for oysters through food pathway. These authors could not detect Ag accumulation in oysters but increased Ag contents were observed in faeces. In the mentioned study also decreased meat condition indexes were measured, suggesting that algae were not ingested.

However, in the present work a progressive increase in CI values compared to T0 in all experimental groups indicates that oysters had feeding activity. These results suggest that, given the relatively low Ag and Cu concentrations employed, longer exposure periods would have been needed to detect isotopic changes due to metal accumulation as previously performed in direct exposure experiments (Mikolaczyk et al., 2016). Even if algae accumulated spiked metals (0.755 mg/kg of Ag and 43.6

mg/kg of Cu; Tables 1 and 2) the final exposure concentrations were lower than after exposing directly oysters to metals and, thus, the final exposure through food pathway would be lower than from direct pathway. Moreover, some studies have reported high amounts of undigested cells of *I.galbana* in faeces and pseudofaeces of oysters *Crassostrea ariakensis* attributed to over-feeding (Alexander et al., 2008) suggesting that the measurements of faeces and pseudofaeces could be of major interest in future food pathway experiments.

On the other hand, it cannot be excluded that the biphasic digestion of oysters divided in the extracellular and intracellular digestion can also affect metal assimilation. On the first phase, digested materials are rapidly processed with little absorption, whereas in the intracellular digestion, higher absorption rates occur because particles are processed slower (Wildgust et al., 2000). In the present experiment, a dominance of the extracellular over intracellular digestion is suggested as an explanation of both CI increase and low metal accumulation.

Together with metal accumulation, different biological responses were measured from biochemical to individual level. In this context, metallothionein synthesis is regarded as one of the most important metal exposure biomarker (Le et al., 2016). Metallothioneins are low molecular weight proteins involved in metal sequestration and detoxification and their induction after Cu exposure has been previously observed in bivalves (Ringwood et al., 1998; Santovito et al., 2015). However, several factors such as metal, biological species, organ and type of exposure can affect MT synthesis (Le et al., 2016). Among these factors, possible effects caused by feeding activity should not be discarded. Rapid induction of MT (on day 7) occurred in the present experiment in $^{107}\text{Ag}+^{63}\text{Cu}$ -exposed oysters, which was also detectable in the ^{107}Ag -exposed group after 14 days. After 21 days, MT contents were again close to T0 values, suggesting that the new MT produced during the first 14 days of exposure were enough to cope with metal exposure. A similar response

pattern was observed after direct exposure to a range of Cu and Ag mixtures in sea and brackish waters (Rementeria et al., 2016a,b).

The process of metal detoxification in which MTs take part is quite well known. Firstly, these proteins sequester the metals by complexation on to deprotonated thiol groups with subsequent transfer to the lysosomes of digestive gland cells. Then these lysosomes are excreted to the lumen of digestive gland tubules through exocytosis and end up in the faeces (Amiard et al., 2006; Marigómez et al., 2002). Therefore, the observed increase in BSD in the lysosomes of digestive gland cells through the experiment is in concordance with the described detoxification mechanism. Although Control group oysters also showed high BSD during the experiment, except for day 21, when they diminished to values lower than T0 values, the BSD measurements produced two additional interesting results: the obtained BSD values in digestive gland lysosomes were higher than those observed after direct exposure (Rementeria et al., 2016a,b), probably because of a higher activity of digestive cells during to algae exposure. On the other hand, the activation of two different storage compartments for metal accumulation was detected (Fig. 10): Ag was mainly stored in the haemocytes, whereas Cu appeared in the digestive gland epithelium. Accumulation of Ag in haemocytes is not surprising, since it has been already reported by several authors (Amiard-Triquet et al., 1991). However, these authors also observed metal accumulation in the basal lamina of digestive tubules. Surprisingly, in the present experiment, very low accumulation of BSD in basal lamina of digestive gland occurred in comparison to field and direct exposure observations (Rementeria et al., 2016a; Rodríguez-Iruretagoiena et al., 2016). These results may indicate that Cu and Ag have limited accumulation capacity in the basal lamina and/or that the haemocytes are able to cope with metal exposure levels as employed in the present work, suggesting that metal accumulation in the

basal lamina of digestive gland tubules only occurs, when the limit of metal storage capacity in haemocytes is reached.

Regarding lipofuscin levels in oysters digestive gland cells, a similar distribution pattern to BSD accumulation in the lysosomes of digestive cells was expected (Rementeria et al., 2016a,b; Zorita et al., 2006). Lipofuscins are produced by the peroxidation of unsaturated neutral lipids and their increased intracellular accumulation in marine molluscs has been related to pollutant exposure, age and season (Raftopoulou and Dimitriadis, 2012). In previous experiments, the increase of lipofuscin contents was attributed to the accumulation of oxidised cell debris in lysosomes as a consequence of the increased oxidative stress provoked by Ag (Rementeria et al., 2016a,b). In the present work, all the exposure groups including the Control group had higher values of lipofuscins than those observed in previous experiments (Rementeria et al., 2016a,b). This result was attributed to the feeding activity in accordance with Blanco-Rayon, (2013) who observed higher amounts of lipofuscins in mussels fed with *I. galbana* than in unfed mussels. This author suggested that digested algae pigments could transform to lipofuscins accumulated in lysosomes. However, the possible deleterious effects of metal exposure on lipofuscin accumulation cannot be discarded, especially for Ag, because lipofuscin contents at day 7 increased in particular for ^{107}Ag -exposed individuals and at day 14 for the $^{107}\text{Ag}+^{63}\text{Cu}$ exposure group, indicating certain levels of oxidative damage in the mentioned groups.

Similar to previous experiments, neutral lipids mainly occurred in the connective tissue of oysters (Rementeria et al., 2016a,b). This tissue is known to be an energy reservoir for oysters, composed by vesicular cells storing glycogen to be used in energy demanding processes such as reproduction, or to face stress conditions (changes in salinity, detoxification onset...) (Berthelin-Heude et al., 2000; Jouaux et al., 2013; Rementeria et al., 2016a; Séguin et al., 2016; Thompson et al., 1996).

Higher amounts of neutral lipids in the connective tissue were detected during this experiment than in previous experiments (Rementeria et al., 2016a,b), which is in agreement with the gamete developmental stage suggesting that the oysters were storing energy reserves for gametogenesis (Dridi et al., 2007). However, a decrease in neutral lipid contents compared to the Control group occurred particularly on day 14 in $^{107}\text{Ag}+^{63}\text{Cu}$ -exposed oysters, suggesting that oysters could have started expending part of their energy budget to face metal exposure stress.

The gamete developmental stage of oysters along the experiment was in concordance with the season when the experiment was held: winter (Fabioux et al., 2005; Ortiz-Zarragoitia and Cajaraville, 2010; Rodriguez-Iruretagoiena et al., 2016). Thus, the majority of individuals were in resting phase (sexually undifferentiated) or in very early phases of development, indicating that the treatment did not have any relevant influence on gamete development. Accordingly, the possible influence of the gonad development as confounding factor for some biomarkers (MT content determination, neutral lipids...) would be negligible.

Digestive epithelium of bivalves is known to be influenced by the feeding activity; in particular the digestive gland atrophy is an indicator of starvation (Couch, 1984; Kim et al., 2006). Properly fed bivalves increase their epithelial thickness, whereas stress factors such as pollution can reduce it (Zaldibar et al., 2007). In this experiment, animals from the Control groups showed a reduction of their digestive gland atrophy through the experiment. In contrast, oysters exposed to Ag alone or in combination with Cu tended to show increasing atrophy, indicating a higher stress level. The reduction in size and amount of digestive tubules, increasing the connective tissue between them is also a stress response measured in bivalves (Brooks et al., 2011; Garmendia et al., 2011). The ratio between digestive gland tubules and connective tissue, i.e. the CTD ratio, is a pertinent biomarker in oysters (Rementeria et al., 2016a,b). The increase in CTD detected through the experiment in all experimental

groups even in the Control group, points towards an increasing connective tissue proportion and/or shrunk/loss of digestive tubules. In the case of ^{107}Ag -exposed oysters, this increase seemed to be in relation with the increased atrophy values. Furthermore, the obtained CTD values were higher than the ones observed in oysters directly exposed to different combinations of ^{107}Ag and ^{63}Cu (Rementeria et al., 2016a). Conversely, the influence of the feeding regime should not be discarded, as *I. galbana* exposure could have influenced this parameter as suggested by Múgica et al., (2015).

Five of the measured biomarkers from lower to higher biological levels, were integrated into the Integrative Biological Response index (IBR index). Previous studies have demonstrated the validity of this tool in oysters *Crassostrea gigas* (Rementeria et al., 2016a,b) as well as in other sentinel species (Brooks et al., 2011; Cravo et al., 2012; Garmendia et al., 2011; Marigómez et al., 2013a). The IBR index suggested highest toxicity of $^{107}\text{Ag}+^{63}\text{Cu}$ after 7 days, with a particularly clear response of metallothionein contents and the CI. On the other hand, the increased lipofuscin contents in oysters exposed to $^{107}\text{Ag}+^{63}\text{Cu}$ on day 14 is in agreement with the enhanced toxicity exerted by the combination.

Finally, both Ag and Cu single exposure groups showed an increase of the IBR/n values through the experiment, in particular in the case of Ag. The slower increase on IBR/n values of oysters uniquely exposed to Cu probably reflects that Cu is better regulated than Ag due to its nature as an essential element, being properly regulated at the applied low concentrations.

