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Rôles de différents facteurs écologiques sur le transfert trophique des éléments traces chez des téléostéens marins

Simon Pouil

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UNIVERSITÉ DE LA ROCHELLE

Thèse de doctorat pour l'obtention du grade de Docteur de l'Université de La Rochelle
Ecole doctorale : Sciences pour l'Environnement Gay Lussac
Spécialité : Biologie des organismes

Simon POUIL

RÔLES DE DIFFÉRENTS FACTEURS ÉCOLOGIQUES SUR LE TRANSFERT TROPHIQUE DES ÉLÉMENTS TRACES CHEZ DES TÉLÉOSTÉENS MARINS

Soutenue publiquement le 13 octobre 2017

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Marc METIAN	Chercheur IAEA HDR, Directeur de thèse

Crédits photographiques : Juvéniles turbots *Scophthalmus maximus* dans un bassin expérimental du Laboratoire de Radioécologie (Jean-Louis Teyssié, IAEA)

2017

Rôles de différents facteurs écologiques sur le transfert trophique des éléments traces chez des téléostéens marins

Ce travail de thèse a été réalisé au sein des équipes :

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“Je pense sincèrement que la pollution de la planète ce n'est pas aussi grave qu'on le dit...
C'est beaucoup plus grave.”

Le Chat, Philippe Geluck

“Les hommes et les poissons ont ceci en commun que les grands hommes comme les grands poissons,
ont tous disparu.”

La montagne de l’âme, Gao Xingjian

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A toute ma famille,

A Barbara,

PRÉFACE

L'ensemble des publications réalisées dans le cadre de cette thèse figure en Annexes.

Articles inclus dans la thèse

Annexe 1 **Pouil S**, Bustamante P, Warnau M, Metian M (soumis) Overview of trace elements trophic transfer in fish through the concept of assimilation efficiency. *Marine biology*.

Annexe 2 **Pouil S**, Warnau M, Oberhänsli F, Teyssié J-L, Bustamante P, Metian M (2017) Comparing single-feeding and multi-feeding approaches for experimentally assessing trophic transfer of metals in fish. *Environmental Toxicology and Chemistry* 36:1227-1234.

Annexe 3 **Pouil S**, Warnau M, Oberhänsli F, Teyssié J-L, Bustamante P, Metian M (2016) Influence of food on the assimilation of essential elements (Co, Mn, and Zn) by turbot *Scophthalmus maximus*. *Marine Ecology Progress Series* 550:207-218.

Annexe 4 **Pouil S**, Warnau M, Oberhänsli F, Teyssié J-L, Metian M (2015) Trophic transfer of ^{110m}Ag in turbot *Scophthalmus maximus* through natural and compounded feed items. *Journal of Environmental Radioactivity* 150: 189-194.

Annexe 5 **Pouil S**, Teyssié J-L, Rouleau C, Fowler SW, Metian M, Bustamante P, Warnau M (2017) Comparative study of trophic transfer of the essential metals Co and Zn in two tropical fish: A radiotracer approach. *Journal of Experimental Marine Biology and Ecology* 486: 42–51.

Annexe 6 **Pouil S**, Clausing R, Metian M, Bustamante P, Dechraoui Bottein M-Y (en préparation) How ingestion of algal toxins is influencing transfer of essential nutrients (Mn and Zn) in fish.

Annexe 7 Jacob H, **Pouil S**, Lecchini D, Oberhänsli F, Swarzenski P, Metian M. (2017) Trophic transfer of essential elements in the clownfish *Amphiprion ocellaris* in the context of ocean acidification. *PLoS One* 12(4):e0174344.

Annexe 8 **Pouil S**, Oberhänsli F, Bustamante P, Metian M (sous presse) Investigation of temperature and pH variations on the metal trophic transfer in turbot (*Scophthalmus maximus*). *Environmental Science and Pollution Research*. DOI: 10.1007/s11356-017-8691-4.

Annexe 9 **Pouil S**, Oberhänsli F, Bustamante P, Swarzenski P, Metian M (en préparation) Contrasting effects of water salinity on essential metal assimilation efficiency in a euryhaline teleost, the turbot *Scophthalmus maximus*.

Annexe 10 **Pouil S**, Oberhänsli F, Bustamante P, Metian M (2017) Dietary Zn and the subsequent organotropism in fish: No influence of food quality and environmental conditions (pH and temperature). *Chemosphere* 183C: 503-509.

Autres articles qui ne sont pas liés à la thèse

Article 1 Kuranchie-Mensah H, Teyssié J-L, Oberhänsli F, Tumnoi Y, **Pouil S**, Warnau M, Metian M (2016) Bioconcentration of Ag, Cd, Co, Mn and Zn in the Mangrove oyster (*Crassostrea gasar*) and preliminary human health risk assessment: A radiotracer study. *Bulletin of Environmental Contamination and Toxicology* 97(3): 413-417.

Article 2 Metian M, **Pouil S**, Hédouin H, Oberhänsli F, Teyssié J-L, Bustamante P, Warnau M (2016) Differential bioaccumulation of ^{134}Cs in tropical marine organisms and the relative importance of exposure pathways. *Journal of Environmental Radioactivity* 152: 127-135.

Article 3 **Pouil S**, Bustamante P, Warnau M, Oberhänsli F, Teyssié J-L, Metian M (2015) Delineation of ^{134}Cs uptake pathways (seawater and food) in the variegated scallop *Mimachlamys varia*. *Journal of Environmental Radioactivity* 148: 74-79.

Article 4 Metian M, **Pouil S**, Boustany A, Troell M (2014) Farming of bluefin tuna: Reconsidering global estimates and sustainability concerns. *Reviews in Fisheries Science & Aquaculture* 22(3): 184-192.

Une partie des résultats obtenus au cours de cette thèse a fait l'objet de communications orales ou de posters présentés lors de congrès scientifiques.

Communications orales et posters

Metian M, **Pouil S**, Giraud E, Townsend A, Swarzenski P, Fowler S (2017). Radiocesium accumulation in aquatic organisms: A global synthesis from an experimentalist's perspective. Communication orale. *4th International Conference on Radioecology and Environmental Radioactivity (ICRER)*. 3-8 Septembre, Berlin, Allemagne.

Pouil S, Warnau M, Oberhänsli F, Teyssié J-L, Bustamante P, Metian M (2016). Investigation of metal assimilation efficiencies in marine fish. Communication orale. *Colloque 2016 de la Société Française d'Ecotoxicologie Fondamentale et Appliquée*. 29-30 Juin, Reims, France.

Metian M, **Pouil S**, Jacob H, Oberhänsli F, Teyssié J-L, Lecchini D (2016). Studying multi-stressors effects in coral reef organisms: Perspectives related to the use of radiotracer techniques. Communication orale. *13th International Coral Reef Symposium*. 19-24 Juin, Honolulu, Hawaii.

Metian M & **Pouil S** (2016). Nuclear applications to study potential effects (directs or indirects) of ocean acidification on marine organisms. Poster. *4th International Symposium on the Ocean in a High-CO_2 World*. 3-6 Mai, Hobart, Australia.

Pouil S, Metian M, Giraud E, Bustamante P, Dechraoui Bottein MY (2016). Effects of brevetoxin pre-exposure on Mediterranean mussel *Mytilus galloprovincialis*: Insights into feeding physiology. Communication orale. *GdR Phycotox 2016*. 15-16 Mars, Villefranche-Sur-Mer, France.

Lacoue-Labarthe T, **Pouil S**, Oberhänsli F, Teyssié J-L, Bustamante P, Metian M (2015). Effects of metabolic inhibitors on metal accumulation in juveniles of the cuttlefish *Sepia officinalis*. Communication orale. *Cephalopod International Advisory Council*. 10-15 Novembre, Hakodate, Japon.

Metian M, **Pouil S**, Oberhänsli F, Teyssié J-L, Dechraoui Bottein MY, Warnau M (2015). Overview of nuclear application to study aquaculture-environment interactions and aquaculture nutrition. Communication orale. *Aquaculture 2015: Cutting Edge Science in Aquaculture*. 23-26 Août, Montpellier, France.

ACRONYMES ET ABRÉVIATIONS

Eléments traces

Ag	Argent
Am	Américium
As	Arsenic
Al	Aluminium
Ba	Baryum
Cd	Cadmium
CH_3Hg	Méthyl-mercure (ou MeHg)
Co	Cobalt
Cu	Cuivre
Cr	Chrome
Cs	Césium
Fe	Fer
Hg	Mercure
Mn	Manganèse
Pb	Plomb
Po	Polonium
Se	Sélénium
Zn	Zinc

Variables biologiques

A_0	Activité initiale (*Initial activity*)
A_t	Activité restante (*Remaining activity*)
AE	Efficacité d'assimilation (*Assimilation efficiency*)
FCE	Efficacité de conversion alimentaire (*Food Conversion Efficiency*)
IR	Taux d'ingestion (*Ingestion rate*)
GTT	Temps de transit intestinal (*Gut transit time*)
k_e	Taux de perte (*Efflux rate*)
k_u	Taux d'accumulation (*Uptake rate*)
$T_{b1/2}$	Temps de vie biologique (*Biological half-time*)
TAM	Fraction métallique biodisponible (*Trophic available metal*)
TTF	Facteur de transfert trophique (*Trophic transfer factor*)
g	Constante de vitesse de croissance (*Growth rate constant*)

Autres abréviations

ETM	Elément trace métallique
TBT	Tributylétain

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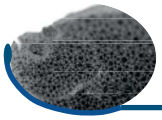

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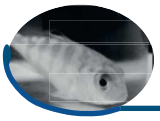

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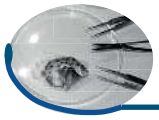

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Chapitre 1
Introduction

CHAPITRE 1
INTRODUCTION

1.1. LES MÉTAUX DANS LES ÉCOSYSTÈMES MARINS

1.1.1. Les métaux : définitions

Le terme métal désigne un élément chimique capable de former des liaisons métalliques ou ioniques. Les métaux représentent la majorité des éléments terrestres et constituent 91 des 118 éléments chimiques connus. Au sein du tableau périodique des éléments, les métaux se situent à gauche et au centre (**Fig. 1A**). Ce sont essentiellement des solides cristallins, néanmoins le mercure (Hg) se présente à l'état liquide dans les conditions normales (20°C sous pression atmosphérique).

De nombreuses formes de classification des métaux existent et sont basées sur des critères variés. Ainsi, il s'avère difficile d'appréhender la complexité de cette classe d'éléments. Cependant, certains termes dominent la littérature scientifique en lien avec les métaux. En effet, il est courant de lire « métaux lourds » ou « éléments traces métalliques » quand il s'agit de désigner un métal. Ces termes résultent de deux façons de classer les métaux toutes deux basées soit sur des caractéristiques et propriétés physiques de certains éléments soit sur leurs quantités présentes dans l'environnement. Ainsi, le terme « métal lourd » désigne les éléments du tableau périodique ayant des propriétés d'éléments métalliques et dont la masse atomique est élevée (Holister & Porteous 1976) ou encore supérieure à celle du sodium (Bennet 1986) ou dont la masse volumique supérieure à 3,5 ou 7 g cm^{-3} selon les auteurs (Duffus 2002). Cependant, ce concept reste mal défini et est parfois utilisé de façon abusive avec l'association des métaux réellement lourds avec d'autres l'étant moins (e.g. l'Al dont la masse volumique est 2,7 g cm^{-3}). En raison de ces limites, les termes « élément trace » ou encore « élément trace métallique » (ETM) tendent à supplémenter celui de « métal lourd » dans la littérature. Ces termes définissent les éléments aux propriétés métalliques (métaux et métalloïdes) présents en quantités très faibles dans l'environnement (de l'ordre du µg g^{-1} ou moins ; Pais & Benton Jones 1997). Dans ce manuscrit, le terme « métaux », plus usuel, est privilégié pour désigner les éléments traces métalliques étudiés. Par ailleurs, il existe d'autres formes de classification des métaux et notamment une basée sur l'affinité des ions métalliques (Rainbow 1997). Selon cette classification les métaux dits de classe A présentent une forte affinité avec les ligands contenant de l'oxygène (e.g. Al^{3+}, Ba^{2+}, Mg^{2+}) tandis que ceux de la classe B présentent une plus forte affinité pour les ligands contenant du soufre ou de l'azote (e.g. Ag^{2+}, Au^{2+}, Hg^{2+}).

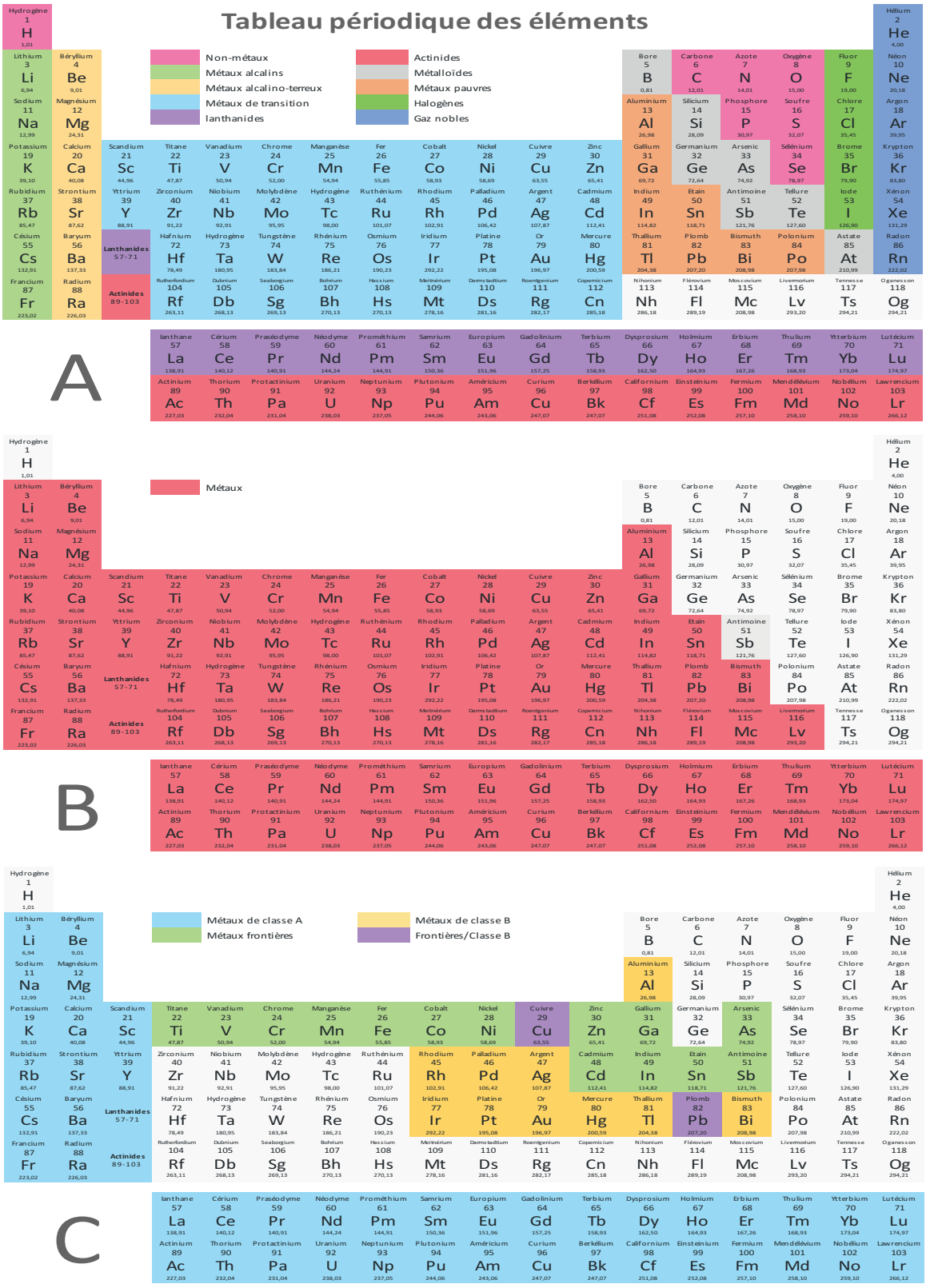


Figure 1. Classification des éléments (A) par groupes (B) selon s'ils sont considérés comme étant des métaux ou (C) par « famille de métaux » d'après Nieboer & Richardson (1980) et Rainbow (1997)

Enfin, un groupe intermédiaire, les métaux de transition, regroupe les éléments qui présentent des caractéristiques communes aux métaux des deux précédentes classes (Nieboer & Richardson 1980, Rainbow 1997 ; **Fig. 1**). Cette classification a pour avantage de mettre en exergue l'affinité des métaux qui est un facteur important intervenant dans leur comportement dans l'environnement et le biote. Les métaux sont des éléments non conservatifs (i.e. leurs concentrations dans l'eau et les particules ne sont pas constantes), non dégradables, ni biologiquement, ni physico-chimiquement (Clark 1989). De par leurs propriétés intéressantes telles que leur capacité de conduire l'électricité et la chaleur, leur malléabilité ou encore leur ductilité, les métaux font l'objet d'utilisations très diverses par l'Homme.