CONCLUSION

Although, organotropism studies have not been able to quantify metal accumulation kinetics, the integration of a battery of biomarkers was useful to discriminate between oyster's health status in the different experimental groups. Even if the experimental set-up worked properly with a successful accumulation of metals by algae and ingestion by oysters the obtained results were not the expected ones. The strongly changed isotope ratios in algae clearly show that the metals were probably ingested by the oysters. The absence of a clear accumulation signal in oysters suggest that (i) longer exposure times would be necessary to obtain sufficient metal accumulation and/or (ii) efficient excretions mechanisms prevent from Ag and Cu accumulation via the trophic pathway, for the applied low exposure levels. Thus, longer exposure periods or higher exposure levels would be necessary for the proper understanding of food pathway exposure effects in *Crassostrea gigas* and to assess the importance of this pathway in the contamination of wild oysters used for biomonitoring. On the other hand, the biomarkers used in the experiment provided relevant information on Ag and Cu toxicity after trophic exposure. A first increase in MT in metal exposed oysters, followed by increased lipofuscin accumulation and atrophy in the digestive gland after longer exposure periods indicate a cellular damage, probably linked to oxidative stress. Moreover, the presence of Cu in addition to Ag exposure seems to have an additive/synergistic effect on the Ag toxicity, at least at short term (14 days) exposure.

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IV. CONCLUSION AND THESIS

CONCLUSIONS

- I. Highest metal exposure was detected in the Gironde Estuary, compared to the other estuaries. Although biomarkers differed in their response magnitude they showed a similar response pattern for mussels and oysters. In this context, the IBR index appeared as a relevant tool not only to integrate the measured biomarkers but also to allow comparisons between species.
- II. The ability of oysters to accumulate Ag at higher levels combined with the relatively low amount of pathological alterations (infiltrations, parasites...) that may interfere on tissue level biomarkers (MLR/MET, CTD) make them a very suitable organism for the determination of environmental Ag pressure and toxicity.
- III. The exposure Cu and Ag alone at levels that can be found in the environment, and especially in combination, resulted in a diminished health status (quantified through IBR) in oysters.
- IV. Reduced salinity conditions did not affect biomarker response after exposure to Ag and Cu. The IBR index seemed to be a relevant parameter even in reduced salinity conditions and allowed to detect the onset of detoxification processes that occurs earlier in brackish waters. Higher toxicity of Ag species prevailing at lower salinity levels compared to Cu was observed. However, further research should focus on the role of salinity-dependent speciation of dissolved Ag and the uptake kinetics of this metal in different oyster tissues.
- V. Although, organotropism studies have not provided information on metal accumulation kinetics, the integration of a battery of biomarkers was useful to discriminate between oyster health status, with those oysters exposed to Ag and the Ag-Cu mixture after dietary exposure showing

altered health condition. The presence of Cu during exposure to Ag seems to have an additive/synergistic effect in the Ag toxicity at least at short term exposure.

- VI. Even if the accumulation of metals in microalgae was efficient, the metal accumulation in oysters fed with these contaminated microalgae was not measurable. The use of longer exposure times or higher concentrations would be necessary in order to properly understand the food pathway exposure effects of Cu and Ag in *Crassostrea gigas* via the food pathway.

THESIS

The field collected oysters present a dissimilar health status with those from Gironde Estuary being significantly more affected than Oka and Ibaizabal and this affection can be at least partially related to the higher Ag accumulations detected. The exposure to Ag and Cu in the laboratory indicated that Ag toxicity is enhanced in the presence of Cu while decreased seem to accelerate biological responses. The integration of biomarkers in the IBR index allowed a better discrimination of treatments indicating its suitability in environmental health assessment programs.

V. APPENDIX

APPENDIX I

MATERIALS AND METHODS

1. Study area

As described in Chapter 1, three estuaries from the Bay of Biscay (Ibaizabal, Oka and Gironde) were selected for this study. One sampling point per estuary was selected: Arriluze (Ibaizabal Estuary), Murueta (Oka Estuary) and La Fosse (Gironde Estuary) (Fig. 1).

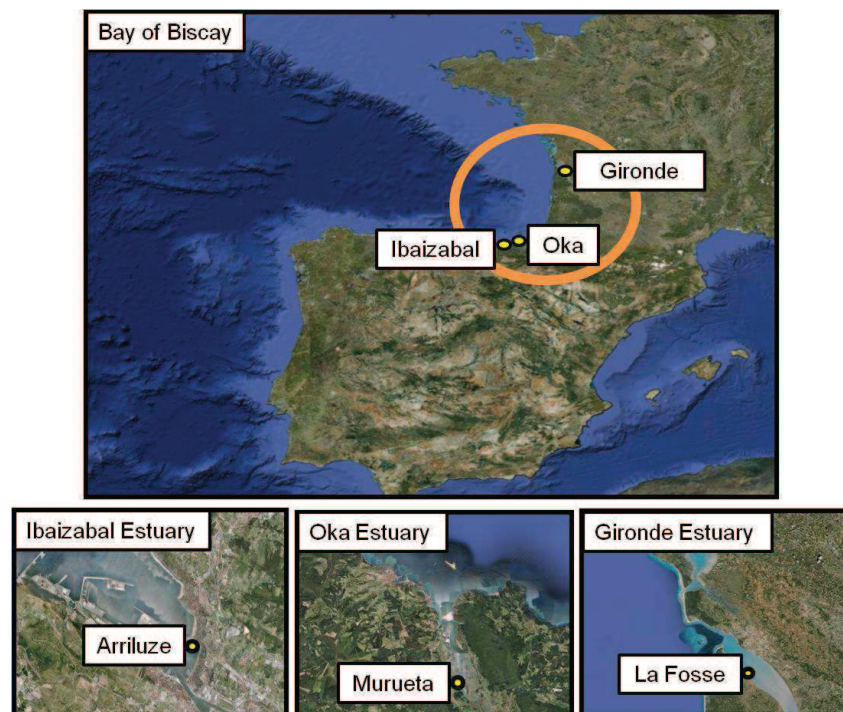


Figure 1: The 3 selected estuaries from the Bay of Biscay: Ibaizabal, Oka and Gironde Estuary. The selected points were Arriluze (Ibaizabal Estuary), Murueta (Oka Estuary) and La Fosse (Gironde Estuary).

2. Sample collection and processing

In June 2012, October 2012 and February 2013, oysters *Crassostrea gigas* (Thunberg, 1973) were collected in the three different sampling points: Arriluze (Ibaizabal Estuary), Murueta (Oka Estuary) and La Fosse (Gironde Estuary) from the Bay of Biscay.

After collection, oyster samples were rapidly transferred to Plentzia Marine Station (PiE UPV/EHU, Basque Country, Spain) and dissected. All the samples were processed for biological measurements ($n=10$). For histology, a ~5 mm dorsoventral cross-section which included all main organs and tissues (gills, digestive gland and gonad) was obtained in all the individuals. Sections were introduced into histological cassettes and immersed in formalin (4% formaldehyde diluted in natural filtered sea water) for 24 h. Then fixative was removed and samples were stored in 70% ethanol until being dehydrated and embedded into paraffin following the standard histological procedure.

2.5.1 Digestive gland and gonad histology

From the cross section that included digestive gland and gonads, 4 μm thick sections were obtained in a microtome and stained with haematoxylin-eosin. Afterwards, microscopic slides were analysed under a light microscope (Olympus BX 61) (Olympus, Japan).

2.5.2 Gamete developmental stage

Animals sex and gamete developmental stage as well as Gonad Index (GI) were determined for each individual following the procedure described by Kim et al., (2006), which is based on a subjective scale of the developmental stage of follicles and gametes after examination under the light microscope. Eight different developmental stages were defined, from sexually undifferentiated to spawned, with their corresponding value ranged from 1 to 8. Finally, a mean gonad index value (GI) was calculated per sampling point and season.

2.5.3 Histopathological alterations

Histopathological analyses through oyster's whole body transections at different microscopical magnifications were completed. Different pathologies indicative of bivalve's health status were identified including: Granulocytomes (GRN),

Haemocytic infiltrations (HAE), Haemocytic infiltrations in Gonads (HAE gn), Inflammations (INF) and Parasites (PAR) (including *Mytilicola intestinalis*, *Nematopsis*, *Martelia* and copepods). Prevalence values for each alteration were obtained in terms of percentages.

2.5.3.1 Digestive gland atrophy

A semi-quantitative procedure based on Kim et al., (2006) was followed in order to determine the thinning of digestive tubule epithelium. Individuals were observed under an Olympus BX-61 microscope (Olympus, Japan) light microscope and a score based on digestive gland atrophy degree was assigned to each one. Values ranged from 0 (normal tubule wall thickness) to 4 (wall extremely thin, nearly all tubules affected).

2.5.3.2 Tissue integrity in digestive gland (CTD ratio)

The integrity of the digestive gland tissue was determined by calculating the connective-to-diverticula (CTD) ratio. For this, 5 fields of digestive gland tubules were obtained per individual with a digital camera attached to an Olympus BX-61 microscope (Olympus, Japan) and processed through Image J program (Image Processing and Analysis in Java, Maryland, USA). CTD ratio is defined as the extent of the interstitial connective tissue relative to the area occupied by digestive diverticula. The following formula was applied to obtain CTD ratio: $CTD\ ratio = c / (b + d + l)$. Where: (c) interstitial connective tissue, (b) basophilic cells, (d) digestive cells and (l) diverticular lumen (Brooks et al., 2011).