1.1.2. Les métaux dans l'environnement marin : sources et devenirs

Les métaux sont naturellement présents dans la biosphère et font partie des constituants de la lithosphère (Garrett 2000). Leur dynamique dans l'environnement terrestre et océanique suit des cycles biogéochimiques qui expliquent l'hétérogénéité observée dans la distribution des métaux (Garrett 2000). L'apport métallique dans les océans résulte de la combinaison de plusieurs processus qui se soldent par des apports d'origines telluriques et atmosphériques. Des apports provenant des sources hydrothermales ont également été mis en évidence (Edmond et al. 1979, Elderfield & Schultz 1996). Il est admis que l'origine naturelle des métaux contenus dans les sédiments et les eaux naturelles est constituée à 80% de l'altération des roches sédimentaires et à 20% de l'altération des roches primaires (Martin & Meybeck 1979). Pour dresser le bilan des apports métalliques, il est nécessaire de considérer également l'influence des activités humaines qui ne cesse de croître. En effet, les activités domestiques, industrielles, militaires, minières et agricoles sont à l'origine de relargage de métaux dans l'environnement. Pour bien appréhender l'importance des métaux pour l'Homme, il faut considérer le fait que 7 à 8% de l'énergie mondiale qui est consommée sont consacrés à leur production primaire (van der Voet et al. 2013). Les effets de ces activités se font ressentir depuis très longtemps. A ce titre, Monge et al. (2015) ont récemment mis en évidence des pollutions métalliques dues aux activités anthropiquesdatant de l'Age de Pierre. Aujourd'hui, les métaux sont également employés dans de nouveaux secteurs d'activité et sont, par exemple, utilisés dans la fabrication de matériels électroniques, d'accumulateurs électriques ou encore de diodes électroluminescentes.

Leur consommation s'est ainsi très fortement accrue depuis les années 1980, au point que certains métaux rares dits « métaux stratégiques » sont désormais inclus dans les « matières premières minérales critiques » (European Commission 2014). La démographie mondiale ne cesse d'augmenter et certains auteurs prédisent que jusqu'à 1,4 milliards d'habitants vivront dans des zones littorales ne dépassant pas 10 m d'élévation d'ici 2050 (Neumann et al. 2015). Cet accroissement de la pression anthropique entraîne une augmentation des apports métalliques d'origine humaine dans les écosystèmes côtiers. Or, il s'agit de zones de transition inévitables entre les fleuves et les océans qui sont le siège d'interactions d'une grande diversité de par les fortes variations des facteurs physicochimiques, hydrodynamiques, sédimentologiques, climatiques mais aussi biologiques auxquelles elles sont sujettes. Ces zones constituent par ailleurs des habitats permanents ou temporaires pour de nombreux organismes marins et notamment les poissons.

Les métaux peuvent être présents dans l'eau sous forme ionique ou sous forme de complexes chimiques mais également être conservés et accumulés dans les écosystèmes associés aux particules telles que les sédiments, les matières en suspension ou les colloïdes (Phillips 1977). Les effets écotoxicologiques des métaux peuvent donc persister pendant des décennies après les incidents de pollution et ils peuvent être dommageables lorsque les sédiments ont remis en suspension.

1.2. L'ACCUMULATION DES MÉTAUX DANS LES ORGANISMES MARINS

1.2.1. Les effets sur les organismes : notions d'essentialité et de toxicité

Avant d'aborder les notions d'essentialité et de toxicité des métaux pour les organismes il est important de définir certains termes. En effet, des confusions sont possibles dans les concepts de biodisponibilité, de bioaccumulation, de bioconcentration et de bioamplification appliqués aux métaux. La biodisponibilité est définie comme étant la quantité de métaux disponible pour l'assimilation par les organismes (Newman & Jagoe 1994). Il est important de noter que la biodisponibilité des métaux peut être impactée par les interactions entre les métaux et la matière carbonée qui conduisent à la formation de composés organométalliques.

En effet, certains métaux tels que le Cd et le Hg ont la capacité de se lier à des composants non métalliques de macromolécules tels que les groupes sulfhydriles (thiol, -SH) de protéines contenant de la cystéine par différents mécanismes tels que la bioalkylation ou la biométylation. Par exemple la méthylation du Hg conduit à la formation d'un composé toxique, le méthyl-Hg (CH_3Hg), susceptible de se bioaccumuler plus facilement que le Hg inorganique dans les organismes et de se bioamplifier dans les réseaux trophiques aquatiques. La biodisponibilité dépend de nombreux facteurs tels que l'espèce, le métal ou encore sa spéciation dans l'environnement ambiant. Cette définition met en évidence l'intérêt en toxicologie aquatique, de tenir compte de la fraction biodisponible des métaux et ne pas se limiter à leurs concentrations totales dans le milieu et dans les organismes.

La bioaccumulation désigne la capacité des organismes à concentrer dans leurs tissus un métal par voie dissoute ou particulaire (EPA 2000). La bioconcentration est un cas particulier de la bioaccumulation. Elle désigne une augmentation de la concentration d'un métal dans un organisme par rapport à celle observée dans l'eau (Hédouin 2006).

La notion de bioamplification désigne l'augmentation, tout au long de la chaîne trophique, des concentrations d'un métal. La bioamplification chez les poissons a été étudiée dans des environnements naturels, mais les relations entre concentration de métaux dans les proies et celles retrouvées chez les consommateurs / prédateurs sont difficiles à établir dans ces conditions. En effet, les concentrations de métaux dans les proies, sont souvent comparées à celles de certains tissus et organes (foie et muscles) du prédateur, en particulier chez les prédateurs de grande taille. Par ailleurs, il est toujours complexe d'isoler les voies d'accumulation des métaux en adoptant une approche de terrain (Gray 2002) d'où l'intérêt de l'approche expérimentale dans des conditions contrôlées (voir Chapitre 2).

L'ensemble des mécanismes évoqués précédemment peut avoir des conséquences différentes sur les organismes selon la nature des métaux et les concentrations atteintes dans le biote. Bien souvent, les métaux sont caractérisés en fonction de leur essentialité ou non pour les organismes. Les métaux dit essentiels, ne pouvant être synthétisés par les organismes, sont directement impliqués dans les processus métaboliques. A titre d'exemple, le Co est un composant clé de la cobalamine (vitamine B12), et en tant que tel est essentiel notamment pour la formation et le maintien du tissu nerveux.

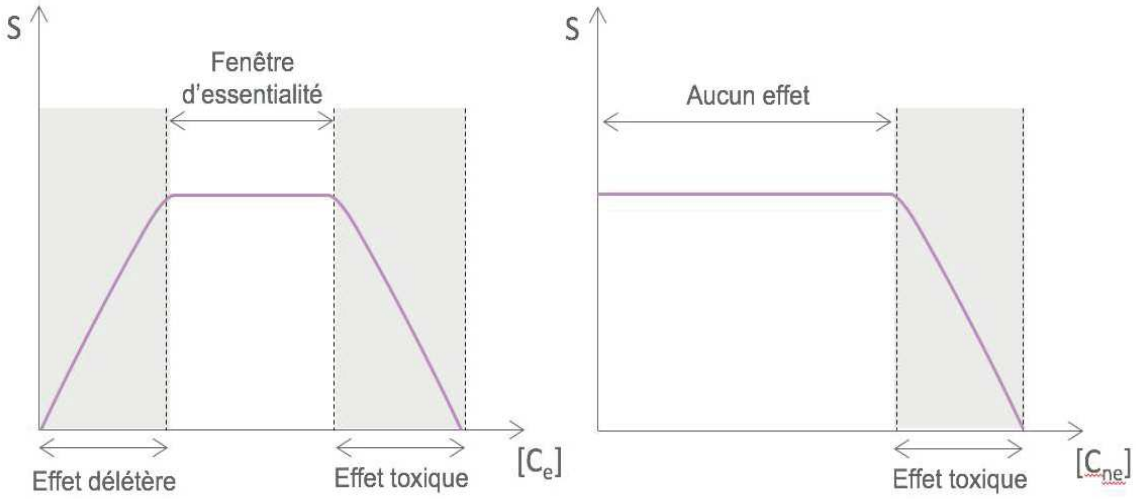


Figure 2. Relation entre l'état de santé d'un organisme (S) et les concentrations en éléments essentiels [C_e] ou non essentiels [C_{ne}] pour les organismes. Modifiée d'après Hopkin (1989).

En tant que cofacteur ou composant de plusieurs systèmes enzymatiques clés, le Mn est essentiel à la formation osseuse et des tissus de soutien (synthèse des mucopolysaccharides), à la régénération des globules rouges, au métabolisme des glucides et au cycle de reproduction. Le Zn est quant à lui impliqué dans de nombreux systèmes enzymatiques jouant un rôle vital dans le métabolisme des lipides, des protéines et des glucides (Simkiss 1979, Williams 1981). Si les besoins métaboliques en éléments essentiels sont satisfaits alors la santé et le développement des organismes peuvent être optimaux. Ces effets ont des implications importantes à la fois pour les écosystèmes et également en aquaculture. Cependant, lorsque ces mêmes éléments sont présents en quantités insuffisantes ou à l'inverse, en excès, des troubles tels que des altérations physiques ou pathologiques liés à des problèmes de carence ou de toxicité peuvent survenir. C'est pourquoi il convient de nuancer le terme d'élément d'essentiel en considérant la notion de « fenêtre d'essentialité » développée par Hopkin (1989) et reprise en d'autres termes par Langley et al. (1997) (**Fig. 2**). Un même élément dit essentiel peut être toxique en fonction de sa concentration dans l'organisme.Cette notion de dose est fondamentale, car la plupart des éléments ne sont toxiques qu'à partir d'une certaine concentration (Rainbow 2002). La toxicité dépend donc de la concentration du métal mais également de la durée de l'exposition, de la forme physico-chimique du métal (spéciation), de la présence d'autres métaux, de la sensibilité des espèces aux métaux et de nombreux autres facteurs (âge, état de santé, statut reproducteur, etc ; Wang 1987).

La toxicité des métaux est aussi directement liée à leur accumulation par les organismes, à la réactivité de ces métaux vis-à-vis de molécules d'importance biologique, et à l'aptitude des organismes à métaboliser ou à détoxiquer les métaux. Certains métaux sont considérés comme non-essentiels (e.g. Hg et Pb), car ils n'ont aucune fonction biologique reconnue et des effets néfastes sur les organismes sont détectables à de faibles concentrations (**Tableau 1**). Souvent, les effets toxiques de ces éléments résultent, ou sont renforcés par, les interactions entre d'autres métaux. Par exemple, le Cd peut induire des changements dans l'homéostasie du Zn, ce qui entraîne une rétention accrue de Zn dans le foie et/ou les reins (Brzóska & Moniuszko-Jakoniuk 2001). Certains éléments sont capables de former des composés organométalliques, déjà évoqués précédemment, qui se comportent différemment de la forme élémentaire, et certains peuvent être très toxiques (e.g. CH_3Hg et le tributylétain TBT, utilisé comme biocide dans les peintures *antifouling*). La distinction entre métaux essentiels et non-essentiels n'est pas figée et certains métaux considérés comme non-essentiels peuvent s'avérer avoir un rôle dans les fonctions biologiques de certains organismes. Ainsi, au début des années 2000, des études ont mis en évidence le rôle du Cd dans l'activation d'une enzyme, l'anhydrase carbonique chez des diatomées marines océaniques dans des conditions de déficience en Zn (Lane & Morel 2000, Lane et al. 2005). L'exposition des organismes à des concentrations toxiques d'un élément peut entraîner l'apparition d'effets tels que des changements morphologiques, des altérations dans le développement ou dans la physiologie ou encore des modifications de comportements conduisant à une baisse de la valeur sélective d'un individu et dans certains cas à sa mort. Ainsi, bien que les potentiels effets toxiques des métaux prennent leurs origines à l'échelle subcellulaire, il est possible d'en observer les effets à l'échelle écosystémique.

L'intérêt des études portant sur la bioaccumulation des métaux chez les poissons ne se limite pas à des perspectives écologiques. En effet, les poissons constituent une ressource alimentaire importante pour de nombreuses populations humaines. Il s'agit d'ailleurs d'une des ressources alimentaires les plus échangées au monde (Pauly et al. 2002). A la fois pour leur potentielle toxicité pour les consommateurs finaux que nous sommes et pour leur essentialité conditionnant les performances de croissance en aquaculture (Watanabe et al. 1997), il apparaît important d'étudier le transfert des métaux chez les poissons, notamment par la voie trophique.

Tableau 1. Eléments traces considérés dans les études expérimentales concernant l'assimilation chez les poissons. Les éléments considérés dans les travaux de cette thèse sont indiqués en gras.

Elément		Numéro atomique	Masse atomique	Essentialité
Ag	**Argent**	**47**	**108**	**Non**
Am	Américium	95	243	Non
As	Arsenic [1]	33	75	Oui
Cd	**Cadmium**	**48**	**112**	**Non [2]**
Co	**Cobalt**	**27**	**59**	**Oui**
Cr	Chrome	24	52	Oui
Cs	Césium	55	133	Non
Cu	Cuivre	29	64	Oui
Hg	Mercure	80	201	Non
Mn	**Manganèse**	**25**	**55**	**Oui**
Po	Polonium	84	209	Non
Se	Sélénium [1]	50	109	Oui
Zn	**Zinc**	**30**	**65**	**Oui**

[1] L'As est un métalloïde et le Se est classé parmi les non-métaux et est souvent étudié en écotoxicologie notamment en relation avec le Hg.
[2] Cependant, un rôle biologique du Cd a été identifié chez certaines diatomées marines dans des conditions particulières, notamment de déficience de Zn (Lane & Morel 2000).

1.2.2. Les différentes voies d'exposition aux métaux

Les métaux, tout comme les composés organiques, peuvent pénétrer dans les organismes marins directement à partir de l'eau de mer en traversant les membranes perméables des cellules des organismes marins. Dans les tissus, ils sont compartimentés, stockés et liés à des ligands pour en réduire la réactivité toxique (Deb & Fukushima 1999). Ces procédés éliminent les métaux des fluides tissulaires et permettent le maintien de gradients de diffusion indispensables aux processus osmorégulateurs. La bioconcentration des métaux dans les organismes, induite par l'ensemble de ces processus, peut être estimée en utilisant le facteur de concentration (FC). En radioécologie, le FC se calcule par la mesure de la radioactivité de l'organisme exprimée en Bq kg^{-1} divisée par la radioactivité environnementale exprimée en Bq kg^{-1}. Ainsi, dans certains cas des FCs supérieurs à 1000 peuvent être observés. De la même façon un facteur de transfert (TF) peut être calculé pour quantifier l'accumulation des métaux chez les organismes par l'intermédiaire du sédiment. Les métaux bioaccumulés à partir de l'eau ou du sédiment sont susceptibles d'être ensuite transférés par la voie trophique à d'autres échelons trophiques. Les organismes marins, dont les poissons, sont donc exposés aux métaux via trois sources d'accumulation majoritaires (eau de mer, sédiments et nourriture) et les capacités d'accumulations des organismes sont différentes selon chaque source.

L'importance de chaque source dans la bioaccumulation totale du métal dans l'organisme dépend de la concentration du métal dans chacune des voies et des aptitudes biologiques de l'organisme (e.g. taux d'ingestion, métabolisme...). De nombreuses expériences se sont intéressées à déterminer quelle était la voie majoritaire d'accumulation des métaux dans les organismes et ont démontré l'importance de la voie trophique dans l'accumulation des métaux chez de nombreux organismes marins (e.g. Metian et al. 2009, Mathews & Fisher 2009, Hédouin et al. 2010b).

1.3. LE TRANSFERT TROPHIQUE DES MÉTAUX : DU PHYTOPLANCTON AUX POISSONS

De nombreuses études expérimentales se sont focalisées sur le transfert trophique des métaux par la mise en place de chaînes alimentaires simplifiées. Ainsi, dans les années 1990, le transfert trophique des métaux depuis des producteurs (phytoplancton) à des consommateurs primaires tels que du zooplancton (Fisher & Reinfelder 1991, Wang & Fisher 1998, Xu & Wang 2001, Yu & Wang 2004) ou des mollusques bivalves (e.g. Wang & Fisher 1996, Ettajani et al. 2001, Blackmore & Wang 2002, Hédouin et al. 2010a,b,c) a été largement étudié. Ces études ont mis en évidence que le transfert trophique des métaux chez les copépodes et les bivalbes est notamment régi par la répartition subcellulaire de ces mêmes métaux dans les cellules de phytoplancton ingérées. Certains de ces travaux ont conduit ensuite aux investigations plus poussées sur l'importance de considérer la répartition subcellulaire des métaux dans les proies pour comprendre leur assimilation par les prédateurs. Par la suite, les chaînes alimentaires reproduites en laboratoire se sont complexifiées avec l'ajout de consommateurs secondaires. Ainsi les exemples de chaînes alimentaires basées sur du phytoplancton et des mollusques bivalves eux-mêmes consommés par des gastéropodes prédateurs ont permis d'apporter de nouvelles connaissances quant au transfert trophique des métaux (Cheung & Wang 2005, Rainbow et al. 2007). En effet, ces études ont démontré l'importance du type de nourriture et de la physiologie des prédateurs dans les écosystèmes aquatiques. Par la suite, les chaînes alimentaires simulées en laboratoire se sont diversifiées et ont atteint jusqu'à quatre maillons comportant un producteur et trois consommateurs successifs incluant des poissons (Mathews & Fisher 2008).

Chez les poissons (au sens de téléostéens), dès les années 1970, certains auteurs ont suggéré expérimentalement que la voie alimentaire constitue la voie d'accumulation majeure des métaux (Pentreath 1973, 1976, Willis & Sunda 1984, Dallinger et al. 1987). Ces observations ont par la suite été confirmées le calcul de la contribution de chacune des voies d'exposition (Mathews & Fisher 2009). A partir de là, il est devenu clair que la nourriture constitue la voie majeure d'acquisition des métaux chez les poissons. Le transfert trophique des métaux peut être estimé par une approche de modélisation basée sur un modèle bioénergétique développé dans les années 1990 (Landrum et al. 1992). Ainsi, la bioaccumulation des métaux dans les poissons peut être expliquée comme un équilibre entre les taux d'absorption et de perte de métal et peut être décrite par l'équation suivante :

$$C_{ss} = [(k_u \times C_w) + (AE \times IR \times C_f)] / (k_e + g)$$

où C_{ss} est la concentration en métal dans un prédateur à l'équilibre, k_u est le taux d'absorption du métal, C_w est la concentration en métal dissous dans l'eau, AE est l'efficacité d'assimilation du métal ingéré (voir Chapitre 2 pour une définition de ce concept), IR est le taux d'ingestion, C_f est la concentration en métal dans la proie, k_e est le taux d'efflux du métal hors du prédateur, et g correspond à la vitesse de croissance du prédateur (Thomann 1981, Landrum et al. 1992, Thomann et al. 1995). Le réarrangement de cette équation permet d'évaluer le potentiel d'un métal à se bioamplifier le long de la chaîne alimentaire. Ainsi, pour un maillon de la chaîne alimentaire dans lequel un prédateur ingère du métal provenant d'une proie, il est possible de calculer un facteur de transfert trophique (TTF) comme suit :

$$TTF = (AE \times IR) / (k_e + g)$$

Un TTF>1 indique une possibilité de bioamplification (Reinfelder et al. 1998). Cette approche montre l'importance de la quantification de l'assimilation des métaux chez les poissons pour comprendre le transfert trophique de ces éléments au sein de ce taxa. Pourtant, contrairement à ce qui a été fait chez les invertébrés, le nombre d'études relatives au transfert trophique, et plus encore à l'assimilation des métaux chez les poissons, reste encore relativement faible. Ainsi, notre compréhension des facteurs influençant le transfert trophique des métaux chez les poissons présente encore certaines lacunes.

Il est possible de caractériser les facteurs influençant le transfert trophique en trois compartiments (**Fig. 3**) : les proies, les prédateurs et leur environnement. La majorité des études relatives au transfert trophique des métaux chez les poissons se sont intéressées aux facteurs relatifs aux proies susceptibles d'impacter leur assimilation. Ainsi, différents travaux ont montré que, chez une même espèce prédatrice, l'assimilation des métaux peut varier selon le type d'aliment ingéré (e.g. Wang & Wong 2003, Dutton & Fisher 2011, Wang et al. 2012).

Par une approche mécanistique, certains auteurs ont étudié les facteurs intrinsèques aux proies qui pourraient expliquer les différences d'assimilation observées. La relation entre le répartition subcellulaire des métaux dans la nourriture et l'assimilation observée chez les prédateurs a été étudiée chez plusieurs espèces, suite notamment aux travaux initiés sur des invertébrés (Wallace & Lopez 1996, 1997, Wallace & Luoma 2003). Les résultats de ces travaux sont encore contrastés. Certains auteurs ont mis en évidence une relation positive entre la répartition subcellulaire du Cd, du CH_3Hg et du Zn dans les proies et l'assimilation de ces éléments chez différentes espèces de poissons (Seebaugh et al. 2005, Zhang & Wang 2006, Dang & Wang 2010) mais cela n'a pu être démontré que pour un nombre réduit de métaux par un nombre limité d'études. Les concentrations métalliques dans les proies sont également susceptibles d'impacter l'assimilation des métaux par les prédateurs. En effet, Wang et al. (2012) ont démontré qu'avec des niveaux croissants de Cd et de Zn ajoutés dans un aliment commercial, leurs efficacités d'assimilation diminuent de façon significative chez le pagre à tête noire *Acanthopagrus schlegeli*, alors que l'assimilation du Hg inorganique n'a pas été affectée. Cependant, dans une autre étude menée chez une espèce de poisson herbivore le sigan *Siganus canaliculatus*, l'augmentation des concentrations métalliques dans les macroalgues n'a pas eu d'effet significatif sur l'assimilation des métaux.

Le transfert trophique des métaux chez les poissons est également fonction de l'espèce considérée. Ainsi, des comparaisons interspécifiques ont été faites sur l'assimilation chez plusieurs prédateurs. Les différences observées ont été mises en relation avec l'écologie trophique des organismes (Ni et al. 2000) et la diversité phylogénique (c'est-à-dire les caractéristiques anatomiques, physiologiques et biologiques; Mathews et al. 2008). L'influence de la taille des prédateurs (allométrie) sur l'assimilation des métaux a également été étudiée chez le pagre à tête noire *A. schlegeli*.

Dans cette étude, l'assimilation du Se et du Zn augmentait avec la taille des poissons alors qu'aucun effet significatif n'a été mis en évidence pour le Cd (Zhang & Wang 2007).

Il existe encore peu d'études concernant le comportement alimentaire des prédateurs alors que ce paramètre semble être important dans la compréhension de l'assimilation des métaux. L'approche expérimentale a permis l'étude de facteurs biologiques susceptibles d'influencer le transfert trophique des métaux chez les poissons. A titre d'exemple, Van Campenhout et al. (2007) ont étudié l'influence de la composition de la nourriture mais également l'état physiologique du prédateur étudié, la carpe commune *Cyprinus carpio*. Par ailleurs, cette étude se distingue par la prise en compte des effets de la température sur l'assimilation des métaux chez cette espèce. En effet, bien que les facteurs environnementaux tels que la température et le pH soient connus pour affecter la physiologie digestive des organismes aquatiques et notamment des poissons dans leurs jeunes stades de vie (e.g. Pimentel et al. 2015), peu d'études relatives au transfert trophique des métaux ont considéré ces facteurs.

1.3. LES OBJECTIFS DE LA THÈSE

Les poissons accumulent les métaux (essentiels et non-essentiels) au travers différentes voies. Aujourd'hui de nombreuses études ont mis en évidence le rôle majeur que peut avoir le transfert trophique sur l'accumulation des métaux essentiels et non-essentiels chez ce groupe. Toutefois, malgré le fait que l'importance de cette voie d'accumulation des métaux soit reconnue, il existe de nombreuses lacunes concernant le transfert trophique des métaux chez les poissons. En effet, l'implication de nombreux facteurs reste encore non élucidée. Parmi les facteurs écologiques susceptibles d'influencer le transfert trophique des métaux chez les poissons, une division en deux catégories a été opérée dans le cadre de cette thèse :

Les facteurs biologiques qui regroupent l'ensemble des actions du vivant sur le vivant. Il s'agit donc des facteurs écologiques d'un écosystème qui dépendent des organismes qui y vivent. Dans le cas du transfert trophique des métaux chez les poissons, il s'agit principalement des interactions proies-prédateurs qui n'ont été étudiées que sous certains aspects dans la littérature. En particulier, il subsiste, entre autres, des lacunes concernant l'influence du type de proies sur le transfert trophique, notamment des éléments essentiels chez les poissons prédateurs ou encore l'effet de la taille ou du stade physiologique d'un prédateur sur sa capacité à assimiler ces mêmes éléments (**Fig. 3**).

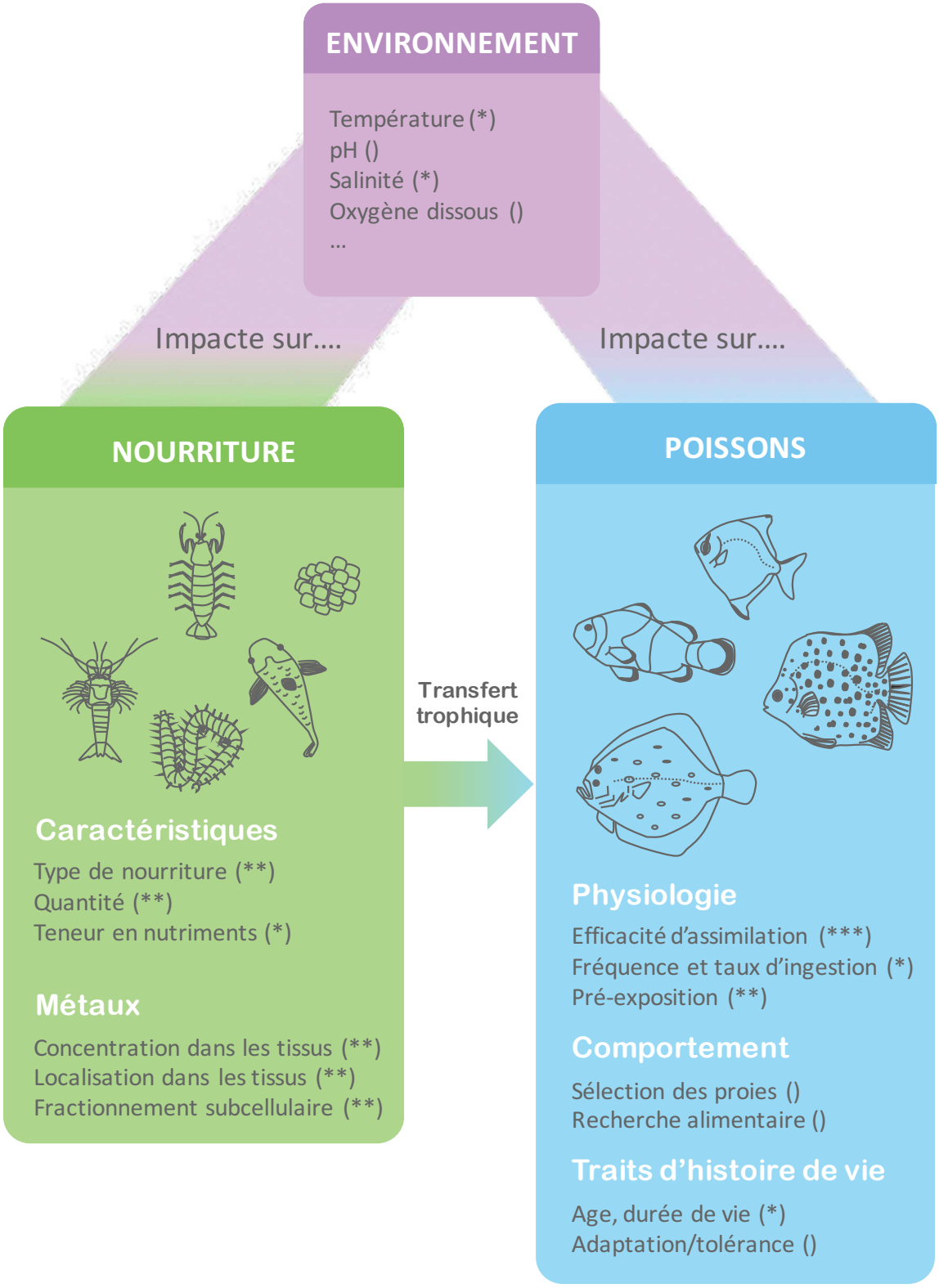


Figure 3. Processus contrôlant le transfert trophique des métaux chez les poissons. Les étoiles entre parenthèses indiquent les processus déjà étudiés en conditions expérimentales. Le nombre d'étoiles est proportionnel à l'information disponible dans la littérature. L'absence d'étoile indique que le processus en question n'a pas été encore étudié. Créée d'après la revue de la littérature présentée dans l'**Article 1**.

Les facteurs environnementaux qui sont liés aux conditions physico-chimiques du milieu. Cette catégorie regroupe les facteurs physico-chimiques d'un écosystème influençant sa biocénose. En milieu marin, dans le contexte des changements globaux où les activités anthropiques sont à l'origine de modifications environnementales sans précédent, il est primordial d'appréhender l'influence de facteurs tels que l'acidification des océans, l'élévation des températures des eaux de surface ainsi que les variations de salinité des eaux côtières sur le transfert trophique des métaux chez les poissons. Cependant, jusqu'à présent, cette catégorie de facteurs n'a été que très peu considérée en écotoxicologie des métaux (**Fig. 3**). Dans ce contexte, les objectifs de cette thèse sont :

1) d'apporter de nouvelles informations ainsi qu'une validation expérimentale de méthodologies clés dans l'étude en conditions contrôlées du transfert trophique des métaux chez les poissons ;

2) d'étudier d'étudier notamment l'influence de la ressource trophique, de l'espèce, du stade physiologique et de la taille sur l'assimilation des métaux chez différentes espèces de poissons marins ;

3) d'étudier l'influence de facteurs environnementaux clés tels que le pH, la température, ou encore la salinité sur l'assimilation des métaux chez plusieurs espèces de poissons marins.

Cette thèse s'articule en plusieurs parties. L'approche méthodologique adoptée dans ce travail est présentée dans le chapitre 2 qui inclut les principales conclusions d'une revue de la littérature concernant l'efficacité d'assimilation des métaux chez les poissons (**Article 1**, voir Annexes) ainsi que les résultats d'un article méthodologique concernant la validation de l'approche dite du *single-feeding* utilisée lors de l'ensemble des expériences réalisées durant cette thèse (**Article 2**). Les chapitres 3 et 4 constituent des parties intégratives qui visent à présenter et à discuter des principaux résultats respectivement concernant l'influence des facteurs biologiques (**Articles 3** à **6**) et des facteurs environnementaux (**Articles 7** à **9**) obtenus au cours de ce travail de thèse, y compris les résultats des travaux non publiés. Les principales conclusions et perspectives mise en évidence durant cette thèse (notamment au travers des résultats rassemblés dans l'**Article 10**) seront présentées au chapitre 5.

Chapitre 2
Approches méthodologiques

CHAPITRE 2
APPROCHES MÉTHODOLOGIQUES

2.1. LES AVANTAGES DES EXPÉRIENCES EN CONDITIONS CONTROLÉES

La compréhension du transfert trophique des métaux dans les poissons est essentielle pour quantifier précisément leurs capacités d'accumulation (Luoma & Rainbow 2005). Ainsi le transfert trophique des métaux chez les poissons a été étudié dans des environnements naturels (e.g. Canli & Atli 2003, Copat et al. 2012, Metian et al. 2013), mais la relation entre la concentration des métaux dans les proies et la bioaccumulation par leurs prédateurs est difficile à établir dans ces conditions. En effet, il n'est pas possible, sur le terrain, d'isoler les différentes voies d'accumulation des métaux (i.e. par l'eau, le sédiment ou la nourriture). Seules des expériences menées en laboratoire permettent d'isoler la voie alimentaire comme unique source d'exposition aux métaux permettant une quantification précise du transfert trophique car les doses ingérées et retenues peuvent être mesurées. Par ailleurs, en laboratoire, il est possible de contrôler les conditions du milieu et ainsi faire varier les valeurs prises par les différents facteurs testés susceptibles d'influencer le transfert trophique des métaux chez les organismes vivants. Il peut s'agir par exemple de facteurs biologiques tels que l'ingestion de différentes nourritures ou encore de facteurs environnementaux tels que le pH ou la température. Pour ces derniers, le contrôle des conditions présente également l'avantage de pouvoir simuler les variations prévues pour les siècles à venir par les modèles tels que ceux utilisés par le Groupe d'experts Intergouvernemental sur l'Evolution du Climat (GIEC) et ainsi mieux appréhender les effets potentiels des changements globaux sur les organismes vivants. Ainsi, l'approche expérimentale dans des conditions contrôlées, même si elle ne peut prétendre reflèter la complexité des relations observées au sein d'un écosystème naturel, apparaît comme une excellente option pour évaluer sans ambiguïté le transfert trophique des métaux chez les poissons. C'est pourquoi ce type d'approche a été retenu dans le cadre des travaux présentés dans ce manuscrit (**Articles 2** à **10**).

2.2. L'EFFICACITÉ D'ASSIMILATION (AE) : UN PARAMÈTRE CLÉ DANS L'ÉTUDE DU TRANSFERT TROPHIQUE DES MÉTAUX

2.2.1. L'assimilation et sa détermination expérimentale

L'un des paramètres les plus pertinents pour quantifier le transfert trophique des métaux chez les poissons est l'efficacité d'assimilation (AE pour *Assimilation Efficiency*). L'AE est un paramètre physiologique qui présente l'avantage de pouvoir être comparé quantitativement entre les métaux, les espèces de poissons, les aliments utilisés et les conditions environnementales (Wang & Fisher 1999, Croteau et al. 2007). Ces raisons expliquent pourquoi ce paramètre est largement utilisé dans les études d'écotoxicologie. Cependant, la définition de ce concept apparaît parfois peu claire dans la littérature. En effet, comme l'ont déjà souligné Wang & Fisher (1999), il existe encore des divergences dans les études expérimentales concernant la définition de l'AE. Selon ces auteurs, « Dans les études bioénergétiques, l'absorption d'un élément ou d'un composé équivaut à l'ingestion totale de la substance moins sa quantité dans la matière fécale et est la somme de l'assimilation et de l'excrétion soluble » (Wang & Fisher 1999). C'est cette définition que nous retiendrons dans ce manuscrit (**Article 1**). Il est important de différencier l'efficacité d'absorption de l'efficacité d'assimilation, qui sont respectivement la fraction du composé ingéré qui traverse l'épithélium intestinal, et le résultat de la différence entre l'absorption et l'excrétion, i.e. le processus par lequel les déchets métaboliques - endogènes - et autres matériaux non utiles - exogènes - sont éliminés d'un organisme (Brett & Groves 1979). Cette définition de l'AE est conforme à Warnau et al. (1996b) qui, en substance, indique que l'AE peut être définie comme la fraction du matériau ingéré qui est étroitement liée (i.e. incorporée) dans les organes et les tissus d'un organisme donné. D'un point de vue théorique, la différence entre absorption et assimilation est claire, mais dans la pratique, il est extrêmement difficile de dissocier ces deux mécanismes au niveau de l'organisme entier.

Pour déterminer correctement l'AE, il est également important de considérer un autre paramètre physiologique : le temps de transit intestinal (GTT pour *Gut Transit Time*). En effet, c'est au cours de cette phase, qui est, par définition, le temps de passage d'un aliment ingéré dans le tube digestif, que l'absorption des métaux a lieu. Ainsi, certains auteurs utilisent ce paramètre physiologique pour déterminer le temps requis pour évaluer avec précision l'AE. Cette méthode présente certaines limites qui doivent être prises en compte.