2.6 Intralysosomal metal accumulation

Histochemical detection of metals was carried out in 4 µm thick sections of paraffin embedded samples ($n=10$ per treatment). After embedding, sections were dewaxed in xylene and hydrated, then, the slides were left at 37°C oven at least 24h for

drying. Samples were covered with the commercial silver enhancement kit (Silver enhancing kit for light and electron microscopy, BB International) at room temperature, development was checked under light microscope and stopped after 15-20 min. Slides were washed with tap water and metals were developed as black silver deposits (BSD). Finally, slides were mounted in Kaiser's glycerine gelatine and volume density of BSDs ($V_{V_{\text{BSD}}}$) in the lysosomes of digestive gland cells was measured with an image analysis system (Soto et al., 2002).

2.7 Statistics

All data was statistically analysed with SPSS v 22.0 statistical package (SPSS Inc., Chicago, Illinois, USA). Results were showed as mean values \pm standard deviations (SD). Homogeneity of variances (Levene's test) and data normality (Kolmogorov-Smirnov test) was checked before performing one-way analysis of variance (ANOVA), which determined significant differences between sampling points and months. Differences were considered statistically significant when $p < 0.05$, thus. Duncan's test was used for multiple range comparison between pairs. In those cases where data has not normal and homogeneous distribution, Kruskal Wallis non parametric test was used.

RESULTS

1. Gamete developmental stage

The oysters collected at three different estuaries showed a similar gamete developmental stage varying according to the season. In June 2012 (Fig. 2 A) oysters from Gironde Estuary showed a retarded gamete developmental stage with lower GI values and almost the 50% of oysters in the earliest phases of gamete development (sexually undifferentiated, early development and mid development). In contrast, oysters from Ibaizabal and Oka Estuary had a GI value ~ 5 , with the majority of oysters (80% in Oka Estuary) and (60% in Ibaizabal Estuary) fully developed and thus close to spawning phase (Fig. 2 A and B). In October 2012, oysters from three sites were mainly in spawned phase (85% in Oka, 80% in Ibaizabal and 60% in Gironde Estuary) with the remaining oysters in spawning phase (Fig. 2 B). Finally, in February 2013 most of the oysters from the 3 sites were in early developmental stage (60% in Oka and Ibaizabal Estuaries and 55% in Gironde Estuary) (Fig. 3 F). However, some oysters in Oka Estuary (10%) were in spawned phase. Overall, oysters from the three estuaries followed the annual cycle for gamete development, but oysters from Gironde Estuary in particular, had a more retarded gametogenesis compared to oysters from Ibaizabal and Oka.

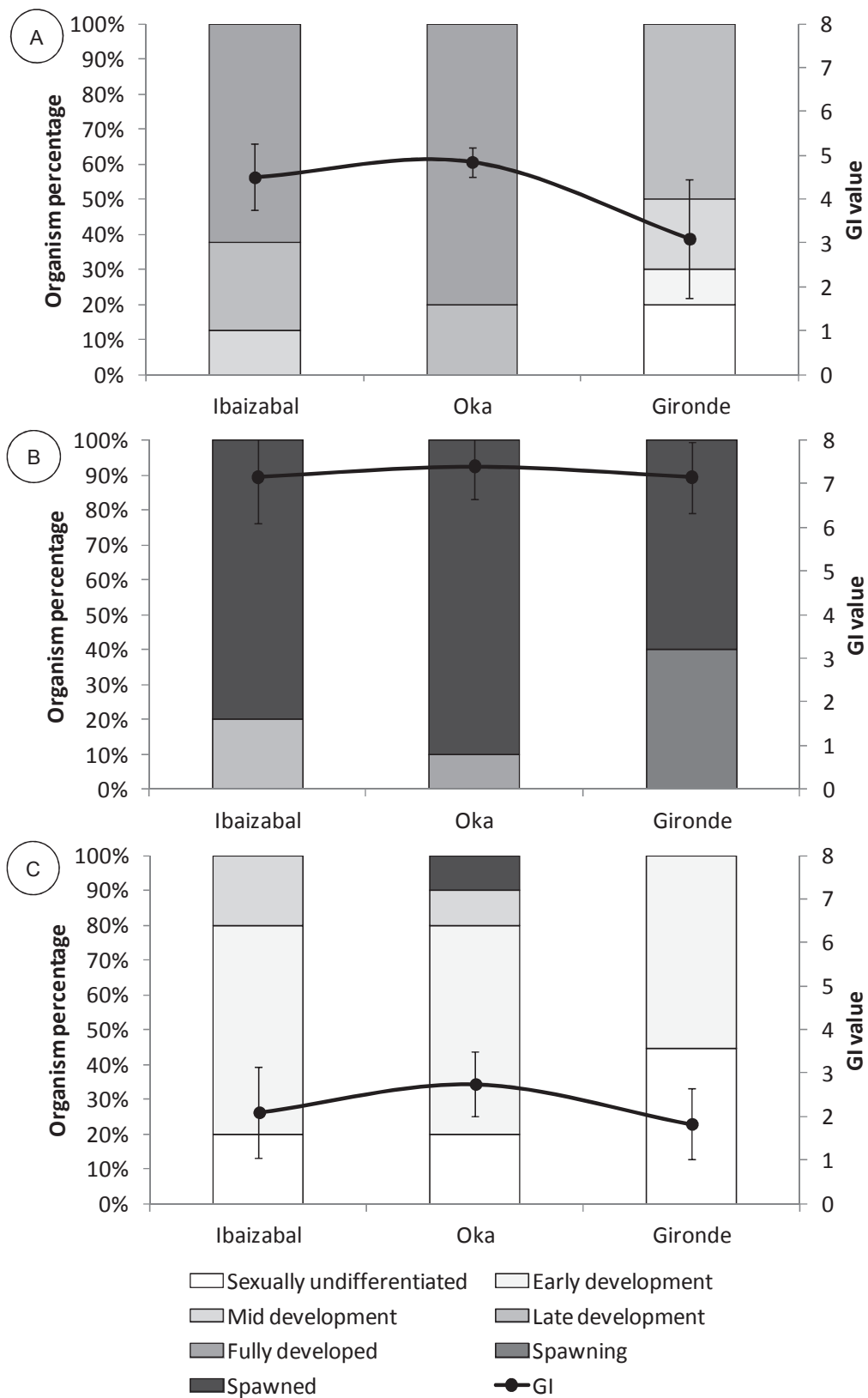


Figure 2: Percentage of annual gamete developmental stage for oysters (stacked bars) and mean values (\pm standard deviations) of the Gonad Index (represented with a line) of oysters in: June 2012 (A), October 2012 (B) and February 2013.