Toutefois, pendant le GTT, il est extrêmement difficile de garantir que seule l'absorption des composés ingérés a lieu. En effet, les éléments absorbés, tels que les métaux, sont transportés par la circulation sanguine jusqu'au foie et peuvent être excrétés rapidement (i.e. durant le GTT) directement par la bile ou encore par les branchies et les reins (Wood 2011). Cette situation pourrait donc avoir une incidence sur l'exactitude de la détermination de l'AE (**Article 1**). De plus, certaines observations indiquent que l'élimination peut se produire ultérieurement par les sucs digestifs ou encore dans des entérocytes détachés qui sont évacués par la voie intestinale (Wood 2011). En outre, il est possible qu'une partie de la fraction non assimilée ne soit pas évacuée et soit encore présente dans le mucus intestinal qui peut jouer un rôle régulateur dans l'absorption des éléments ingérés tels que les métaux (Bury et al. 2003). Cette situation pourrait donc avoir une incidence sur l'exactitude de la détermination de l'AE. Ce fait soulève la question cruciale de l'influence de la durée des expériences (i.e. le temps de dépuration après ingestion de nourriture). Ce point clé est traité plus en détails dans la section 2.5.

2.2.2. le calcul de l'efficacité d'assimilation (AE)

Deux méthodes sont couramment utilisées pour calculer l'AE des métaux chez les poissons à partir d'expériences menées avec des radiotraceurs émetteurs gamma. Pour les deux méthodes, la radioactivité dans les poissons est suivie après un nourrissage et la période de dépuration par comptages réguliers des animaux vivants.

Dans la première méthode, l'AE est déterminée à un moment donné (juste après le GTT) et exprimée comme étant le pourcentage de la fraction métallique ingérée initialement qui est retenu. Dans ce cas, la dépuration est généralement courte (i.e. quelques heures ou quelques jours). Cette méthode permet d'obtenir une réponse rapide quant au transfert trophique des métaux chez les poissons. La deuxième méthode est basée sur un suivi cinétique de la dépuration sur une plus longue période de temps (i.e. plusieurs semaines). Cette technique, largement utilisée dans les études en radioécologie, est couplée à l'utilisation de modèles non-linéaires qui permettent une description précise des différentes phases de la dépuration. Ainsi, la dépuration des métaux dans les poissons est exprimée comme étant le pourcentage de la radioactivité restante (radioactivité au temps t divisée par la radioactivité initiale mesurée dans l'organisme au début de la période de dépuration x 100).

Chez les poissons, il est habituellement possible de modéliser les cinétiques obtenues par un modèle non-linéaire à deux composantes exponentielles :

$$A_t = A_{0s} x\ e^{-k_{es}\ x\ t} + A_{0l} x\ e^{-k_{el}\ x\ t}$$
$$\text{avec } A_{0l} = AE$$

Où A_t et A_0 sont les activités restantes (%) aux instants t et 0; k_e est la constante de dépuration (i.e. efflux rate constant) exprimée en j^{-1}. Les indices «s» et «l» sont liés respectivement à la composante décrivant la perte rapide (i.e. *short-term*) et à la composante décrivant la perte lente (i.e. *long-term*). La composante «s» représente la dépuration de la fraction de radiotraceur faiblement associée aux organismes et éliminée rapidement (c'est-à-dire la proportion éliminée par les fécès). La composante «l» décrit la dépuration de la fraction de radiotraceur qui est effectivement absorbée par l'organisme et éliminée lentement (Hubbell et al. 1965, Reichle 1967, Reichle et al. 1970, Whicker & Schultz 1982). C'est cette dernière qui permet d'estimer l'efficacité d'assimilation (ainsi AE = A_{0l}; Reichle 1967, Fowler & Guary 1977, Miramand et al. 1982). Dans certains cas, la perte de la fraction métallique assimilée peut être extrêmement lente (**Articles 2-4**, **7** et **8**) ce qui se traduit mathématiquement par une valeur de k_{el} qui n'est pas significativement différente de 0. Ainsi la modélisation de la cinétique de perte peut être simplifiée et l'équation devient :

$$A_t = A_{0s} x\ e^{-k_{es}\ x\ t} + AE$$

Cette méthode nécessite une durée de dépuration suffisamment longue pour obtenir une détermination précise de la phase de perte lente. Habituellement, dans cette méthode, la dépuration du poisson dure plusieurs semaines. Étant donné que tous les processus d'excrétion (urinaire, branchiale et biliaire) sont pris en compte, c'est la méthode la plus robuste pour déterminer avec précision l'AE et c'est pourquoi elle a été retenue dans le cadre des travaux menés durant cette thèse.

2.3. LA SPECTROMÉTRIE GAMMA : UN OUTIL PERTINENT POUR LA DÉTERMINATION DE L'AE DES MÉTAUX

Par rapport aux approches classiques, la spectrométrie gamma offrent plusieurs avantages. En effet, l'utilisation de radiotraceurs à émission gamma permet le comptage régulier de même organismes vivants sur des périodes d'observation relativement longues (supérieures à un mois ; e.g. Hédouin et al. 2010a, Metian et al. 2016, Kuranchie-Mensah et al. 2016) de sorte que la quantité totale de radioactivité ingérée et la perte subséquente observée chez les organismes peuvent être quantifiées avec précision. Cette technique ne nécessite pas une récupération complète des matières fécales. Ainsi, il est possible de générer des données plus rapidement avec une variabilité biologique réduite et de diminuer sensiblement le nombre d'organismes expérimentaux devant être sacrifiés. En raison de la sensibilité extrêmement élevée de cette technique de mesure, la spectrométrie gamma permet également d'effectuer des expériences de bioaccumulation dans des conditions d'exposition réalistes, c'est-à-dire en utilisant des concentrations faibles, aux mêmes niveaux que les concentrations naturelles des métaux dans l'environnement (Warnau & Bustamante 2007), ce qui permet de ne pas entraîner d'effets toxiques sur les organismes suivis et de ne pas perturber les propriétés physico-chimiques du milieu expérimental. Cette technique s'avère tout à fait appropriée pour étudier les paramètres cinétiques de bioaccumulation des métaux.

Dans le cas de l'étude d'un transfert trophique des métaux, ces paramètres sont le taux de perte et l'efficacité d'assimilation. Grâce à ces avantages uniques, la technique de spectrométrie gamma est largement appliquée en écotoxicologie marine expérimentale sur de nombreux organismes (e.g. Warnau et al. 1996a, Bustamante et al. 2002, Metian et al. 2009, Wang et al. 2012). Dans les travaux réalisés durant cette thèse, le transfert trophique des métaux a donc été réalisé en faisant appel à cette technique. La radioactivité des traceurs a été systématiquement mesurée à l'aide d'un système de spectromètre gamma à haute résolution composé de 5 détecteurs équipés d'un crital de germanium permettant la détection simultanée de plusieurs radiotraceurs. La radioactivité dans les organismes vivants et les échantillons de tissus a été déterminée par comparaison avec des échantillons de référence (appelés « standards » ou « fantômes ») de géométrie appropriée et d'activité connue conçus pour étalonner les détecteurs. Les mesures ont été corrigées afin de tenir compte de la désintégration radiolabelled et du bruit de fond environnant.

Pour les comptages, les poissons vivants ont été placés dans des tubes de comptage de polystyrène cristal transparent (**Article 7** : 42 mm x 65 mm, **Article 2** à **5** et **10** : 80 mm x 50 mm, **Article 6** et **Articles 8** à **9** : 116 mm x 80 mm), remplis d'eau de mer non contaminée. Si nécessaire, des dispositifs pour limiter les mouvements des individus ont été ajoutés permettant de maintenir une géométrie constante pendant la période de comptage sans altérer le bien-être des organismes. Les durées de comptage (toujours <60 min) ont été maintenues aussi courtes que possible et ajustées pour obtenir une erreur de comptage <5% (e.g. Rodriguez y Baena et al. 2006). Afin de valider les conditions de comptages, des essais ont été systématiquement effectués avant les expériences. Pour cela, les organismes ont été placés dans des conditions de comptage similaires à celles appliquées en expérience afin d'observer leur comportement, c'est-à-dire dans une boîte de comptage pendant un maximum de 60 min dans l'obscurité. La concentration d'O_2 dissous a été suivie tout au long de ces essais et a toujours été >3 mg L^{-1}. Par ailleurs, aucune altération de la santé ou du comportement des organismes n'a été observée par la suite.

2.4. LA TECHNIQUE DU *SINGLE-FEEDING*

Pour mesurer l'AE, il est essentiel de quantifier avec précision la quantité totale de radioactivité ingérée par les animaux ce que permet de faire la technique de *single-feeding* (aussi appelée *pulse-chase feeding*). Dans cette technique, les organismes sont nourris avec un aliment radiomarqué pendant une courte période de temps (plus courte que le GTT) pour s'assurer que l'ingestion totale puisse être quantifiée avec précision. En effet, lorsque le temps d'alimentation est plus long que le GTT, la quantité de radioactivité ingérée peut être sous-estimée conduisant finalement à une surestimation de l'AE. Les animaux sont ensuite nourris avec des aliments non radioactifs dans des conditions identiques pour purger leurs intestins des matières radioactives non digérées. Après le *single-feeding* et régulièrement durant la dépuration, les organismes sont comptés pour déterminer la cinétique de dépuration des métaux ingérés. Dans une expérience de *single-feeding*, impliquant un nourrissage unique des organismes avec un aliment radiomarqué suivi d'une purge des matières ingérées avec des aliments non radioactifs, il est donc important que les organismes soient exposés à l'aliment radiomarqué pendant une période plus courte que le GTT.

Pour produire des aliments radioactifs destinés aux poissons, des proies naturelles (mollusques, crustacés, polychètes ou d'autres poissons) ou des aliments composés (i.e. granulés) sont typiquement radiomarqués par voie dissoute pendant une période de temps suffisamment longue pour obtenir un signal détectable dans les poissons prédateurs tout au long des expériences. En effet, l'utilisation de radioisotopes pour déterminer l'AE implique d'avoir des quantités suffisantes de radioactivité dans les organismes pour permettre des mesures précises en des temps de comptage raisonnablement courts (< 60 min). La nourriture est systématiquement rincée dans une eau non-contaminée afin d'éliminer les radiotraceurs faiblement liés qui pourraient être désorbés lors du nourrissage. Le rinçage préalable permet donc de garantir que l'exposition des organismes prédateurs aux radiotraceurs se fait uniquement par la nourriture. Un schéma du protocole expérimental est présenté ci-après (**Fig. 4**). La technique du *single-feeding* suppose que le processus d'assimilation n'est pas affecté par la fréquence d'ingestion de nourriture. Afin de tester cette hypothèse et ainsi apporter une validation expérimentale de l'approche *single-feeding*, deux expériences ont été conduites en parallèle où des juvénile de turbots *Scophthalmus maximus*, maintenus dans des conditions de laboratoire contrôlées, ont été exposés aux métaux par un (*single-feeding*) ou quatre nourrissages (*multi-feeding*) avec des granulés radiomarqués (^{54}Mn, ^{57}Co, ^{65}Zn et ^{109}Cd, **Article 2**). Les données cinétiques issues de l'expérience de *single-feeding* ont ensuite permis de reconstituer un *multi-feeding* théorique basé sur l'hypothèse d'une AE constante pour chacun des nourrissages. Les résultats obtenus indiquent que la reconstruction d'un *multi-feeding* théorique est cohérente avec les données fournies par le *multi-feeding* effectué dans les mêmes conditions. Ces résultats fournissent une validation expérimentale de l'approche de *single-feeding*. Il serait cependant intéressant d'étudier l'influence de nourrissages répétés sur une plus longue période de temps afin de confirmer les tendances observées sur quatre nourrissages.

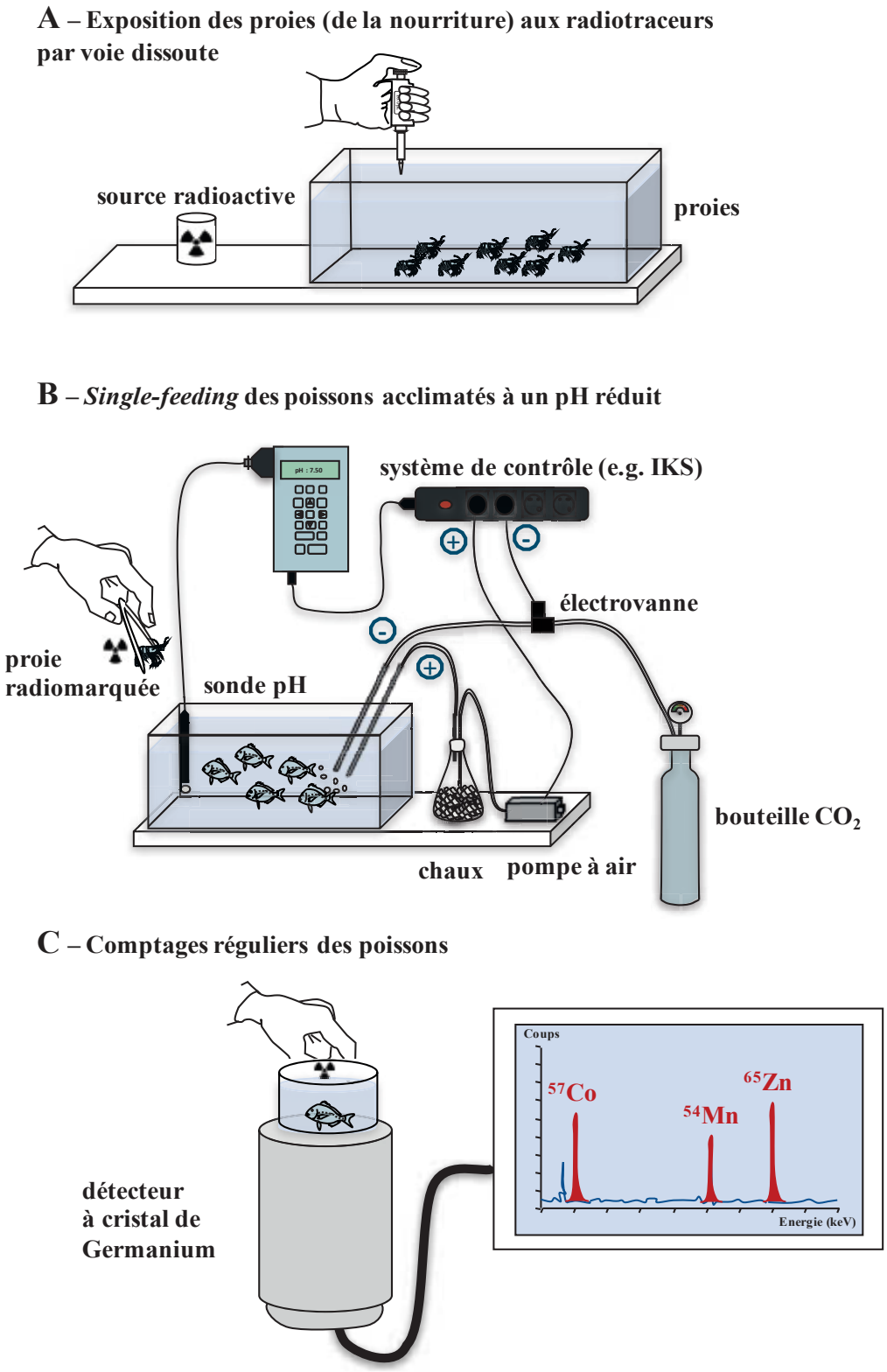


Figure 4. Schéma simplifié d'un protocole expérimental de *single-feeding* basé sur l'utilisation de radiotraceurs. Ici est illustré comme exemple une expérience fictive visant à étudier l'effet d'une réduction de pH de l'eau sur le transfert trophique d'éléments traces essentiels (Co, Mn et Zn). Plus de de précisions concernant les méthodologies utilisées pour le contrôle et la régulation des facteurs étudiés sont fournies dans les sections « Materials and Methods » des articles disponibles en Annexes (voir **Articles 2-9**).

2.5. LA DURÉE DE LA DÉPURATION : UN CHOIX MÉTHODOLOGIQUE CRUCIAL

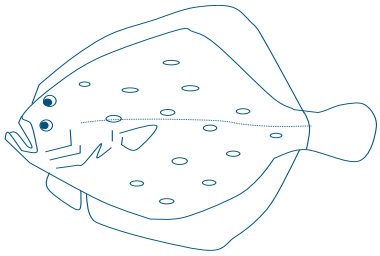
Chez les poissons, la dépuration peut être habituellement décrite en trois phases différentes. La première phase (habituellement quelques heures après l'alimentation), est très courte car elle correspond au passage de l'aliment ingéré de l'estomac à l'intestin où le processus d'absorption a lieu. Par la suite, les éléments non absorbés sont évacués. La deuxième phase (habituellement dans la première semaine de dépuration, Baines et al. 2002, Dutton & Fisher 2011, **Articles 2** à **9**) est caractérisée par les processus d'absorption et d'excrétion rapide. Au cours des deux premières phases, comme nous l'avons démontré dans l'**Article 5** en réalisant des dissections successives dans le temps chez deux espèces de poissons tropicaux, la quasi-totalité des métaux ingérés se trouvent dans l'estomac et l'intestin. Enfin, la troisième phase se caractérise essentiellement par les échanges des métaux absorbés entre les différents organes et tissus. La perte est, pendant cette phase plus lente et la quantité de métaux présente dans le corps, provenant de la nourriture radiomarquée ingérée initialement, se stabilise. De par la complexité de la dépuration chez les poissons, qui comprend plusieurs phases distinctes où de nombreux processus biologiques prennent place, le choix de la durée des expériences apparaît décisif pour déterminer précisément l'AE. En effet, il est nécessaire d'avoir une période de dépuration suffisamment longue pour couvrir les processus d'absorption et d'excrétion. Comme expliqué dans la section 2.2, le GTT peut être utilisé pour définir le temps de dépuration retenu par les expérimentateurs. En radioécologie, certains auteurs estiment un GTT par la collecte de matières fécales radioactives. Dans ce cas, le GTT est défini comme le moment où les dernières matières fécales radioactives ont été collectées. Ainsi, la durée de dépuration est choisie pour couvrir le GTT (i.e. dans presque tous les cas une durée variant de 24 à 72 heures ; e.g. Xu & Wang 2002, Van Campenhout et al. 2007). Avec cette méthode, pouvant être qualifiée d'approche « court terme », l'estimation de la fraction restante des métaux ingérés ne tient pas compte de tous les processus d'excrétion qui se sont produits au cours de la troisième phase de la dépuration. Habituellement, dans l'approche « court terme », l'AE est déterminée à un instant donné et exprimé en pourcentage de métaux retenus. Cette approche ne permet pas de décrire la cinétique de la troisième phase de dépuration comme cela est fait habituellement lorsque la durée de la dépuration s'étend sur plusieurs semaines (approche « long terme »).