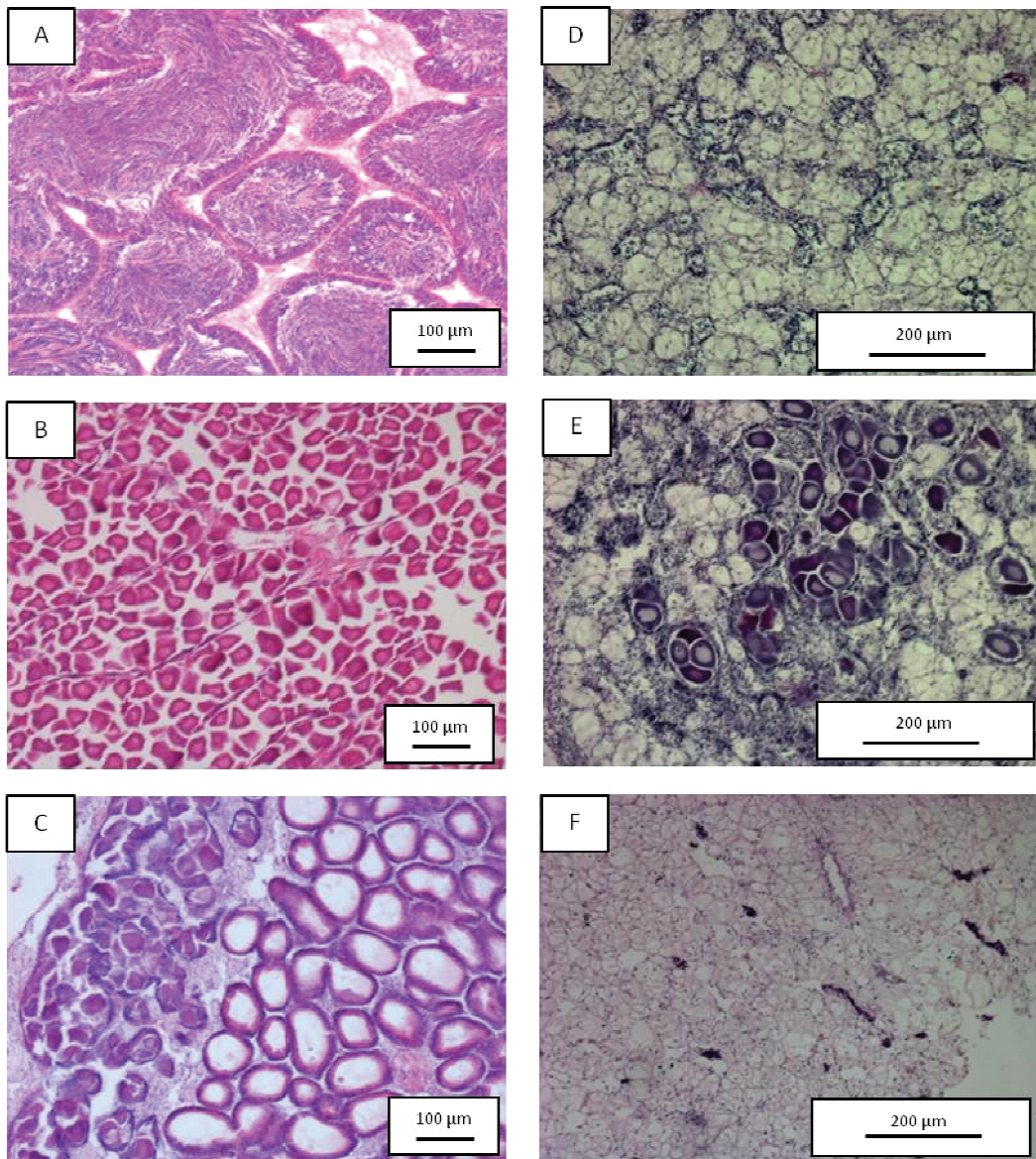


Figure 3: Annual gamete developmental stage for oysters. A, B and C are oysters collected in June 2012; D and E are oysters collected in October 2012 and F are oysters collected February 2013 from the 3 estuaries of the Bay of Biscay (A and D) Ibaizabal, (B and E) Oka and (C and F) Gironde Estuary. Oysters (A, B, C) collected in June 2012 were in the last developmental phases almost ready for spawning. In (D, E) oysters from October 2012 were already spawned. Finally in (F) February 2013 gonads were in the first phases of development.

2. Digestive gland atrophy

2.1 Atrophy

Digestive gland atrophy in oysters showed differences both between sampling sites and months (Fig. 4). In June 2012, significant differences between sampling points were observed between the Gironde Estuary and the other two estuaries, being values from the French estuary significantly higher. Furthermore, in October 2012, oysters from Gironde Estuary had also the highest atrophy values being significantly different from those collected in the estuary of Oka River. In February 2013, no significant differences between estuaries were observed. On the other hand, a seasonal increase of atrophy values was measured from June 2012 to February 2013. In fact, significantly lower values were obtained in Ibaizabal estuary in June 2012 compared to October 2012 and February 2013. Similarly, oysters from Oka Estuary showed significantly higher atrophy values for oysters collected in February 2013 compared to oysters collected in June and October 2012 (Fig. 4).

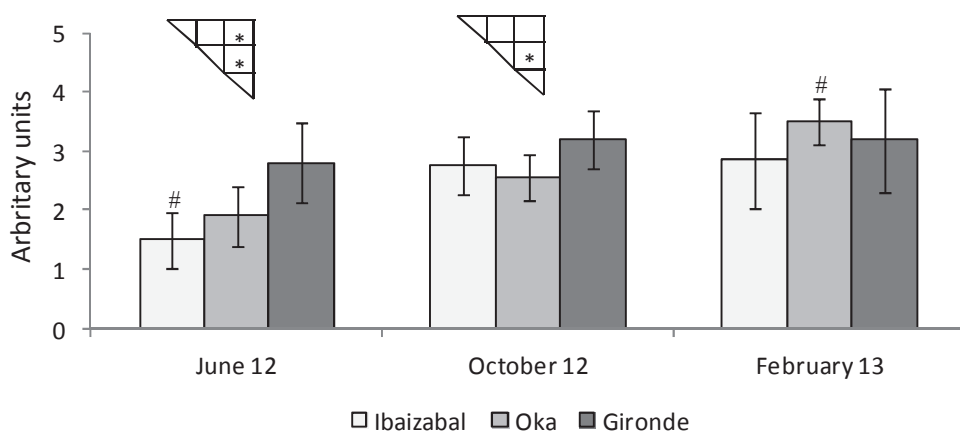


Figure 4: Mean values (\pm standard deviations) of annual digestive gland atrophy values for oysters from the three estuaries collected in June 2012, October 2012 and February 2013. Statistically significant differences ($p < 0.05$) between sampling points from the same month are expressed in (*) while differences between same sampling points at different months are expressed in (#).

3. Tissue integrity of the digestive gland (CTD ratio)

The measured CTD ratio values varied significantly according to the season when oysters were collected. A general increase of CTD values was observed from June

2012 to February 2013, indeed, at this period all sampling points showed statistically significant higher values than in the rest of the months. Moreover, significant differences between sampling points at the same period were observed for oysters, with oysters collected in June 2012 in the Oka Estuary exhibiting lower CTD ratio values whereas in February 2013 significantly higher values were found in oysters coming from Gironde Estuary (Fig. 5).

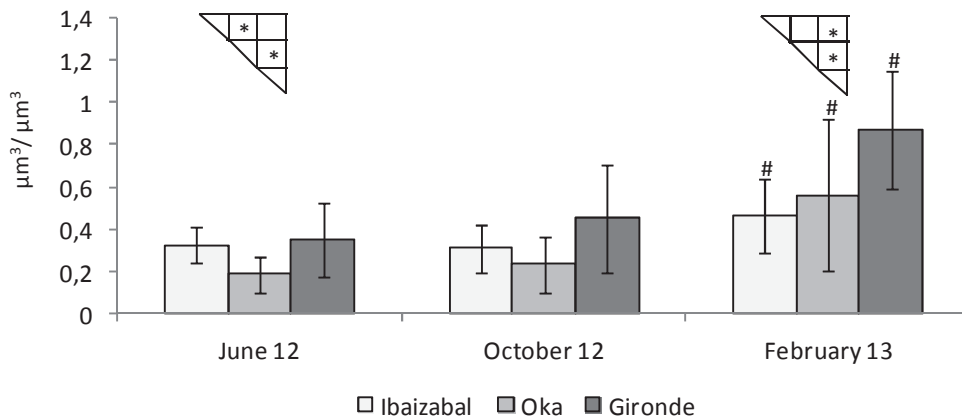


Figure 5: Mean values (\pm standard deviations) of annual CTD values for oysters at three sampling points. Statistically significant differences ($p < 0.05$) between sampling points from the same month are expressed in (*) while differences between same sampling points at different months are expressed in (#).

4. Histopathological alterations

The most prevalent histopathological alterations detected in oysters were the haemocytic infiltrations and then the inflammations (Table 1). The prevalence of histopathological lesions varied through sampling months. In June 2012 oysters from Oka Estuary showed highest prevalence values while in the other two estuaries almost no histopathological alterations were observed. Moreover, in some individuals strong HAE infiltration was recorded in Oka Estuary (Fig. 6 B) as well as an inflammation of digestive gland tubules (Fig. 6 E). Additionally, a hermaphrodite oyster was found in June 2012 at Ibaizabal Estuary (Fig. 6 A). In October 2012 annual highest prevalence values were obtained in all sampling points. Finally, it is also noticeable that in February 2013 lowest prevalence values were recorded for all the sampling points (Table 1).

Table 1: Histopathological alterations in oysters collected in three different estuaries from the Bay of Biscay along the year.

Oysters	Estuary	GRN(%)	INF(%)	HAE(%)	PAR(%)
June 2012	Ibaizabal	0	0	0	0
	Oka	0	30	30	10
	Gironde	0	0	10	0
October 2012	Ibaizabal	0	0	20	0
	Oka	0	0	20	0
	Gironde	0	20	30	10
February 2013	Ibaizabal	0	0	0	0
	Oka	0	0	10	0
	Gironde	0	0	0	0

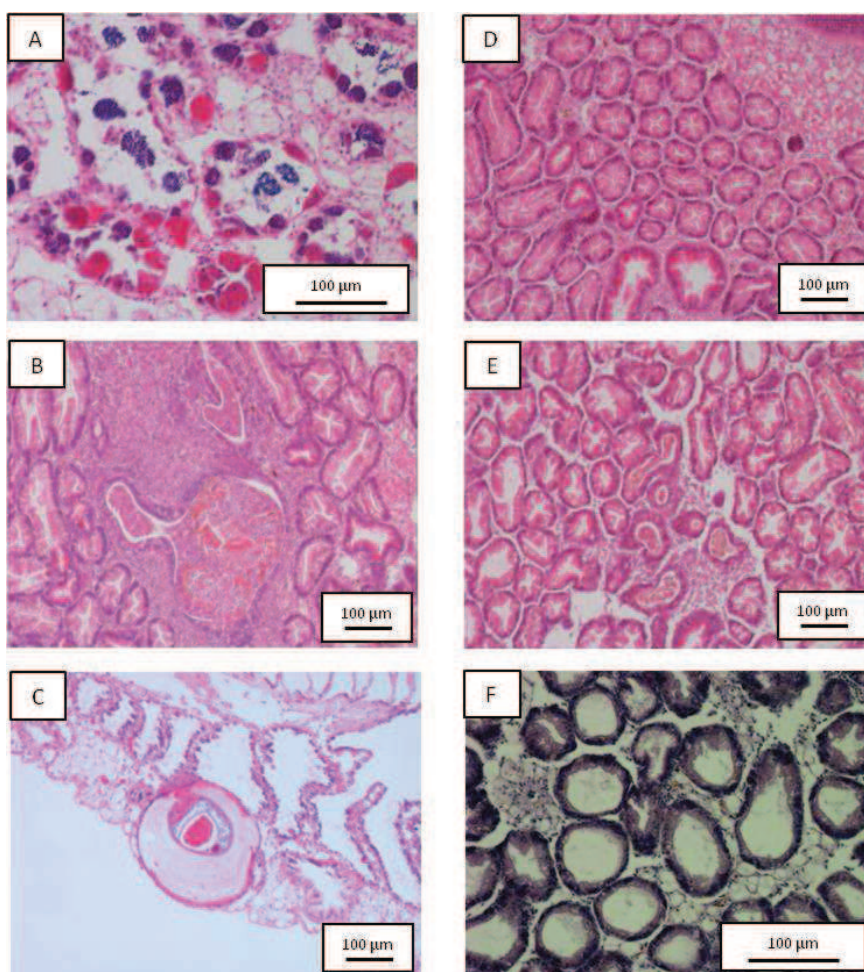


Figure 6: Hematoxylin-eosin stained samples indicating different histological alterations. (A) Abnormal gonad development in a hermaphrodite oyster collected in Ibaizabal Estuary in June 2012. (B) Haemocytic infiltration in the digestive gland of an oyster collected in Oka Estuary in June 2012 probably surrounding a parasite infection. (C) Parasite infection in the mantle of an oyster collected in June 2012 at Gironde Estuary. (D) Healthy digestive gland with good digestive tubule epithelium thickness in oyster collected in June 2012 at Ibaizabal Estuary. (E) Haemocytic inflammation into the digestive gland tubules of an oyster collected in June 2012 in Oka Estuary, note that slight atrophy of digestive gland tubules is also recorded. (F) Severe atrophy of digestive gland tubules in an oyster collected in Gironde Estuary in October 2012.

5. Intralysosomal metal accumulation (BSD)

Distribution and amount of BSD in oysters' tissues and cells varied according to the estuary of origin. The digestive gland of oysters collected in Gironde Estuary showed clearly higher levels of BSDs whereas individuals coming from Oka and Ibaizabal Estuaries showed lower amounts. The basal lamina of digestive gland tubules appeared stained in all sampling sites and months, however lower intensity was seen in February 2013 for oysters from Oka and Ibaizabal Estuaries compared to Gironde Estuary (Fig. 7). Additionally, a stronger staining in oysters from Gironde Estuary was also observed for digestive gland epithelium (Fig. 7 E and F). Finally, in this sampling point BSDs were also present in the interstitial connective tissue of digestive gland, whereas nearly no staining was observed for this tissue in oyster coming from the rest of estuaries (Fig. 7).

The quantification of intralysosomal accumulation of BSDs was clearly different according to the sampling point. In all the measured months values obtained for oysters coming from Gironde Estuary were significantly higher than values from Oka and Ibaizabal Estuaries. In general, similar accumulation values were detected in oysters from these two last estuaries; however in June 2012 obtained values were markedly higher in Oka and Ibaizabal Estuaries than at the following months. In fact, values for oysters from Oka Estuary in June 2012 were significantly higher than the ones observed in October 2012 and February 2013 at the same sampling point (Fig. 8).

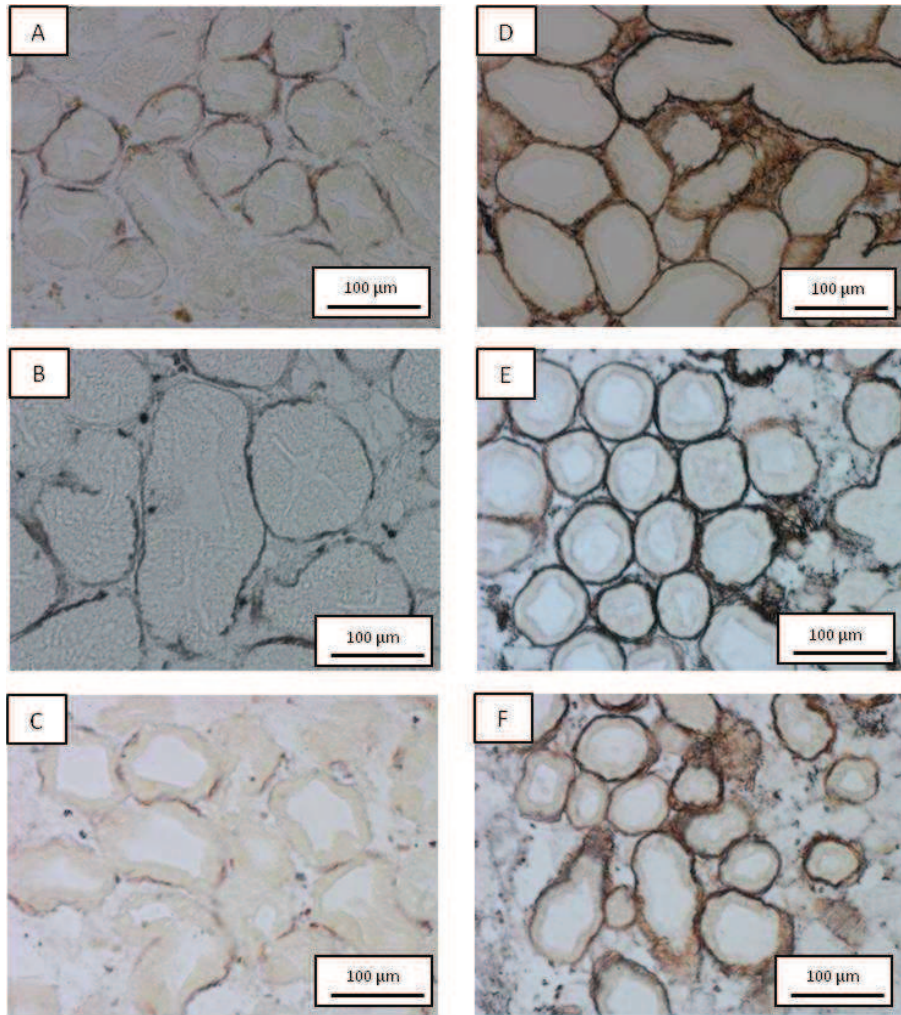


Figure 7: Paraffin sections of oysters stained by autometallography. Oysters collected in (A) Ibaizabal, (B and C) Oka and (D, E and F) Gironde Estuaries in (A and D) June 2012, (B and E) October 2012 and (C and F) February 2013. Note that oysters from Gironde Estuary showed during the whole year higher amounts of BSD particularly in the basal lamina of digestive gland tubules.

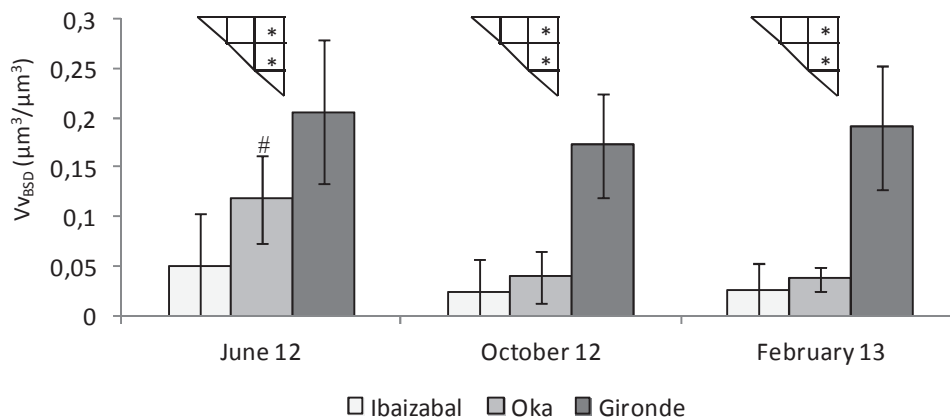


Figure 8: Mean values (\pm standard deviations) of annual intralysosomal accumulation of BSD for oysters in three estuaries from the Bay of Biscay. Statistically significant differences ($p < 0.05$) between sampling points from the same month are expressed in (*) while differences between same sampling points at different months are expressed in (#).