Afin de comparer les AE obtenues à l'aide des approches « court terme » et « long terme », à partir des données fournies issues du **Supplementary Data** de l'**Article 2**, une comparaison statistique (test non paramétrique de Wilcoxon-Mann-Whitney) a été effectuée sur les activités restantes de juvéniles de turbot *S. maximus* nourris avec des granulés radiomarqués avec du ^{54}Mn. Cette comparaison révèle qu'à partir de deux jours de dépuration (c'est-à-dire moins de 24h après le GTT) et jusqu'à 21 jours (approche « long terme »), l'activité restante est stable ($p > 0,05$). Néanmoins, la comparaison statistique révèle une différence significative ($p = 0,04$) entre les AE individuelles estimées comme étant le pourcentage de l'activité restante après deux jours (approche « court terme ») et les AEs individuelles obtenues en ajustant un modèle (approche « long terme »). Cet exemple montre que, sur un ensemble de données, les approches « court terme » et « long terme » peuvent conduire à des estimations d'AE légèrement différentes. Un tel biais peut être évité en utilisant une période de dépuration suffisamment longue qui englobe à la fois les processus d'absorption et d'excrétion et permet une détermination précise de l'AE. Dans l'approche de dépuration « court terme », une partie des processus d'excrétion intervenus au cours de la dernière phase de la dépuration est supposée négligeable, ce qui n'est pas toujours vrai. L'approche « court terme » ne peut donc être envisagée qu'après une investigation des processus de dépuration dans des conditions expérimentales données. Pour l'ensemble de ces raisons, c'est une approche « long terme » qui a été adoptée pour l'ensemble des expériences présentées dans cette thèse.

2.6. LES MODÈLES BIOLOGIQUES CHOISIS

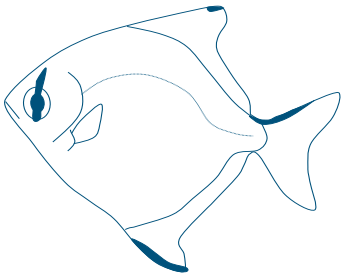
Plusieurs espèces de poissons ont été considérées dans les travaux présentés dans ce manuscrit en fonction des questions traitées. Pour chaque espèce étudiée, une description rapide est fournie ci-après accompagnée d'une justification du choix de chaque modèle biologique.

Le turbot *S. maximus* est une espèce de la famille des Scophthalmidés et de l'ordre des Pleuronectiformes. Cette espèce de poisson plat vit dans les eaux côtières européennes soumises à des variations des conditions environnementales (température, pH, salinité...) et sensibles aux activités anthropiques. Par ailleurs, il s'agit d'un prédateur de niveau trophique de 4,4 ± 0,0 (Froese & Pauly 2017) se nourrissant d'une grande variété de proies (crustacés, polychètes et autres poissons ; Sparrevohn & Støttrup 2008, Florin & Lavados 2010).

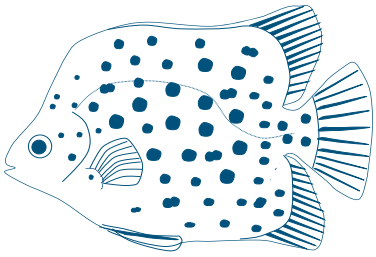


Nom scientifique : *S. maximus*
Nom commun : turbot
Stade(s) utilisé(s) : juvéniles
Masse : 10 - 45 g

Cette espèce est consommée par l'Homme et est produite à grande échelle en aquaculture (72 000 tonnes produites dans le monde en 2014, FAO 2017). L'utilisation de poissons d'aquaculture présente par ailleurs plusieurs avantages. En effet, les traits biologiques et la génétique de ces individus, élevés depuis plusieurs générations en captivité, sont bien connus. Par ailleurs, de tels individus supportent mieux le stress généré par les manipulations lors des expériences que les individus sauvages. L'ensemble des raisons évoquées précédemment alliées à sa disponibilité sur le marché, et un régime alimentaire varié (des granulés à une large variété de proies naturelles) expliquent qu'il s'agit de l'espèce la plus étudiée dans les travaux présentés dans ce manuscrit.

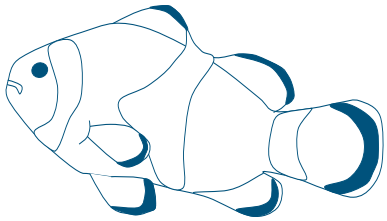


Nom scientifique : *M. argenteus*
Nom commun : poisson-lune argenté
Stade(s) utilisé(s) : juvéniles & adultes
Masse : 6 - 100 g



Nom scientifique : *S. argus*
Nom commun : pavillon tacheté
Stade(s) utilisé(s) : juvéniles & adultes
Masse : 3 - 200 g

Le poisson-lune argenté *Monodactylus argenteus* et le pavillon tacheté *Scatophagus argus* sont deux espèces appartenant toutes deux à l'ordre des Perciformes et respectivement à la famille des Monodactylidés et des Scatophagidés. Ces deux espèces vivent dans les zones côtières et plus particulièrement les mangroves dans le Sud-Est asiatique. Il s'agit de deux espèces omnivores opportunistes (niveaux tropiques de 3,0 ± 0,33 et de 3,0 ± 0,35 respectivement pour *M. argenteus* et *S. argus*, Froese & Pauly 2017) dont l'écologie trophique est très proche ce qui en fait des candidats intéressants pour les expériences de comparaisons interspécifiques. Par ailleurs, l'utilisation d'espèces tropicales répond également à un besoin de données sur le transfert de métaux et de radionucléides chez les organismes tropicaux afin de faire des comparaisons valables à l'échelle mondiale (Fowler & Fisher 2005).



Nom scientifique : *A. ocellaris*
Nom commun : poisson-clown
Stade(s) utilisé(s) : juvéniles
Masse : 0.3 - 0.5 g

Le poisson-clown à trois bandes *Amphiprion ocellaris* est une espèce appartenant à la famille des Pomacentridés et à l'ordre des Perciformes. Cette espèce vit dans les récifs coralliens du Sud-Est asiatique jusqu'au Nord de la Grande Barrière de Corail australienne. Il s'agit d'une espèce omnivore (niveau trophique de 2,8 ± 0,3 ; Froese & Pauly 2017) de plus en plus étudiée pour déterminer la sensibilité et la réponse physiologique des poissons coralliens face aux changements globaux.

2.7. LES RADIOTRACEURS ÉTUDIÉS

Les radioisotopes (ou isotopes radioactifs) ne diffèrent de leurs équivalents stables que par leur nombre de neutrons et possèdent donc les mêmes propriétés chimiques que ces derniers et c'est pourquoi leur utilisation est pertinente pour étudier le transfert trophique des métaux chez les organismes aquatiques et notamment les poissons. Une revue de la littérature en radioécologie relative au transfert trophique des métaux chez les poissons (**Article 1**) révèle qu'en excluant les travaux présentés dans ce manuscrit, il n'y a que peu d'études qui se sont focalisées sur les métaux essentiels tels que le Co et le Mn. Beaucoup d'études ont porté sur l'assimilation du Zn, souvent associé au Cd, un métal non-essentiel (e.g. Ni et al. 2000, Chan et al. 2003, Zhang & Wang 2007). Une des raisons au fait que ces deux éléments sont omniprésents dans la littérature est d'ordre méthodologique. En effet, en radioécologie, les principales raies d'émission des radioisotopes utilisées pour suivre le devenir de ces métaux se situent à des énergies très éloignées (respectivement 88,03 et 1115,55 keV pour le ^{109}Cd et le ^{65}Zn, **Tableau 2**) facilitant ainsi leur détection lors d'une même expérience. Ce constat est particulièrement vrai lors de l'utilisation de détecteurs à scintillation à iodure de sodium (NaI). Dans le cadre des travaux présentés dans ce manuscrit, les éléments étudiés étaient majoritairement des éléments essentiels (Co, Mn et Zn, **Tableau 2**) et des éléments non-essentiels (Cd et Ag, **Tableau 2**). Ce dernier élément, très étudié dans les années 1990 et au début des années 2000 (e.g. Garnier & Baudin 1990, Baudin & Garnier-Laplace 1994, Adam et al. 2002), fait l'objet d'un nouvel attrait dans la recherche en écotoxicologie lié notamment à l'utilisation de plus en plus conséquente de nanoparticules d'argent dans de nombreux secteurs d'activités (Savery et al. 2013).

Dans certains cas, en raison notamment de la difficulté d'obtenir un signal suffisant lors du premier comptage, les incertitudes de comptage se sont avérées trop élevées pour une utilisation des données et c'est pourquoi une sélection des éléments dont les données étaient valables a été systématiquement opérée avant traitement.

Tableau 2. Métaux étudiés dans les travaux présentés dans ce manuscrit et détails concernant les radioisotopes utilisés comme traceurs.

Elément		Radioisotope	Temps de demi-vie (j)	Energie (keV)	% Emission gamma
Ag	Argent	^{110m}Ag	252	88,03	3,65
Cd	Cadmium	^{109}Cd	464	122,06	85,68
Co	Cobalt	^{57}Co	271	657,76	94,37
Mn	Manganèse	^{54}Mn	312	834,84	99,98
Zn	Zinc	^{65}Zn	244	1115,55	50,75

Chapitre 3
Influence des facteurs biologiques

CHAPITRE 3 : INFLUENCE DES FACTEURS BIOLOGIQUES

La sélection des différents facteurs biologiques susceptibles d'affecter le transfert trophique des métaux chez les poissons a été faite afin de couvrir un large éventail de variables à la fois au niveau de la ressource alimentaire (proies naturelles et aliment d'aquaculture) et des consommateurs. Ainsi les facteurs suivants ont été considérés dans les travaux présentés dans le contexte de cette thèse, certains concernent la ressource alimentaire et d'autres les consommateurs :

• Facteurs liés à la ressource :

- Type de nourriture
- Présence de biotoxines dans la nourriture

• Facteurs liés aux consommateurs :

- Variations interspécifiques
- Stades physiologiques
- Taille des organismes (i.e. âge)

3.1. L'ASSIMILATION DES MÉTAUX DÉPENDANTE DU TYPE DE NOURRITURE

3.1.1. Etat de l'art

De nombreuses études ont mis en évidence que le type de nourriture influence l'AE des métaux chez des poissons. A titre d'exemple, Dutton & Fisher (2011) ont démontré que l'AE du choquemort *Fundulus heteroclitus heteroclitus* pour le Hg pouvait être multipliée par 2 en fonction du type de proies ingérées (un amphipode benthique *Leptocheirus plumulosus* ou un oligochète *Lumbriculus variegatus*). De nombreuses études ont conduit également à l'obtention d'AEs très variables en fonction du type de nourriture pour de nombreux métaux essentiels (Cu et Zn) et non-essentiels (Cd et Hg qu'il soit sous forme inorganique -Hg^{2+}- ou organique -MeHg-) chez d'autres espèces marines telles que le pagre à tête noire *A. schlegeli* (Dang et al. 2009, Wang et al. 2012), le vivaneau des mangroves *Lutjanus argentimaculatus* (Xu & Wang 2002) ou encore le gaterin noir *Plectorhinchus gibbosus* (Wang & Wong 2003). Cependant, dans la majorité des études, les proies utilisées ne correspondent pas toujours aux proies naturelles ingérées par les espèces étudiés comme le reflète l'utilisation d'artémies *Artemia salina* comme aliment radiomarqué (e.g. Ni et al. 2000, Xu & Wang 2002). Par ailleurs, pour une même espèce, il est rare que les différents taxons de proies utilisés en expérience reflètent la diversité de son régime alimentaire naturel. Malgré ces lacunes, notamment identifiées dans l'**Article 1**, le type de nourriture reste un des facteurs influençant l'AE des métaux chez les poissons les plus étudiés. Au vu de l'ensemble de ces résultats, certains auteurs ont tenté d'identifier, par une approche mécanistique, quels étaient les facteurs dans la nourriture pouvant expliquer de telles différences.

Ainsi, il existe différentes hypothèses rapportées dans la littérature scientifique pour expliquer les différences d'AEs observées. La première hypothèse suppose que l'AE du prédateur peut être estimée à partir du pourcentage de métal contenu dans les parties molles (i.e. sans le squelette, l'exosquelette ou la coquille) des proies ingérées (Reinfelder & Fisher 1994). Une telle relation a été rapportée pour le Cd, le Co, le Se et le Zn chez les capucettes *Menidia menidia* et *M. beryllina* nourris avec du zooplancton. La deuxième hypothèse suppose que la proportion de métaux biodisponibles pour les prédateurs est liée à la quantité de métal associée à la fraction cytosolique des proies (Wallace & Lopez 1996). Plus récemment, les mêmes auteurs ont démontré chez une espèce de crevette *Palaemon macrodatylus*, que la fraction de Cd assimilée est équivalente à la quantité de métaux contenue dans le cytosol et les organelles des bivalves servant de nourriture. C'est ainsi que la notion de *Trophic Available Metal* ou TAM a été définie (Wallace & Luoma 2003). Concernant les poissons, Zhang & Wang (2006) ont trouvé une relation positive entre la TAM de plusieurs types de nourriture (copépodes, bivalves, viscères de poissons et zooplancton) et l'AE du Zn et du Se chez le térapon *T. jarbua*. Néanmoins, aucune équivalence stricte entre la TAM des proies et l'AE des métaux de leurs consommateurs n'a été démontré chez les poissons contrairement aux invertébrés. Ainsi, la détermination de la fraction biodisponible des métaux d'un niveau trophique à un autre est encore intensément étudiée ou discutée dans la littérature scientifique (e.g. Rainbow et al. 2011, 2015).

3.1.2. Objectifs et hypothèses de recherche

L'objectif principal a été d'étudier l'influence du régime alimentaire sur l'AE des métaux d'un poisson marin de haut niveau trophique (4,4 ± 0,0 ; Froese & Pauly 2017), le turbot *Scophthalmus maximus*. Les AEs de métaux essentiels (Co, Mn et Zn) et d'un métal non-essentiel (Ag) ont été mesurées chez le turbot *S. maximus*, nourris avec trois proies naturelles différentes (crustacé, poisson et polychète) reflètant son régime alimentaire naturel (Sparrevohn & Støttrup 2008, Florin & Lavados 2010) ainsi qu'un aliment composé utilisé en aquaculture de cette espèce. En complément de l'estimation des AEs, des dissections des différentes proies radiomarquées ont été réalisées afin d'isoler les parties molles des parties dures (squelette et exosquelette) ainsi qu'un fractionnement subcellulaire des métaux contenu dans les différents aliments a également été effectué. Ainsi, il a été possible de tester les hypothèses suivantes :

1) Les AEs d'un poisson sont corrélées à la quantité de métaux dans les parties molles des proies (Reinfelder & Fisher 1994) ;

2) La proportion de métaux biodisponibles pour les consommateurs est liée à la quantité de métal associée à la fraction cytosolique des proies (Wallace & Lopez 1996) ;

3) La TAM des proies reflète l'AE chez le consommateur (Wallace & Luoma 2003).

3.1.3. Résultats et Discussion

Les cinétiques de dépuration du Co, du Mn et du Zn ayant permis la détermination des efficacités d'assimilation (AEs) chez le turbot ont été ajustées par un modèle exponentiel comprenant une constante (voir section 2.2.2, R^2= 0,76-0,98). L'AE est dépendante du métal et du type de nourriture (**Fig. 5**). L'AE du Co varie significativement entre les types de nourriture. Ce métal est peu assimilé dans le cas d'un nourrissage avec des granulés et des vers radiomarqués (AE = 2,4 ± 1,4% et 5,1 ± 1,1%, respectivement). A l'inverse, l'AE est élevée lorsque les turbots sont nourris avec des poissons (AE = 43,1 ± 12,0%), et une situation intermédiaire est observée lorsqu'ils sont nourris avec des crevettes (AE = 16,3 ± 4,0%). L'AE du Mn est également variable selon le type de nourriture ingéré, mais dans une moindre mesure. En effet, l'AE de ce métal est significativement plus faible (p <0,001) lorsque les turbots ont été nourris avec des granulés et des poissons radiomarqués (AE = 24,8 ± 7,4% et 23,0 ± 7,7%) que lorsqu'ils sont nourris avec des crevettes ou des vers (AE = 42,0 ± 6,6% et 43,7 ± 2,3%, respectivement). La variation d'AE a été moins prononcée pour le Zn. Pour cet élément, les AEs sont comparables lorsque les turbots sont nourris avec des granulés et des vers (AE = 16-18%, **Fig. 5**).

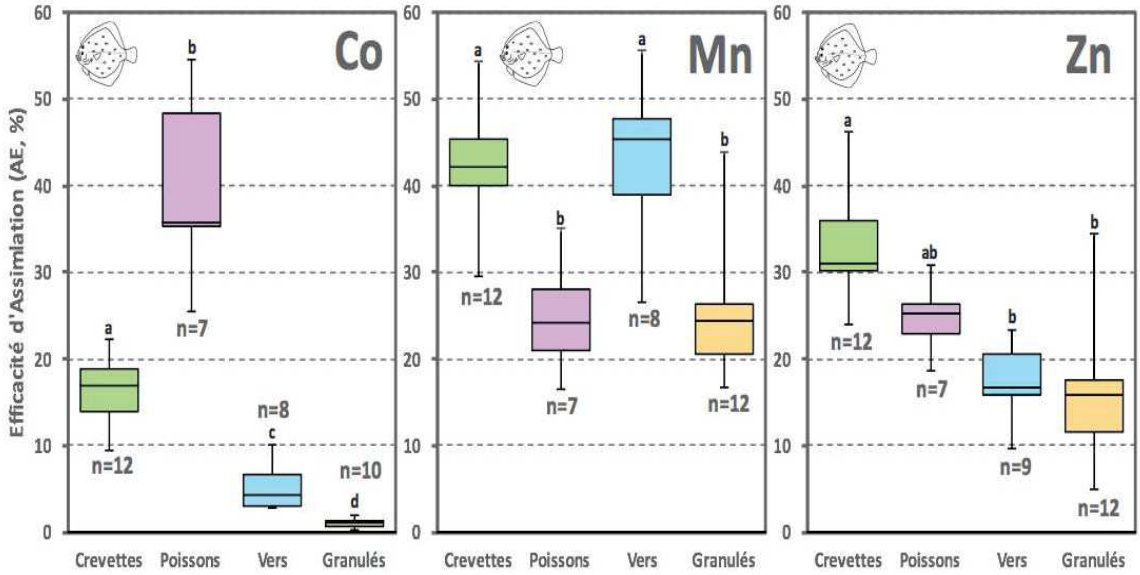


Figure 5. Comparaison des efficacités d'assimilation (AEs) calculées pour chaque individu du Co, du Mn et du Zn chez des turbots juvéniles après un *single-feeding* avec des crevettes, des poissons, des vers et des granulés radiomarquées. Les lettres désignent les différences significatives (p<0,05). Données issues de l'**Article 3**.

Le Zn est toutefois plus efficacement assimilé lors d'un nourrissage avec des crevettes (AE = 32,2 ± 6,0%). L'AE du Zn est intermédiaire lorsque les turbots sont nourris avec des poissons (AE = 24,8 ± 3,3%). La dépuration du Ag n'a pu être décrite que dans le cas d'un nourrissage des turbots avec des granulés et des vers radiomarquées. Les cinétiques ont été ajustées par un modèle exponentiel comprenant une constante (R^2=0,99).

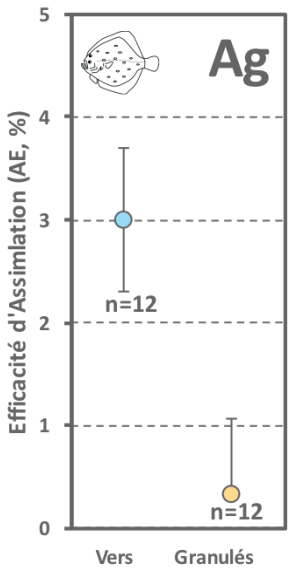


Figure 6. Comparaison des efficacités d'assimilation (AEs, moyennes ± écarts-types), du Ag chez des turbots juvéniles après un *single-feeding* avec des granulés et des vers radiomarqués. Données issues de l'**Article 4**.

Pour l'Ag, lorsque les turbots ont été nourris avec des poissons et des crevettes, les activités ingérées ont être mesurées avec précision pour chaque individu lors du premier comptage. Toutefois, après 48 h de dépuration, les incertitudes des comptages sont devenues très élevées (> 20%). Après deux semaines, les activités étaient inférieures aux limites de détection. Par conséquent, la cinétique de dépuration ne pouvait pas être modélisée pour ces deux types de nourriture. Un suivi cinétique a cependant pu être effectué lorsque les turbots ont été nourris avec des granulés et des vers. Dans ces deux cas, l'Ag n'a que très peu été assimilé par les turbots (AE <4%, **Fig. 6**).

Nos résultats montrent ainsi que les efficacités d'assimilation (AE) dépendent des métaux. Par ailleurs, excepté dans le cas de l'Ag, très peu assimilé par les poissons quel que soit le type de nourriture, les AEs sont affectées par le type de nourriture. Il est bien documenté dans la littérature que les formes de stockage et la localisation des métaux dans la nourriture déterminent la biodisponibilité de ces éléments pour les prédateurs (e.g. Reinfelder & Fisher 1994, Wallace & Lopez 1996, Wallace & Luoma 2003, Meyer et al. 2005) et ont ainsi une incidence sur l'AE. Dans cette étude, aucune relation claire n'a été détectée entre les AEs et le fractionnement des métaux dans la proie au niveau des organes et tissus (dissection) ou au niveau subcellulaire (fractionnement).

Par conséquent, de tels résultats ne soutiennent pas les hypothèses de Reinfelder & Fisher (1994) et de Wallace & Lopez (1996) dans le cas du turbot. En effet, pour le Co et le Zn, les valeurs d'AE pour le prédateur étaient inférieures à celles attendues des deux hypothèses qui admettent l'équivalence entre AE et la fraction métallique dans les parties molles ou dans le cytosol des proies. La fraction de Co et Zn contenue dans les compartiments présumés biodisponibles de la proie (c'est-à-dire les tissus mous et la fraction soluble) n'a pas été assimilée en totalité par le turbot. L'expérience réalisée avec les granulés conduit à des conclusions similaires. En effet, dans ce cas, les radiotraceurs n'ont pas été biologiquement mais physiquement et chimiquement liés à la matrice alimentaire. Ainsi, les radiotraceurs se trouvent virtuellement dans leur totalité sous une forme « soluble » supposée biodisponible. Or, malgré cela, une partie des radiotraceurs n'est pas assimilée par les juvéniles de turbot nourris avec cette matrice alimentaire. En d'autres termes, la théorie de la « fraction TAM » ne permet pas d'expliquer les différences observées. Une explication potentielle pourrait venir de l'écologie du turbot. En effet, la température optimale du turbot juvénile (environ 15°C pour des individus de la taille utilisée pour cette expérience) est inférieure à la température utilisée lors des précédentes expériences réalisées avec d'autres modèles biologiques (*Menidia* sp. et *Terapon jarbua*, respectivement maintenues à 18 et 20°C). Compte tenu de la relation positive entre la température et l'activité des enzymes digestives chez les poissons (Xiong et al. 2011), une faible température peut conduire à une plus faible activité enzymatique chez le turbot pouvant expliquer qu'une partie de la fraction supposée biodisponible ne soit pas assimilée. Fait intéressant, nos résultats indiquent que le Mn est le seul élément essentiel pour lequel les AEs mesurées, lorsque les juvéniles turbots ont été nourris avec des crevettes ou des poissons, étaient plus élevées que ce qui était attendu par la théorie. Dans ce cas particulier, la fraction métallique contenue dans le cytosol est inférieure la fraction assimilée par le turbot (% soluble <AE). Par conséquent, la théorie de TAM (i.e. cytosol+organites cellulaires) parait cohérente bien que nos résultats ne puissent le prouver. Par ailleurs, nos données suggèrent que le Mn associé à d'autres compartiments cellulaires insolubles présents dans les tissus mous des proies peuvent être assimilés par le turbot comme cela l'a déjà été démontré chez des invertébrés prédateurs (i.e. néogastropodes nourris avec plusieurs espèces de mollusques et de crustacés ; Cheung & Wang 2005, Rainbow et al. 2007). Dans ce contexte, d'autres études sont nécessaires pour évaluer quelles sont les parties des compartiments de la fraction insoluble (qui comprend les organites, les débris cellulaires et les granules riches en métaux) doivent être prise en compte pour évaluer avec précision la fraction réellement biodisponible du Mn pour un poisson prédateur comme le turbot.

3.2. COMPARAISON DE L'ASSIMILATION DES MÉTAUX CHEZ DES ESPÈCES D'ÉCOLOGIE TROPHIQUE PROCHE

3.2.1. Etat de l'art

Comme l'a révélé l'analyse de la littérature disponible sur l'AE des métaux chez les poissons (**Article 1**), il existe des variations considérables de ce paramètre pour un même élément en fonction des espèces étudiées. Ainsi, Ni et al. (2000) ont mis évidence que l'AE du Zn est jusqu'à 4 fois plus élevée chez le poisson de verre *Ambassis urotaenia* que chez le périophthalme *Periophthalmus modestus* nourri avec les mêmes proies radiomarquées. Une autre étude expérimentale a mis en évidence une AE du Mn deux fois plus élevée chez le turbot *Scophthalmus maximus* comparée à l'AE de ce même élément chez la daurade *Sparus aurata* nourri avec la même proie (Mathews et al. 2008). Toutefois, la compréhension des facteurs à l'origine des différences interspécifiques d'AEs des métaux reste encore limitée. Parmi ces facteurs, les stratégies d'alimentation ont été jugées cruciales dans l'assimilation différentielle des métaux chez des espèces vivant au sein d'un même écosystème (e.g. Ni et al. 2000) se traduisant notamment par des compositions d'enzymes digestives différentes et, ainsi des différences dans leurs potentiels d'oxydoréduction (Mathews et al. 2008).

3.2.2. Objectifs de l'étude

Une comparaison de l'AE du Co et du Zn a été faite entre deux espèces vivant dans un même écosystème et ayant une écologie trophique proche a été réalisée. A cette fin, des juvéniles de deux espèces de poissons tropicaux omnivores, le poisson-lune argenté *Monodactylus argenteus* et le pavillon tacheté *Scatophagus argus* ont été exposés par un *single-feeding* à des artémies *Artemia salina* radiomarquées. La dépuration a ensuite été suivie afin de comparer les AEs des métaux étudiés chez les deux espèces.

3.2.3. Résultats et Discussion

Chez les deux espèces étudiées, les AEs ont été déterminées à partir d'un modèle exponentiel à deux composantes (voir section 2.2.2, R^2=0,94-0,99). L'AE est dépendante des métaux et des espèces. En effet, chez les deux espèces le Co est très peu assimilé (AE<6%). Aucune différence interspécifique n'a été observée pour ce métal ($p>0.05$). A l'inverse, le Zn est plus fortement assimilé avec une AE significativement plus élevée chez le pavillon tacheté *S. argus* (AE = 24,2 ± 1,0% contre 14,7 ± 0,9% pour le poisson-lune argenté *M. argenteus* $p<0,05$).

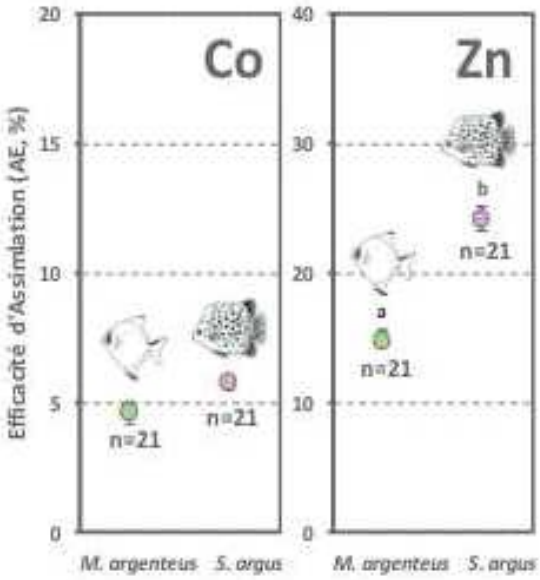


Figure 7. Comparaison des efficacités d'assimilation (AEs, moyennes ± écarts-types) du Co et du Zn chez le poisson-lune argenté *M. argenteus* et le pavillon tacheté *S. argus* après un *single-feeding* avec des artémies radiomarquées. Les lettres désignent les différences significatives (p<0,05). Données issues de l'**Article 5**.

Les résultats indiquent que les AEs du Co sont similaires pour les deux espèces étudiées tandis, qu'à l'inverse, des différences significatives ont été obvervées pour le Zn avec, pour ce métal, une AE plus élevée chez *S. argus*. Puisque les conditions d'exposition (i.e. paramètres de l'eau et nourriture) étaient identiques pour les deux espèces, parmi les nombreux paramètres influençant l'AE, seuls la physiologie digestive ou les mécanismes d'excrétion peuvent expliquer les différences observées. Or, bien que les deux espèces soient omnivores, comme l'indiquent leurs niveaux trophiques identiques (3,0 pour les deux espèces, (Froese & Pauly 2017), des différences existent dans leur écologie trophique. En effet, les analyses de contenus stomacaux indiquent une tendance détritivore marquée chez *M. argenteus* (Blaber 1980, Rainboth 1996), alors que *S. argus* se nourrit principalement de zoobenthos ou encore de poissons (Mills & Vevers 1989, Monkolprasit 1994, Kuiter & Tonozuka 2001, Allen et al. 2002). Les différences de comportement alimentaire suggèrent que la physiologie digestive peut également différer entre les deux espèces notamment par l'activité de certaines enzymes digestives. Par exemple, parmi six espèces de poissons, Hidalgo et al. (1999) ont démontré que l'amylase affichait une activité enzymatique plus élevée chez les espèces omnivores que chez d'autres espèces carnivores. Par ailleurs, Hofer & Schiemer (1981) ont également démontré que l'activité des enzymes protéolytiques, qui brisent les liaisons peptidiques des protéines, est plus élevée chez les espèces de poissons omivores et carnivores que chez les herbivores. Or, le Zn est un constituant majeur des protéines (Watanabe et al. 1997). Une activité plus importante de certaines enzymes digestives et notamment des enzymes protéolytiques chez *S. argus* pourraient donc expliquer l'AE plus élevée du Zn observée chez cette espèce.

3.3. LES EFFETS DU STADE PHYSIOLOGIQUE SUR LE TRANSFERT TROPHIQUE DES MÉTAUX

3.3.1. Etat de l'art

Le passage à l'âge adulte des poissons s'accompagne de changements physiologiques importants. En effet, pour une même espèce, le tube digestif des larves est morphologiquement, histologiquement et physiologiquement moins élaboré que celui des adultes (Govoni et al. 1986). En concomitance avec des changements de régime alimentaire, des changements s'opèrent sur la physiologie digestive afin que l'organisme soit à même d'exploiter de nouvelles ressources alimentaires (Hunter 1981, Govoni et al. 1986). Cependant, il n'existe que peu d'information dans la littérature concernant l'influence du stade de vie sur la bioaccumulation des métaux chez des organismes aquatiques. Cependant, des investigations ont déjà été menées chez les invertébrés. Ainsi, Miramand et al. (2006) ont notamment déjà mis en évidence, par des mesures effectuées sur le terrain, des capacités de bioaccumulation de certains métaux plus élevées Ag, Cu, Fe et Zn) chez les adultes de seiche commune *Sepia officinalis* que chez les juvéniles de la même espèce. De telles observations n'ont, jusqu'à présent, pas été clairement mis en évidence chez les poissons.

3.3.2. Objectifs de l'étude

Afin d'étudier l'influence du stade de vie sur le transfert trophique des métaux (Co et Zn) un *single-feeding* de juvéniles et d'adultes de poisson-lune argenté *M. argenteus* et de pavillon tacheté *S. argus* avec des artémies radiomarquées a été effectué. Puisque la taille des organismes adultes n'a pas permis un suivi cinétique de la dépuration des métaux et donc une détermination des AEs, des dissections fines ont été réalisées à différents temps afin de comparer la distribution des métaux dans le temps entre juvéniles et adultes.

3.3.3. Résultats et Discussion

Pour les deux métaux étudiés (Co et Zn), les résultats obtenus en termes de distribution sont similaires entre les juvéniles et les adultes des deux espèces exceptés durant les trois premiers jours. En effet, durant cette première phase, des différences entre adultes et juvéniles notamment de GTT peuvent expliquer les différences de distribution du Co et du Zn entre adultes et juvéniles des deux espèces étudiées durant les premiers jours de dépuration. (**Fig. 8**). Le constat est le même en termes de concentration, estimée par le calcul de l'Indice de Concentration I_C définit par Rouleau et al. (2000).

Les détails sont fournis dans les données du **Supplementary Data** de l'**Article 5**). Les profils de distribution et de concentration comparables entre juvéniles et adultes peuvent résulter d'un métabolisme similaire pour ces deux stades de vie (i.e. une physiologie digestive et une AE similaires), bien qu'il y ait des différences de taille importantes chez les adultes de poisson-lune argenté et de pavillon tacheté qui étaient respectivement 10 et 40 fois plus grands que les juvéniles.