DISCUSSION

Despite Ibaizabal, Oka and Gironde Estuaries have been long studied by the scientific community; works based on cell and tissue level biomarkers of the three estuaries in conjunction are scarce. Moreover, being among the most relevant estuaries of the Bay of Biscay this work may provide an interesting data on the overall health status of the south eastern area of the bay using oysters *Crassostrea gigas* as sentinel organisms since they are present in the three estuaries. The Ibaizabal and Gironde Estuaries represent two estuaries which are very well known for being chronically polluted including heavy metal pollution. Indeed, concentrations of some metals are still high enough that can cause deleterious effects in aquatic biota (Baudrimont et al., 2016; Cajaraville et al., 2016; Cuevas et al., 2015; David et al., 2012; Lanceleur et al., 2011a; Solaun et al., 2013). On the other hand, the Oka Estuary which is located within the Urdaibai Reserve of the Biosphere has been considered as a reference site in previous studies (De los Ríos et al., 2016; Lekube et al., 2013). However, recent studies have revealed the presence of pollutants in the Oka Estuary such as endocrine disruptors that have provoked deleterious effects on the biota (Ortiz-Zarragoitia and Cajaraville, 2010; Puy-Azurmendi et al., 2013) and also slight metal pollution has been detected (Rodriguez-Iruretagoiena et al., 2016) indicating that the Oka Estuary may present some stress sources. The use of oysters as sentinel species for pollution assessment is widely described, however it should be noted that some responses may be affected by the developmental stage of oysters. Oysters showed a seasonal gamete development pattern with bulk of gonads varying from season to season, reaching its maximum shortly before spawning (Galtsoff, 1964). In the present study, the gamete development of oysters through the year in all estuaries was in good agreement with the generally described gamete development for oysters in the area (initiation of the gametogenesis in late winter or early spring, maturity and spawning in summer and

gonad reabsorption phase in autumn) (Enrriquez-Díaz et al., 2009; Fabioux et al., 2005). Small differences were however, observed in oysters with those from Oka Estuary showing an earlier development closely followed by oysters from Ibaizabal Estuary, while oysters in Gironde Estuary had a slower development. This fact was particularly significant in June 2012, when oysters from Oka and Ibaizabal Estuaries were in majority in fully development phase whereas in Gironde Estuary there were still some individuals in the first developmental stages. In this context, the gonad development of bivalves is known to be dominated mainly by two environmental factors: temperature (mainly affecting gametogenesis speed) and nutrient availability (mainly affecting gametogenesis intensity) (Enrriquez-Díaz et al., 2009; Garmendia et al., 2010).

In general, the sampling site of Arriluze in the Ibaizabal Estuary, is regarded to be richer in organic nutrients than Oka Estuary (Cuevas et al., 2015). However, the annual report for water quality assessment of the Basque Government (Borja et al., 2014) identified high concentrations of nutrients for summer in the inner part of the Oka Estuary. In this period, low pluviometry together with decreased water renewal was observed creating a proper scenario for the increase of nutrient concentration. Moreover, nutrient rich effluents coming from Gernika wastewater treatment plant enhanced this situation (Borja et al., 2014). In consequence, oysters from the inner part of the Oka Estuary would benefit from higher amounts of nutrients and had an earlier development of their gonads in June in comparison with oysters from Ibaizabal Estuary.

On the other hand, similar water temperature values would be expected for Oka and Ibaizabal Estuaries, whereas Gironde Estuary located in more northern latitudes would have lower water temperature values, explaining the retarded gametogenesis in comparison to Ibaizabal and Oka (Enrriquez-Díaz et al., 2009; Garmendia et al., 2010). However, apart from the temperature, the influence of pollutants cannot be

discarded since requirements on energy demand for detoxification processes can cause a decrease on energy investment for reproductive purposes (Rementeria et al., 2016; Séguin et al., 2016). In this context, the Gironde Estuary is well known for being among the most polluted estuaries in the French coast, specially by metal pollution (Lanceleur et al., 2011b) and therefore oysters inhabiting Gironde Estuary are constantly exposed to pollutants that may limit or delay the gamete production.

The digestive gland of oysters is known to undergo several changes depending on environmental and stress conditions, including pollutant exposure. Among those changes, the atrophy of digestive gland epithelium (Izagirre et al., 2014; Zaldibar et al., 2007) and the shrink and reduction in size of digestive gland tubules (Brooks et al., 2011; Garmendia et al., 2011) have been widely described. Presently in order to measure the digestive gland atrophy a semi-quantitative scale based on Kim et al., (2006) was applied. In general terms, higher values were recorded in Gironde Estuary in agreement with the high metal pollution levels historically recorded, indicating a general stress situation of oysters from this estuary. Similarly, oysters collected in December 2010 in Oka Estuary also showed an increase of digestive gland epithelium atrophy that was attributed to higher metal concentrations in oysters' tissues (Rodriguez-Iruretagoiena et al., 2016). Additionally, a seasonal trend in atrophy alterations was observed with increasing values from June 2012 to February 2013 particularly in oysters coming from Ibaizabal and Oka Estuaries. A similar seasonal distribution of atrophy values was previously described by Díez, (1996) for oysters collected in Oka Estuary, showing increased atrophy values in winter months whereas in spring the thickness of digestive epithelium increased. This seasonality on atrophy values was attributed to a higher nutrient availability during spring and summer months. Presently, as previously explained high nutrient contents were recorded during spring and summer months in Oka Estuary and therefore the increased epithelium thickness for this period may be explained as a

consequence of the availability of higher nutrient contents. Indeed, previous authors have considered the atrophy of digestive gland tubules to be characteristic of poor nourished bivalves (Garmendia et al., 2010; Séguin et al., 2016). Moreover, the decrease on atrophy values in autumn and winter months has also been attributed to the stress caused by a post spawning situation (Díez, 1996). In the case of the Gironde Estuary, although atrophy levels were higher during the whole sampling period, in agreement with Oka and Ibaizabal also some seasonality is observed, indicating an altered but not totally deteriorated health status.

Together with digestive gland atrophy of sampled oysters, the increase of connective tissue in respect to digestive tissue was measured through the CTD ratio, which has been already successfully applied as a biomarker of tissue integrity in oysters under controlled metal exposures (Rementeria et al., 2016). Presently, oysters from Gironde Estuary showed the highest CTD ratios, again indicating a higher stress pressure suffered by these individuals. A seasonal trend was also observed for this biomarker, since values for February 2013 were higher in all sampling points and thus indicating that digestive gland tubules are reduced in size and amount compared to connective tissue. This fact, could also be connected with the before mentioned lower amounts of nutrients found during winter in estuarine waters in comparison to spring-summer months, In consequence it could be expected that the obtained nutrients will be stored as reserve material in the connective tissue and thus increasing its amounts. However, this tissue could appear destructured and poorly developed in winter periods under nutrient scarcity (Séguin et al., 2016).

Water quality biomonitoring programs have often relied among other parameters in the detection and quantification of histopathological alterations in sentinel species as a key instrument to understand and assess deleterious effects caused by pollutant presence in aquatic biota (Cuevas et al., 2015). During the present study low

prevalence values for histopathological alterations were recorded, particularly in February 2013 when nearly no alterations were detected. Obtained values were lower than the ones observed by Díez, (1996) in oysters from Oka Estuary but followed the same seasonal pattern that this author described with increased prevalence values in autumn. Accordingly, Garmendia et al., 2010 also described a lower response of tissue level biomarkers in winter periods (including histopathological alterations) than in warmer periods. During the experimental period little parasitic prevalence was observed, and thus the haemocytic infiltrations (most relevant alteration detected) could not be related to the parasite presence. Previous works have related the presence of this type of injury with pollutant exposure including heavy metals (Rasmussen, 1986), furthermore a recent study with oysters inhabiting the Oka Estuary linked the presence of haemocytic infiltrations with metal pollution (Rodríguez-Iruretagoiena et al., 2016). The observed increased haemocytic infiltrations observed in Oka estuary during summer months, could be thus linked with a higher human pressure in the estuary in this period.

On the other hand, also very low levels of hermaphrodite oysters were obtained (1 individual in Ibaizabal in June 2012). Although the possible effect of exposure to endocrine disruptors should not be discarded (Cuevas et al., 2015) the presently recorded value could be considered as normal since it was obtained in when oysters are in their sex change period (Enríquez-Díaz et al., 2009) due to their protandric nature (Santerre et al., 2013).

Finally, the detection of metals in tissues of bivalves through autometallography is a widely employed technique (De los Ríos et al., 2016; Raftopoulou and Dimitriadis, 2011; Rodríguez-Iruretagoiena et al., 2016; Soto et al., 2002) that allow heavy metal detection and distribution in bivalve tissues. In the present work, the technique clearly identified higher BSDs content in oysters from Gironde Estuary through the whole year, in clear agreement with the higher metal concentration values found for

this estuary. No seasonal trends in accumulation of BSD was detected for Gironde Estuary oysters, but oysters from Ibaizabal and Oka Estuaries showed increased BSD in June 2012 compared to October and February. In both cases this increase could be related with an increase of human activities (including leisure and boat painting) during summer periods. Indeed, it should be noted that the Ibaizabal sampling point (Arrizule) is located in a leisure marina (Marigómez et al., 2013). On the other hand, population and human pressure in the Oka Estuary increases in summer due to the high touristic activity in the surrounding area (Rodríguez-Iruretagoiena et al., 2016). In fact, higher metal concentration values were found in oyster tissue inhabiting Oka Estuary in summer-autumn seasons (Raposo et al., 2009) which an increase on the concentrations of some metals in oysters tissues in summer months (Cd, Cu, Fe, Pb, V and Zn) (Rodríguez-Iruretagoiena et al., 2016) in agreement with the present data. Regarding the distribution of BSDs in oyster tissues, a clear signal in the basal lamina of digestive gland tubules in all sampling points and months was observed. Metal accumulation in this compartment has been previously described for oysters in field studies and also in oysters under experimental conditions (Amiard-Triquet et al., 1991; Rementería et al., 2016; Rodríguez-Iruretagoiena et al., 2016).

To sum up, the measured cell and tissue level biomarkers through a whole year in three different estuaries from the Bay of Biscay clearly indicated that oysters from Gironde Estuary present altered health status including higher levels of atrophy and CTD and also increased amount of autometallographical BSDs. Noticeably, oysters from Oka Estuary showed similar or even worse condition than oysters coming from Ibaizabal Estuary indicating that human activities exert a deleterious effect in oysters from Oka Estuary mainly in summer although this estuary is part of a UNESCO's Reserve of the Biosphere. Indeed, recently De los Ríos et al., (2016) suggested that Oka Estuary should not be considered as a reference site since mussels

translocated into this site presented a reduction in lysosomal membrane stability, peroxisome proliferation and gamete atresia. On the other hand, the overall good health status of oysters from Ibaizabal can be in agreement with the improvement of water quality in Ibaizabal Estuary (Solaun et al., 2013).

Finally, this appendix could be considered as an attempt for annual characterization of some of the most used cell and tissue level biomarkers in the present work. The obtained results suggests that a seasonal effect on biomarker response occurs, as previously described in other sentinel organisms such as mussels (Garmendia et al., 2010; Múgica et al., 2015). Therefore, the influence of the seasonal change on biomarker response should be taken into account in future biomonitoring studies. In this sense, Cuevas et al., (2015) recently proposed autumn as the ideal period for histopathological studies on sentinel organisms in order to avoid the confounding factors caused by advanced maturation stages. In agreement, our results indicate that this period would also be appropriate since the measured biomarkers showed higher responsiveness during this period than in winter. Although a higher response occurred in summer, the increased human impact occurred in this period in some of the studied estuaries may work as a confounding factor. Moreover, the presence of mature gonads can induce a high scattering on obtained chemical measurements in bivalve's tissue thus autumn should be considered as appropriate season to perform a biomonitoring combining both biological and chemical measurements.

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APPENDIX II: PROTOCOLS OF EXPERIMENTAL PROCEDURES

FIXATIVE PREPARATIONS

1. Formalin

To prepare 2 litres of solution:

- 57.84 g of $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$.
- 5.12 g of NaH_2PO_4 .
- 200 mL of commercial formaldehyde at 35-40%.
- To be completed with distilled water.

Formalin can also be prepared by directly diluting 200 mL of commercial formaldehyde at 35-40% in 1800 mL natural filtered sea water.

2. Baker's fixative

- Neutralise to pH=7 100 mL of commercial formaldehyde 40%.
- Mix the neutralised solution with 100 mL of 10% CaCl_2 solution (10 g of CaCl_2 in 100 mL of distilled water).
- Add 25 g of NaCl .
- Rinse to 1 L with distilled water.
- Neutralise a second time.

The final buffer solution should be stored at 4°C temperature.

SAMPLE PREPARATION FOR HISTOLOGY

1. Fix samples in formalin

Fix fresh dissected samples in 10% neutral buffered formalin (see above) for at least 24 hours. After fixation samples must be kept in 70 % ethanol until next step.

2. Paraffin embedding

Dehydrate samples and embed them in paraffin as follows:

70 % ethanol	1 hour
96% ethanol	1 hour
96% ethanol	1 hour
Absolute ethanol	1 hour
Absolute ethanol	1 hour
Xylene: Absolute Ethanol 1:1 dilution	1 hour
Xylene	1 hour
Xylene	1 hour
Paraffin (55-60°C)	2 hour
Paraffin (55-60°C)	2 hour
Paraffin (55-60°C)	2 hour

Once samples are embedded in paraffin mount them into histological cassettes.

3. Section obtaining

Obtain 4 μm thick sections of paraffin embedded samples in the microtome. Once sections are obtained, leave them into slides with albumine for microscopical observations. Leave slides drying 24 h at 37-40°C.

4. Histological staining

4.1 Hematoxylin- eosin staining

To stain 4 µm thick paraffin sections, the following protocol was applied (Gamble and Wilson, 2002):

Xylene	10 min
Xylene	10 min
100% ethanol	2 min
100% ethanol	2 min
96% ethanol	2 min
70% ethanol	2 min
dH ₂ O	5 min
Hematoxylin	4 min
dH ₂ O	2 min
dH ₂ O	10 s
dH ₂ O	10 s
Acid Alcohol	10 s
dH ₂ O	5 min
Lithium carbonate	10 s
dH ₂ O	1 min
70% ethanol	3 min
80% ethanol	1 min
Eosin	1 min
96% ethanol	2 min
96% ethanol	2 min
100% ethanol	2 min
100% ethanol	2 min
Xylene	2 min
Xylene	2 min

Once sections are stained, they can be mounted using DPX and protected with a coverslip.

SAMPLE PREPARATION FOR HISTOCHEMISTRY (Neutral lipids and Lipofuscin determination)

1. Sample obtaining

During animal dissection a small piece of the digestive gland of each individual is placed into a plastic chuck and then placed into a Cryovial® and immediately frozen into liquid nitrogen. Samples are stored until use at -80°C.

2. Cryosection obtaining

8 µm cryotome sections are obtained in the cryostat at -26°C chamber temperature. Sections are placed into slides for microscopic observations and stored them in the freezer until use at -40°C.

HISTOCHEMISTRY

1. Neutral lipid determination: Lillie and Ashburn's Isopropanol Oil Red O Method (Culling, 1974)

Lipid and fat deposits in cells and tissues can be examined by Oil Red O (ORO) staining. It is a fat-soluble diazo dye which stains in red cellular areas where lipid accumulation occurred. The staining areas are measured using an image analysis program: Image J (Image Processing and Analysis in Java, Maryland, USA)

Procedure

- Take unfixed cryotome sections (8 µm) to room temperature.
- Fix the sections in Baker's fixative containing 2.5% NaCl for 15 minutes at 4°C (see fixative preparation).
- After drying the sections in the air wash them in 60% isopropilic alcohol twice.
- Stain sections for 20 minutes in saturated Oil Red O solution.
- Differentiate sections in 60% isopropilic alcohol twice for 1 minute.

- Wash them in distilled water for 5-10 minutes.
- Counterstain sections with an aqueous solution of 0.1% Faste Green FCF for 30 min.
- Rinse the sections rapidly in distilled water.
- Mount the slides in glycerine gelatine.

To preparation of Oil Red O solution:

- Weight 0.25 g of Oil Red O.
- Dilute Oil Red O into 50 mL of isopropilic alcohol.
- Add to the previous dilution 33 mL of distilled water.
- Filtrate ORO solution.

2. Lipofuscin staining (Schormol's method, Pearse 1985)

This staining is used for the detection of the lipofuscins in cryosections. Lipofuscins are known to increase their presence under exposure to pollutants. The staining reactivity is measured using an image analysis program: Image J (Image Processing and Analysis in Java, Maryland, USA).

Procedure

- Take unfixed cryotome sections (8 μ m) to room temperature.
- Fix the sections in Baker's fixative containing 2.5% NaCl for 15 minutes at 4°C (see fixative preparation).
- Meanwhile prepare the reaction medium and the 1% acetic acid solution.
Reaction medium: Dissolve 1% ferric chloride and 1% potassium ferricyanide in a ratio 3:1. Take care because it is a photosensitive reaction.
- Rinse the sections in distilled water
- Immerse sections in the reaction medium for 5 minutes. Keep them away from direct light.
- Rinse the sections in acetic acid for 1 minute.

- Rinse the sections in distilled water and mount them in glycerine.
- Store sections away from direct light.

3. Autometallography

This technique which allows the detection of ionic metals that are not strongly attached to proteins, as black silver deposits form (Marigómez et al., 2002; Soto et al., 1997). It is based in the autoinduced amplification of Ag, which applying the basic principles of photography, allows the amplification of the metal atoms and molecules present in biological samples (Danscher, 1984).

The staining reactivity is measured using an image analysis program: Image J (Image Processing and Analysis in Java, Maryland, USA).

Procedure

- Dewax and hydrate sections by respective solution of xylene and descending graded ethanol.
- Left samples overnight at 37°C to dry them.
- Mix kit solutions, Initiator and Enhancer solutions, in a 1:1 ratio on an eppendorf tube.
- Add 100 µL of the mixture in to the samples.
- Left samples developing for 15-20 minutes. Check on the microscope the reaction to avoid over expositions.
- Rinse samples in tap water for 5 minutes.
- Mount the slides in Kaiser's glycerine.

METALOTIONEIN QUANTIFICATION (UNEP/RAMOGGE 1999; Viarengo et al., 1997 from Zorita, 2006)

During dissection digestive glands are introduced into Cryovials® and frozen into liquid nitrogen. Samples are stored at -80°C until use.

Preparation of solutions and chemicals

- Homogenisation buffer 0.5 M Sucrose 20 mM TRIS pH 8.6:

Trizma-base: 242 mg

dH₂O: 100 mL

Sucrose: 17.115 g

- Leupeptin stock solution (1 mg/mL):

Dissolve 1 mg leupeptine in 1 mL distilled water

- Ethanolic stock solution of phenylmethanesulphonyl fluoride (PMSF) (58 mg/mL):

Dissolve 58 mg PSMF in 1 mL ethanol

To the desired volume of Sucrose TRIS buffer add 3.0 µL/mL leupeptin, 1.5 µL/mL PSMF and 0.1 µL/mL β-mercaptoethanol (equivalent to 0.01%).

- Reduced glutathione (GSH) stock solution (1 mg/mL)

Before analysis freshly dissolve 20 mg GSH in 20 mL of 0.25 M NaCl.

- 0.25 M NaCl: Dissolve 1.461 g NaCl in 100 mL distilled water.