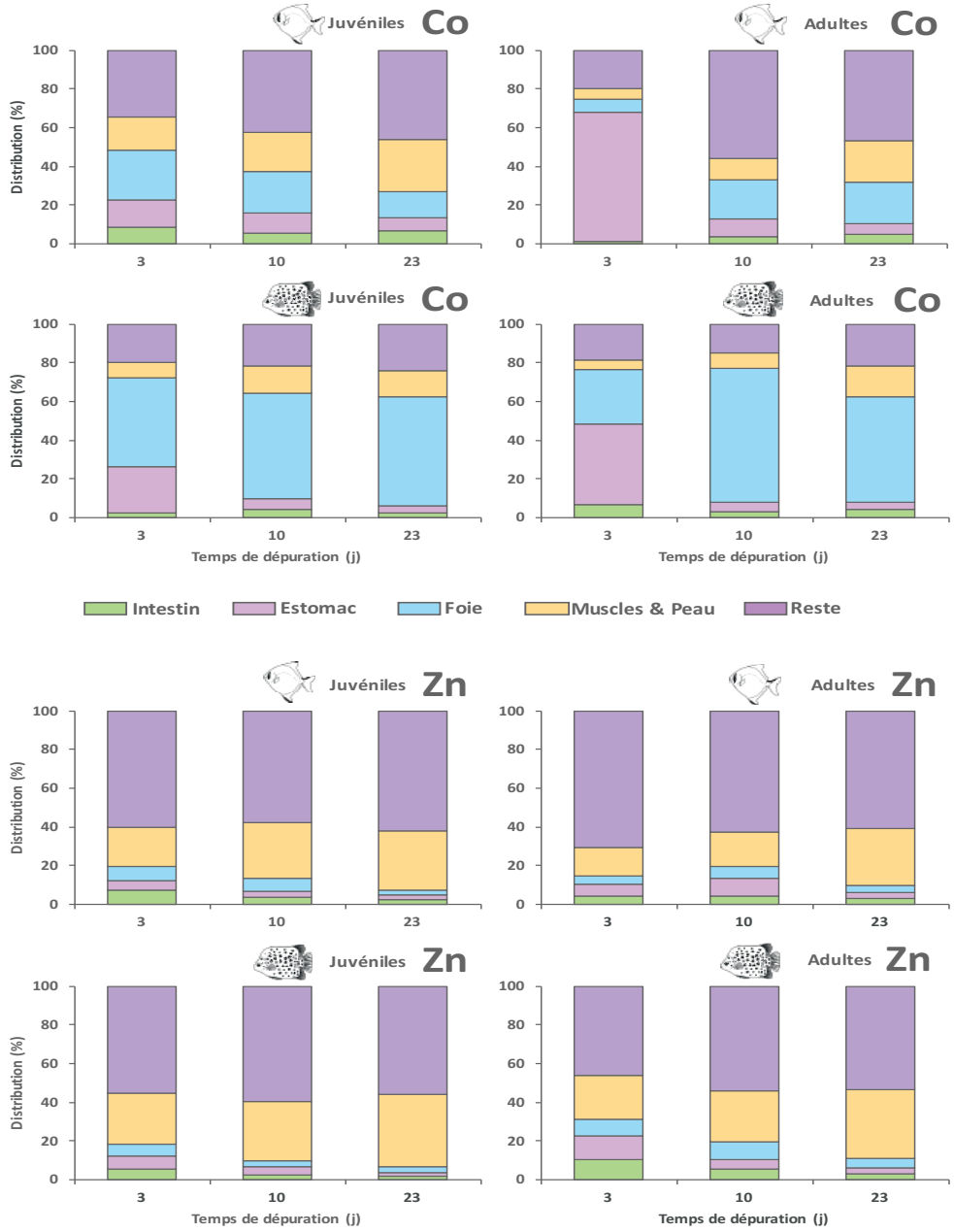


Figure 8. Distribution moyenne du Co et du Zn parmi les 5 compartiments corporels (estomac, intestin, foie, muscles avec peau et reste) chez des juvéniles et des adultes de poisson-lune argenté (*M. argenteus*) et de pavillon tacheté (*S. argus*) au cours de la phase de dépuration après un *single-feeding* effectué avec des artémies radiomarquées. À chaque fois, trois poissons ont été disséqués. Données issues de l'**Article 5**.

Les profils de distribution et de concentration comparables entre adultes et juvéniles peuvent résulter d'un métabolisme similaire chez les juvéniles et les adultes (i.e. une physiologie digestive et une efficacité d'assimilation similaires), bien qu'il y ait des différences de taille importantes (les adultes de poisson-lune argenté et de pavillon tacheté sont respectivement 10 et 40 fois plus grands que les juvéniles). En outre, il est important de noter que les adultes des deux espèces n'étaient pas sexuellement matures au moment de l'expérience. Cela pourrait affecter les résultats en raison de l'attribution non négligeable de certains éléments essentiels aux organes de reproduction, en particulier chez les femelles matures avec production d'ovocytes (e.g. Protasowicki 1986, Rajkowska & Protasowicki 2013).

3.4. DES RÉSULTATS COMPLÉMENTAIRES CONCERNANT L'EFFET DE LA TAILLE (ÂGE) SUR L'ASSIMILATION DES MÉTAUX

3.4.1. Etat de l'art

La taille du corps, induisant des dépenses et une allocation de l'énergie variable peut affecter la bioaccumulation des métaux chez les organismes aquatiques comme cela a été démontré notamment chez des invertébrés (e.g. Hédouin et al. 2006). Toutefois, chez les poissons, les données de terrain n'ont pas permis d'établir de relation sans équivoque entre la concentration corporelle en métaux et la taille des organismes échantillonés (Barak & Mason 1990, Al-Yousuf et al. 2000, Canli & Atli 2003, Farkas et al. 2003). Par exemple, les concentrations en Zn chez les jeunes merlus *Merlangus merlangus* ont été corrélées négativement avec la longueur du corps. Des corrélations similaires ont été trouvées avec les concentration de Cd et Pb dans le foie de la même espèce mais pas dans les autres organes (Kljaković Gašpić et al. 2002). Chez la brème commune, *Abramis brama*, des relations positives, négatives ou constantes entre les concentrations de métaux (Cd, Cu, Hg, Pb) dans les muscles, le foie et les branchies et l'âge des individus ont été mises en évidences (Farkas et al. 2003). Une telle incohérence découle en grande partie de la difficulté de discriminer sur le terrain, où de nombreux facteurs peuvent affecter la bioaccumulation des métaux, les effets de la croissance. Cependant, relativement peu d'études expérimentales ont été menées pour étudier l'effet de la taille sur la bioaccumulation des métaux chez les poissons. Parmi ces études, Zhang & Wang (2005) ont observé une relation négative entre la concentration corporelle de Zn et la taille des individus chez les juvéniles de pagre à tête noire *A. schlegeli*.

3.4.2. Objectifs et hypothèse de recherche

Enfin d'étudier l'influence de la taille sur l'AE des métaux chez les poissons, les données expérimentales issues de l'ensemble des expériences menées chez les juvéniles de turbots *Scophthalmus maximus* nourris avec du granulé radiomarqué et acclimatés aux mêmes conditions expérimentales (température, salinité et pH) ont été compilées afin de tester l'hypothèse selon laquelle l'acquisition d'une digestion et d'une assimilation plus efficace au fur et à mesure du développement des juvéniles conduit à une meilleure AE des métaux chez les individus plus âgés.

3.4.3. Résultats et Discussion

La compilation des AEs du Cd, du Co, du Mn et du Zn chez des juvéniles de turbots maintenus dans les mêmes conditions expérimentales (circuit ouvert, salinité : 38 psu, température : 15 ± 0,5°C, pH : 8,1 ± 0,1, jour/nuit : 12h/12h et nourris avec des granulés indiquent aucune différence significative entre les turbots les plus petits (n=11-12 ; 5,6-14,3 g) et les plus gros (n=4 ; 25,8-28,4 g).

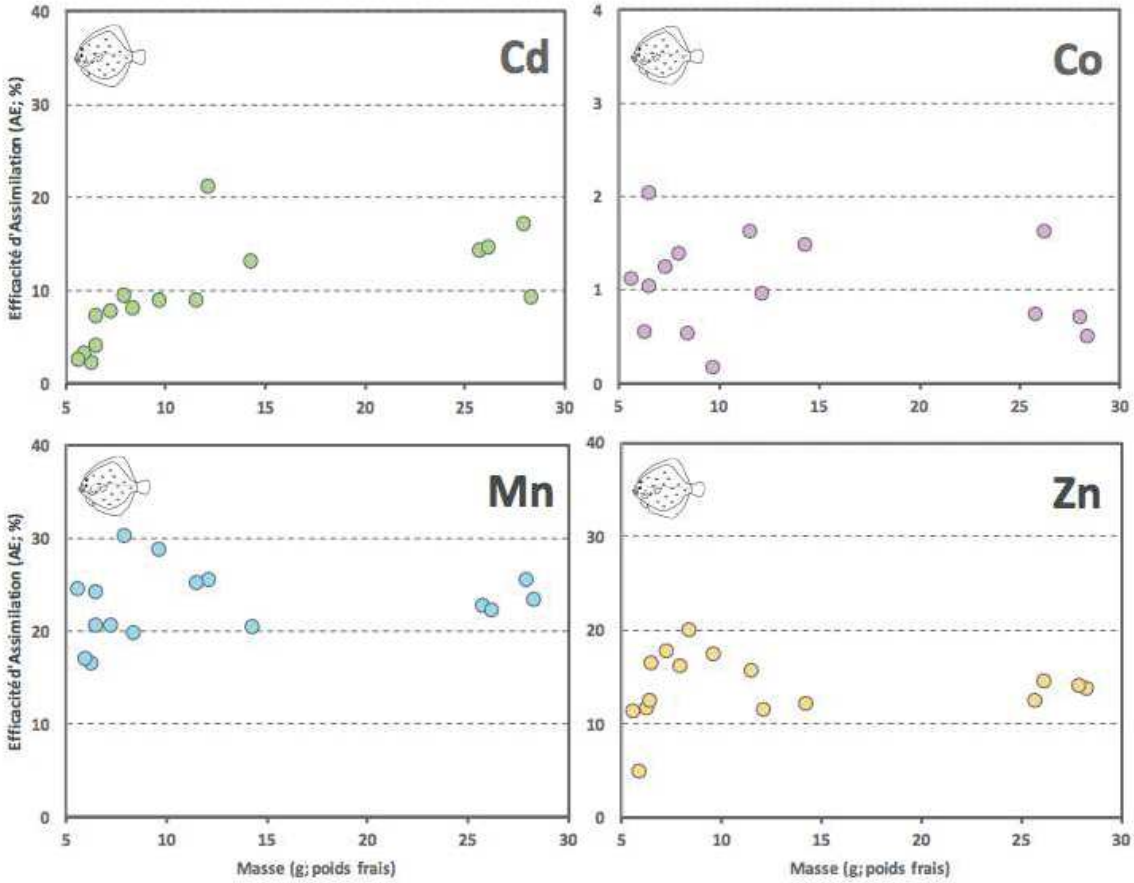


Figure 9. Relation entre l'efficacité d'assimilation du Cd, du Co, du Mn et du Zn et la masse individuelle de juvéniles turbots nourris avec des granulés radiomarqués. Données issues de l'**Article 2**.

Nos résultats indiquent que dans cette gamme de taille (de 5 à 28 g), l'AE reste la même pour les trois métaux essentiels (Co, Mn et Zn) et l'unique métal non-essentiel (Cd) considéré dans cette étude (**Fig. 9**). Il n'y a eu que peu d'études concernant l'infleunce de la taille sur l'assimilation des métaux chez les poissons. Toutefois, Zhang & Wang (2007) ont mis en évidence chez des juvéniles de pagre à tête noire *A. schlegeli* une AE plus élevée du Se et du Zn chez les poissons les plus gros. Ces résultats sont imputables aux rôles essentiels de ces métaux chez les poissons contrairement au Cd pour lequel ces auteurs n'ont démontré aucun effet comme nos résultats l'indiquent également. Contrairement à l'étude présentée ici, Zhang & Wang (2007) ont utilisé dans leur étude des juvéniles dont le processus de différenciation du tube digestif et de développment est encore en cours comme le confirment leurs observations morphologiques et histologiques. Ainsi, il est possible que l'absence de différences d'AE en fonction de la taille soit liée à l'utilisation, dans le cadre de cette thèse, de turbots juvéniles métamorphosés depuis plus de trois mois pour ce qui indique que la différenciation du tube digestif est terminée comme le suggèrent nos observations morphologiques où aucune différence n'a été observée entre individus quelle que ce soit leur taille.

3.5. L'INGESTION DE BIOTOXINES N'INFLUENCE PAS L'ASSIMILATION DES MÉTAUX CHEZ LES POISSON

3.5.1. Etat de l'art

Parmi les plus de 5000 espèces de phytoplancton identifiées, 2% sont connues pour produire des toxines et ce pourcentage semble augmenter (Smayda 1990, Hallegraeff 1993, Sournia 1995). Ces biotoxines peuvent donc être produites en grandes quantités lors des efflorescences de certaines espèces de phytoplancton, il s'agit des évènements de *Harmful Algal Blooms* (HABs). L'augmentation du nombre d'espèces connues comme nocives ou toxiques reflète les améliorations technologiques et de la surveillance accrue dans la détection des HABs et des toxines produites. Les influences anthropiques qui interagissent avec les processus naturels ont contribué à augmenter la fréquence des efflorescences et leur répartition géographique à l'échelle mondiale (Hallegraeff, 1993). Ces interactions ont également entraîné une augmentation du nombre d'espèces anciennement bénignes qui sont devenues toxiques en raison de modifications environnementales et/ou génétiques.

Ainsi les impacts des HABs sur les écosystèmes aquatiques et les organismes qui les composent dont les poissons font l'objet d'un intérêt grandissant (Naar et al. 2007). Parmi les espèces produisant des toxines, la dinoflagellée *Karenia brevis* est à l'origine des marées rouges (*red tides*) dans le Golfe du Mexique. Cette espèce est connue pour produire des brévétoxines sont des neurotoxines notamment à l'origine d'empoisonnements (*Neurotoxic Shellfish Poisoning*, NSP) chez les consommateurs ayant ingérés des mollusques exposés à ces toxines (Landsberg 2002).

Lors des efflorescences de *K. brevis*, les cellules algales et les brévétoxines sont filtrées par les organismes filtreurs qui représentent une voie de passage très importante dans le transfert de ces toxines vers les niveaux trophiques supérieurs et notamment des poissons (Landsberg 2002). Par ailleurs, les mollusques bivalves sont connus pour leur capacité à accumuler d'autres éléments tels que les métaux (e.g. Wang & Fisher 1996, Metian et al. 2009, Hédouin et al. 2010). Pour ces raisons et parce que les poissons accumulent principalement les métaux par la voie trophique, il apparaît important d'étudier les possibles effets de l'ingestion de biotoxines sur le transfert trophique des métaux chez les poissons.

3.5.2. Objectifs et hypothèses de recherche

L'objectif de ce travail a donc été de tester si l'influence potentielle de l'ingestion de brévétoxines sur l'AE des métaux chez le turbot *S. maximus*. Pour ce faire, des juvéniles de cette espèce ont été nourris une seule fois avec des moules radiomarquées (^{54}Mn et ^{65}Zn) et contaminées aux brévétoxines (exposition unique) ou nourris pendant 3 semaines avec des moules contaminées aux brévétoxines (expositions multiples) puis nourris une fois avec des moules radiomarquées (*single-feeding*). Les cinétiques de dépuration ont ensuite été suivies et les AEs ont été déterminées chez les turbots exposés une ou plusieurs foirs aux brévétoxines.

3.5.3. Résultats et Discussion

Les cinétiques de dépuration du ^{54}Mn et du ^{65}Zn chez des turbots exposés par la nourriture une seule fois ou durant 3 semaines à des biotoxines (brévétoxines) ont été mieux ajustées par un modèle exponentiel à deux composantes (R^2 = 0,51-0,96). La comparaison statistique des l'AEs déterminée pour chaque individu indique que, pour les deux métaux essentiels étudiés, il n'y a pas de différence significative entre les différentes conditions expérimentales ($p > 0,05$, **Fig. 10**).

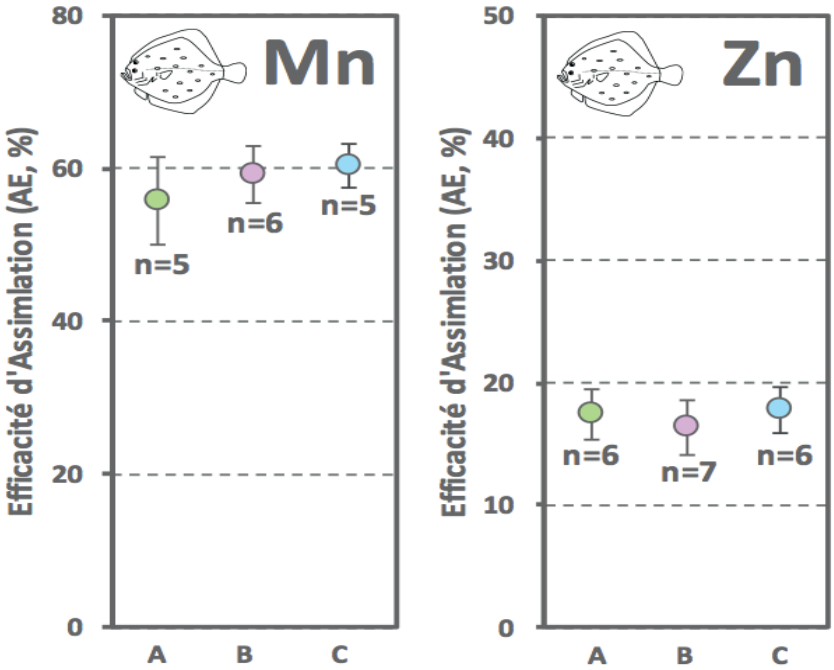


Figure 10. Comparaison des efficacités d'assimilation du Mn et du Zn (AEs, moyennes ± écarts-types), calculées pour chaque individu, chez le turbot *S. maximus* turbots non-exposés (A), exposés par la nourriture une seule fois aux toxines (B, exposition aïgue) ou durant 3 semaines (C, exposition chronique) à des biotoxines (brévétoxines) après un single-feeding avec des moules radiomarquées. Les lettres désignent les différences significatives ($p<0,05$). Données issues de l'**Article 6**.

Étant donné que les brévétoxines sont ichtyotoxiques et causent la mort des poissons lorsqu'elles sont libérées lors des efflorescences, il n'existe jusqu'à présent, que peu d'informations disponibles sur le transfert trophique de ces toxines chez les poissons (Tester et al. 2000, Prince et al. 2006, Landsberg et al. 2009).

Dans cette étude, nous avons démontré que le transfert trophique des brévétoxines de juvéniles de turbots ayant consommées des moules préalablement exposées à des efflorescences simulées de *K. brevis* (approx. 1000 cellules mL^{-1}, Stumpf et al. 2003, Gannon et al. 2009) est limité. En effet, l'analyse des brévétoxines présentes dans les turbots au moment du premier comptage (i.e. 2h après le *single-feeding* avec les moules radiomarquées) indique que quel que soit le mode d'exposition (aïgue ou chronique), les brévétoxines, faiblement retenues par les poissons, se retrouvent essentiellement dans la vésicule biliaire, organe fortement impliqué dans l'excrétion des xénobiotiques de forts poids moléculaires (> 600, Di Giulio & Hinton 2008) tels que les brévétoxines.