- 0.2 M phosphate buffer pH 8 containing 2 M NaCl

Na₂HPO₄ 12H₂O: 5.784 g

NaH₂PO₄ H₂O: 0.53 g

NaCl: 11.688 g

Distilled water: 100 mL

Adjust at pH at 8.0

- 5.5-dthiobis-2-nitrobenzoic acid (DNTB) 0.43 M:

Dissolve 17 mg DNTB in 100 mL phosphate buffer and cover it with aluminium foil.

- RNA (100 mg/mL) store at -20°C
- Cold (-20°C) absolute ethanol
- Chloroform
- 37% HCl
- 1N HCl/EDTA 4mM (store at room temperature)

Distilled water: 91.7 mL

HCl 8.3 mL

EDTA 0.116 g

Procedure

1. Sample homogenisation

- Get digestive gland samples (same sampling point or treatment) from the freezer and maintain them into ice.
- Weight a pool of digestive gland tissue from 10 different individuals. At least 1 g of digestive gland tissue per individual should be obtained.
- Add 3 mL of Buffer dissolution containing β -mercaptoetanol, PMSF and leupeptine, per 1 g of digestive gland tissue.
- Homogenise the pool of samples in a homogeniser.

2. Ultracentrifugation

- Centrifuge samples in the ultracentrifuge (18000 rpm t=20 min T=4°C) to obtain soluble nuclei and mitochondria free fraction containing MTs.

3. Ethanolic precipitation

High molecular weight proteins present in the supernatant are precipitated using cold (-20°C) absolute ethanol.

- Take 1 mL of the supernatant of the previous ultracentrifuged samples. Add 1.05 mL absolute ethanol (-20°C), 80 µL chloroform and mix it on a vortex.
- Centrifuge at (1500 rpm, t=10 min, T=0-4°C): Carefully collect all the supernatant and measure the volume using a pipette. Add to this supernatant 40 µl of 37% HCl, 10 µL of a solution of RNA (1 mg/ 10 µL) and 5.55 mL ethanol absolute (-20°C). Left this solution precipitating during 1 h at -20°C.
- Centrifugate again (1500 rpm, t=10 min, T=0-4°C). Remove obtained supernatant and dry the pellet.

4. Resuspension of the MT enriched fraction

Add into the pellet 2 mL of the following solution (17.4 mL ethanol+0.2 mL chloroform+2.4 mL homogenisation buffer).

Centrifugate again at (1500 rpm, t=10 min, T=4°C) and remove the supernatant.

Add into each sample: 150 µL of 0.25 M NaCl solution, 150 µL of 1 N HCl containing 4 mM EDTA (destabilising solution). Resuspend using a vortex.

5. DNTB assay (Ellman's reaction)

- Prepare the glutathione reference standard curve. For this, prepare a glutathione stock solution at 1 mg/mL concentration in 0.25 M NaCl and store in ice. Prepare at least 3 GSH reference standard concentrations and a blank.
- MT spectrophotometric evaluation: Dissolve 0.43 mM (7.14 mg/42 mL) DNTB in 0.2 M phosphate buffer pH 8 containing 2 M NaCl. Store this solution in darkness at room temperature. Add to blank, standard and samples 4.2 mL of DNTB solutions. Centrifuge metallothionein samples at 750 rpm for 5 minutes at room temperature. Measure the absorbance of

ABS₄₁₂, using a spectrophotometer, using reduced glutathione (GSH) as reference standard.

6. Calculation and result interpretation

- Plot the standard curve. The standard curve is a lineal function of GSH concentration (nmol/mL).

$$ABS_{GSH}^{412} = \epsilon[-SH]$$

In which: ABS_{GSH}⁴¹², (OD₄₁₂), is the Optical Density of GSH samples at 412 nm. ϵ , (OD₄₁₂/nmol/mL), is a constant value that represents extinction coefficient for GSH. [-SH], (nmol/l), is the concentration of sulphhydrylic group in GSH samples.

7. Quantitative determination of MT contents

Obtained ABS_{GSH}⁴¹² values for metallothionein samples are interpolated on the reference curve. The values on the X-axis represent the concentration of sulphhydrylic groups present in the MTs of the samples. Oysters present 21 cysteine residues and molecular weight is 8600 DA, the final volume of DNTB reaction (4.5 mL) and the dilution factor of the homogenate (4), the concentration of mussels metallothionein (ng/g tissue w.w) present in the samples can be obtained using the following formula:

$$[X/21] \times 8600 \times 4.5 \times 4$$

In which: X (nmol/mL) represent the X-axis value coming from interpolation of ABSMT412 value on the reference value.

ALGA CULTURE PROTOCOL

A strain of *Isochrysis galbana* T. Iso clone was grown into previously cleaned 30 L volume methacrylate reactors with natural filtered sea water. Algae were maintained under constant white light exposure (two lamps of 36 W per reactor), room temperature (T=18 °C) and filtered air flow (0.2 µm filters). Culture alga density was daily checked on a Neubaer Counting Chamber and when it reached 8×10^6 cell/mL concentration it was again diluted and enriched with F/2 Algae Food Part A and B (Based on Guillard's formulation, Proline water conditioners) medium.

Procedure

- Gently clean 30 L methacrylate reactors first with bleach during 5 minutes. Then rinse with tap water and soap. Finally rinse with 1% H₂O₂ during 5 minutes and rinse with filtered natural sea water.
- Keep constant white light exposure (two lamps of 36 W per reactor), room temperature (T=18°C) and filtered air flow (0.2 µm filters) into the reactors.
- Add the strain of *Isochrysis galbana* T. Iso clone and dilute with natural sea water.
- Enrich algae with F/2 Algae Food Part A and B (Based on Guillard's formulation, Proline water conditioners) medium in 1:1 proportion. Approximately 500 µL per 4 L of sea water added.
- Routinely check health status of algae and cell concentration under light microscope with a Neubaer chamber.
- Dilute again algae culture once they have reached 8×10^6 cel/mL concentration.
- Clean again culture reactor tanks once per week.

METAL MEASUREMENT PROTOCOL

1. Labware cleaning

Prior using all labware was cleaned:

- 72 h in nitric acid (HNO₃) 10%.
- Rinsed 3 times in des-ionised water.
- Rinsed 3 times in Milli-Q® water.
- Drying under a laminar flow hood.

All the labware is stored in double sealed polyethylene bags until use.

2. Animal obtaining

Animals were collected following OSPAR's commission guidelines (<http://www.ospar.org>) for monitoring contaminants in the biota. After sampling, animals were immediately carried to the laboratory facilities.

Prior dissection:

- Animals were left 24 h in natural sea water for depuration.
- HNO₃ 10% cleaned polyethylene tubes were weight in a precision balance, Precisa XT 220.

3. Animal dissection

- Whole weight of oysters is obtained.
- Animals are opened and the soft body is removed from shells using Teflon scissors. Between individuals tools used for dissection (scissors, scalpel...) are gently rinsed in distilled water for cleaning and prevent any contamination.
- Shell and flesh weights are obtained.
- Each soft body is introduced into a previously cleaned polyethylene tube and weight in a precision balance Precisa XT 220.

4. Freeze drying

- After dissection samples are deep-frozen (-80°C) at least during 24 h.
- Samples are freeze-dried (-60°C) in Heto Powerdry LL3000 Freeze Dryer (Thermo Scientific).
- Samples are weighted in a precision balance Precisa XT 220.

5. Sample homogenisation

- Each sample was individually homogenised with the aid of a mortar until obtaining a homogeneous powder.
- Between individuals, the mortar is gently rinsed in Milli-Q® water and dried to prevent contamination between individuals.
- Each oyster is stored in the corresponding polyethylene tube and weighted in a precision balance Precisa XT 220.

6. Acid digestion

Prior digestion:

- From each sample 200 mg are weighted and introduced in new acid clean polyethylene tubes.
- 200 mg of reference material TORT-2 Lobster hepatopancreas (National Research Council Canada; NRC-CNRC) and NIST 1566b Oyster Tissue (National Institute of Standards and Technology) and IAEE 407 are also weighted and introduced in new clean polyethylene tubes.
- Between individuals the plastic spatula is gently rinsed in Milli-Q® water and dried to prevent contamination between individuals.

The acid digestion of oysters was carried out following the protocol described by Daskalakis, (1996):

- Polyethylene tubes are placed in to a hotplate (DigiPREP MS; SCP SCIENCE).

- In each sample 1.4 mL HNO₃ (14M, PlasmaPur) y 2 mL HCl (12M, PlasmaPur) are added.
- The hotplate is heated at 10 minutes intervals until arriving to 90°C (25°C, 40°C, 60°C, 80°C and 90°C) to prevent gas formation.
- Samples are left digesting during 3 h at 90°C.
- 13.6 mL Milli-Q® water are added into each sample.
- The dilution is introduced into new acid clean polyethylene tubes (14 mL) for their analysis and stock (at 7°C in the fridge).

7. Preparation and analysis at Inductively Coupled Plasma Mass Spectrometry (ICP-MS; Thermo, X Series II)

- Samples are diluted 50 times in HNO₃ Ultrex 2%.
- Blancs samples are diluted 5 times in HNO₃ Ultrex 2%.
- For the preparation of the calibration curve in the ICP-MS (Thermo Scientific, X Series II) the following dilutions should be prepared: SLRS-5 (River water reference material for trace elements, National Research Council Canada; NRC-CNRC), LGC6019 (Certified Reference Material, River water-River Thames) and TM Rain-04 (Certified Reference Material, National Research Council Canada; NRC-CNRC).
- Samples are analysed into the ICP-MS.

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