Le fait que les brévétoxines soient retrouvées uniquement dans cet organe excréteur et ce, même 2h après un nourrissage avec des moules toxiques semblent indiquer que les brévétoxines ingérées ne sont pas forcément distribuées dans l'ensemble du corps des poissons et sont, au contraire, très vite excrétées par voie biliaire. Ces résultats peuvent expliquer l'absence d'effet sur l'assimilation des métaux essentiels étudiés (Mn et Zn).

3.6. MISE EN PERSPECTIVE DES RÉSULTATS

L'ensemble des résultats obtenus dans ces travaux de thèse indique que parmi les facteurs biologiques étudiés, l'influence de la nourriture est prépondérante sur le transfert trophique des métaux chez les poissons. En effet, il s'agit de la seule variable étudiée qui a induit des différences d'AEs pour l'ensemble des métaux considérés à savoir trois métaux essentiels (Co, Mn ou Zn) et un non-essentiel (Ag). Par ailleurs, l'approche mécanistique adoptée pour expliquer les différences observées ne permettent pas encore de comprendre pleinement les raisons de telles différences. Ainsi, des expériences ultérieures sont nécessaires pour comprendre les mécanismes sous-jacents expliquant les différences d'AE en fonction du type de nourriture car les hypothèses existantes ne sont pas toujours démontrées dans le cas de l'utilisation de proies pluricellulaires complexes et de modèles biologiques aussi divers que les poissons. Par ailleurs, il existe encore un manque d'information quant à l'influence de la taille et du stade physiologique des organismes car cette question a été traitée partiellement dans ce manuscrit sans toutefois avoir pu explorer une gamme de taille et de stades suffisamment large pour rendre compte du transfert des métaux chez les poissons tout au long de leur cycle de vie (des larves aux adultes matures des deux sexes). Enfin, ce travail de thèse fournit les premières informations relatives au transfert trophique des métaux chez les poissons dans un contexte de HABs. Il s'agit, à notre connaissance de la première étude expérimentale menée à ce sujet soulevant ainsi un champ relativement vaste d'investigations.

Chapitre 4
Influence des facteurs environnementaux

CHAPITRE 4 : INFLUENCE DES FACTEURS ENVIRONNEMENTAUX

Le champ des facteurs environnementaux suceptibles d'affecter le transfert trophique des métaux chez les poissons est vaste et c'est pourquoi une sélection a été opérée lors de la réalisation des travaux de cette thèse. Une priorité a été donnée à la température en considérant le fait que les poissons marins sont ectothermes conditionnant ainsi leur physiologie et doivent mettre en place des mécanismes d'osmorégulation spécifiques pour assurer leur égulation ionique dans le milieu hypertonique où ils évoluent. Par ailleurs, les activités anthropiques sont à l'origine de modifications drastiques dans les paramètres physico-chimiques des eaux marines dont les effets sur le biote ne sont pas encore complètement élucidés. C'est pour l'ensemble de ces raisons que les facteurs environnementaux suivants ont été considérés dans les travaux présentés dans le contexte de cette thèse :

- Température de l'eau
- pH de l'eau
- Salinité

4.1. LA TEMPÉRATURE, UNE VARIABLE CLÉ DANS LA PHYSIOLOGIE DES POISSONS

4.1.1. Etat de l'art

Parmi les paramètres environnementaux influençant les écosystèmes aquatiques, la température est un des plus important et joue un rôle crucial dans la physiologie des organismes ectothermes tels que les poissons (Fry 1971). Ainsi, la physiologie digestive des poissons est sous l'influence de la température de l'environnment dans lequel ils vivent. En effet, la température de l'eau a un effet important sur le temps de transit des aliments et elle peut affecter l'activité des enzymes digestives (e.g. Temming et Herrmann, 2001 ; Kofuji et al., 2005). Or, ce sont l'ensemble de ces processus qui vont affecter le passage des composés alimentaires au travers l'épithélium intestinal (i.e. absorption). A titre d'exemple, Nakada (2002) a signalé une augmentation significative du temps de transit des aliments chez la sériole *Seriola quinqueradiata* suite à la diminution de la température de l'eau. Une telle observation expérimentale a été confirmée chez une espèce voisine *Seriola lalandi* (Miegel et al. 2010). Par ailleurs, une étude sur le terrain a pu démontré chez la sériole que la digestion des protéines est corrélée à la température de l'eau (Kofuji et al., 2005). En effet, ces mêmes auteurs ont mis en évidence que l'activité des enzymes protéolytiques chez cette espèce est supérieure en été lorsque la température de l'eau est la plus élevée.

D'autres auteurs ont obtenu des résultats similaires chez d'autres espèces de poissons d'eau douce (Hardewig et van Dijk, 2003). Toutefois, il convient de nuancer l'absence des résultats obtenus dans les études mentionnées ci-dessus. Ces travaux menés sur le terrain ou dans un contexte expérimental ont été réalisés à des températures réalistes (i.e. dans les niches écologiques des espèces étudiées). Or, dans un contexte de changement global, il est attendu que la température moyenne des eaux de surface augmente de 3°C (Orr et al. 2005, IPCC 2013) à l'origine de changements importants, notamment au niveau des écosystèmes côtiers (Hoegh-Guldberg et al. 2007). De tels changements de températures pourraient conduire à des effets délétères sur la physiologie digestive des poissons. Dans ce contexte, il apparaît donc important de considérer ce paramètre environnemental dans les études en écotoxicologie. Cependant, bien que l'influence de la température sur la digestion par les poissons de macromolécules telles que les protéines et les lipides ait fait l'objet de nombreuses études dans le cadre de l'aquaculture, peu d'études en écotoxicologie se sont intéressées aux effets de ce paramètre environnemental sur la digestion des éléments traces chez les poissons. Parmi les rares travaux disponibles à ce sujet, Van Campenhout et al. (2007) ont mis en évidence un accroissement de l'AE du Zn chez la carpe commune *Cyprinus carpio* nourrie avec des vers radiomarqués lorsque la température de l'eau augmente.

4.1.2. Objectifs et hypothèses de recherche

Puisque la température est suceptible d'impacter la physiologie digestive des poissons, des juvéniles de turbot *S. maximus* ont été acclimatés durant 8 semaines à deux températures différentes (17°C et 20°C). Ces valeurs correspondent respectivement à la température où l'efficacité de conversion alimentaire (FCE pour *Food Conversion Efficiency*) est optimale (Imsland et al. 2001) et à la projection de la température pour les deux siècles à venir (ΔT°C +3°C; Orr et al. 2005, IPCC 2013). Après un *single-feeding* avec des crevettes radiomarquées (^{110m}Ag, ^{57}Co, ^{65}Zn), les AEs ont été déterminées chez les turbots exposés aux deux conditions de températures afin de tester les hypothèses suivantes :

1) L'efficacité d'assimilation des métaux par les juvéniles de turbots est augmentée par une élévation de la température susceptible d'augmenter l'activité des enzymes digestives ;

2) L'exposition à des températures plus élevées que l'optimum thermique de FCE conduit à une diminution de l'efficacité d'assimilation des métaux chez le turbot.

4.1.3. Résultats et Discussion

Les cinétiques de dépuration du ^{57}Co, ^{65}Zn et ^{110m}Ag chez les juvéniles de turbot exposés à 17°C et 20°C ont été mieux ajustées par un modèle à deux composantes incluant une constante (R^2= 0,90-0,99). Les analyses statistiques effectuées sur les AE estimées individuellement ont révélé que la température n'a pas significativement affectée le transfert trophique de l'Ag et du Co chez les juvéniles de turbots exposés à 17°C et 20°C (p>0,05 ; **Fig. 11**). En revanche, l'AE du Zn est plus élevée chez les turbots exposés à la température la plus haute (p_{ANOVA} = 0,03 ; **Fig. 11**).

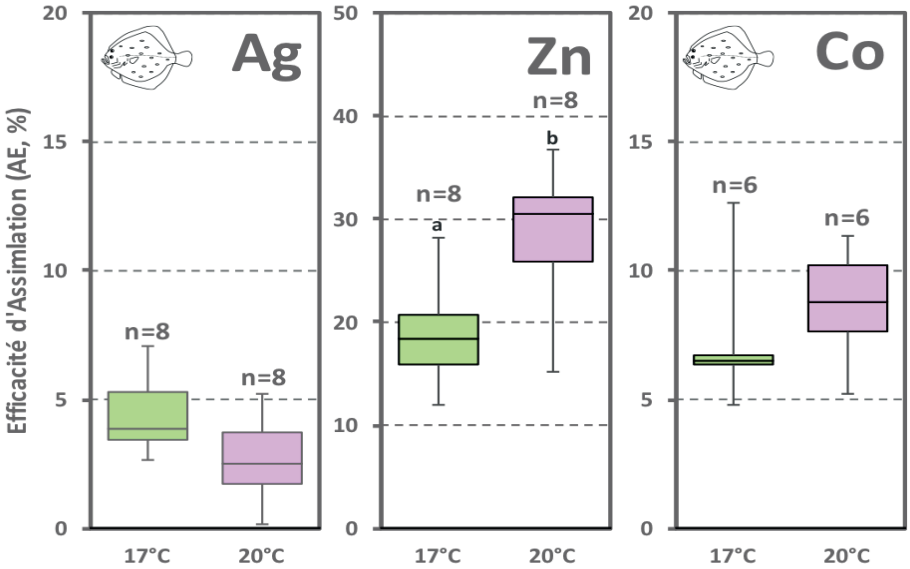


Figure 11. Comparaison des efficacités d'assimilation (AEs, moyennes ± écarts-types) calculées pour chaque individu de l'Ag, du Co et du Zn chez des turbots *S. maximus* acclimatés à deux températures (17°C et 20°C) après un *single-feeding* avec des crevettes radiomarquées. Les lettres désignent les différences significatives (p<0,05). Données issues de l'**Article 8**.

L'AE du Zn est beaucoup plus élevée (>19%) comparée aux autres éléments (Ag et Co) moins assimilés par les juvéniles turbots exposés aux deux températures (AE <9%). Ces résultats pourraient expliquer qu'un effet significatif de la température a été obervé uniquement pour le Zn. En effet, l'AE plus faible pour l'Ag et le Co rend plus difficile la mise en évidence de tout effet significatif. Un effet positif de la température a déjà été démontré sur l'AE du Zn chez la carpe commune *C. carpio* (nourrie avec des proies radiomarquées au ^{65}Zn, Van Campenhout et al. 2007). De tels effets n'ont, à notre connaissance, jamais été démontrés chez les poissons marins. Toutefois, l'utilisation de l'Indice de Concentration I_C a déjà permis de démontrer que l'intestin est impliqué dans le processus d'absorption de Zn le poisson-lune argenté *M. argenteus* (**Article 5**).

Or, comme l'ont expliqué Van Campenhout et al. (2007), l'une des explications possibles pour expliquer les différences d'AE observées sont liées à la concentration plus élevée de transporteurs Zn dans l'intestin des poissons exposés à des températures croissantes ce qui pourrait faciliter l'absorption de cet élément.

4.2. LE PH ET SES EFFETS SUR LA PHYSIOLOGIE DIGESTIVE DES POISSONS

4.2.1. Etat de l'art

Jusqu'à présent, peu d'attention a été portée sur les effets de l'acidification des océans sur la physiologie des poissons (voir la revue de Kroeker et al. 2010). Les effets de la pCO_2 accrue sur les poissons varient en fonction des stades biologiques (larves, juvéniles et adultes) ou encore en fonction des espèces (Munday et al. 2009, 2011, Heuer & Grosell 2014). Ainsi, Moran & Støttrup (2011) ont mis en évidence une croissance sensiblement réduite chez les juvéniles de la morue atlantique *Gadus morhua* exposés à des niveaux croissants de pCO_2. À l'inverse, d'autres études ont montré que des pCO_2 élevées n'avaient aucun effet significatif sur la croissance chez le chromis à épines *Acanthochromis polyacanthus* (Munday et al. 2011) et chez le spare *Stenotomus chrysops* (Perry et al. 2015). En outre, Welch & Munday (2016) ont constaté que le niveau élevé de pCO_2 a augmenté l'efficacité de la reproduction du poisson-clown *Amphiprion percula*, mais l'a diminué chez une espèce voisine, le chromis à épines *A. polyacanthus* dans des conditions similaires.

Parmi les processus biologiques étudiés dans le cadre de l'étude des effets de l'acidification des océans sur les poissons marins, la digestion a reçu peu d'attention à ce jour. Frommel et al. (2012, 2014) ont constaté des altérations morphologiques et physiologiques au niveau du système digestif chez les jeunes stades de vie de deux espèces de poissons sous des conditions de pCO_2 telles que le prévoit les prédictions pour les 100 années à venir, mais aucun effet induit par ces altérations sur les performances digestives chez les espèces étudiées n'a été clairement démontré par ces auteurs concernant les effets sur le transfert trophique des métaux. Cependant, quelques études récentes ont révélé que l'acidification des océans pourrait affecter la physiologie digestive de certaines espèces de poissons.

A titre d'exemple, Rosa et al. (2016) ont montré que les conditions hypercapniques ont entraîné une diminution significative de l'activité enzymatique digestive : 42% pour la trypsine et 50% pour la phosphatase alcaline chez des juvéniles de requin chabot *Chiloscyllium punctatum*. En outre, Pimentel et al. (2015) ont montré que des conditions similaires ont entraîné une diminution de l'activité des enzymes pancréatiques (jusqu'à 26% pour la trypsine et 75% pour l'amylase) et des enzymes intestinales (jusqu'à 36% pour la phosphatase alcaline) de juvéniles de sole du Sénégal *Solea senegalensis*. Toutefois, malgré ces observations, à notre connaissance, aucune étude n'a été menée concernant l'influence de l'acidification des océans sur le transfert trophique des métaux chez les poissons.

4.2.2. Objectifs et hypothèse de recherche

Des juvéniles de poisson-clown *A. ocellaris* et de turbot *S. maximus* ont été acclimatés pendant plusieurs semaines à deux conditions de pH (7,5 et 8,0) soient des pCO_2 respectives de 550 µatm et 1850 µatm. Ces valeurs correspondent respectivement au pH actuel et aux projections pour les deux siècles à venir (ΔpH : -0,5; Orr et al. 2005, IPCC 2013). Après la période d'acclimatation, les juvéniles de poisson-clown et de turbot ont été exposés lors d'un *single-feeding* respectivement à des granulés radiomarqués (^{54}Mn, ^{57}Co, ^{65}Zn) et des crevettes radiomarquées (^{110m}Ag, ^{57}Co, ^{65}Zn) afin, par cette approche intégrative, de tester si les éventuelles altérations de la physiologie digestive des juvéniles de poisson-clown et de turbots réduit l'AE des métaux. L'absorption des métaux se fait au niveau de l'intestin, or cet organe est également impliqué l'équilibre acide-base et l'ionorégulation potentiellement affectés par l'acidification (i.e. augmentation de la pCO_2). Le protocole expérimental décrit ci-dessus a ainsi permis de tester l'hypothèse selon laquelle des changements au niveau de l'intestin pourraient également conduire à une réduction de l'AE des métaux.

4.2.3. Résultats et Discussion

Les cinétiques de dépuration du ^{57}Co, ^{65}Zn et ^{110m}Ag chez les juvéniles de turbot exposés à deux pH (7,5 et 8,0) ont été ajustées par un modèle à deux composantes incluant une constante (R^2= 0,89-0,97). Les analyses statistiques effectuées sur les AE estimés individuellement ont révélé que le pH n'a aucun effet significatif sur l'assimilation des trois éléments étudiés (Ag, Co et Zn) chez les juvéniles de turbots ($p>0,05$; **Fig. 12**).

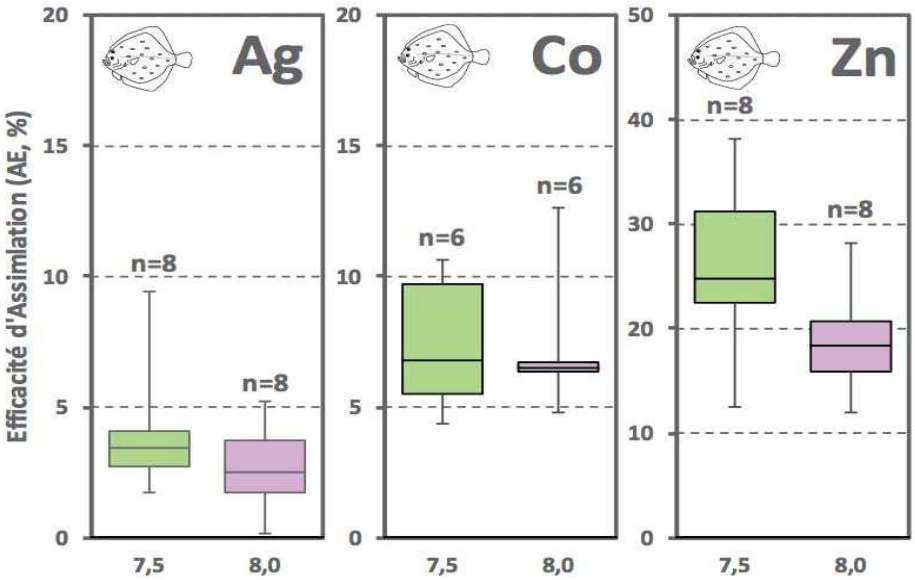


Figure 12. Comparaison des efficacités d'assimilation (AEs, moyennes ± écarts-types) calculées pour chaque individu de l'Ag, du Co et du Zn chez des juvéniles de turbot *S. maximus* acclimatés à deux pH (7,5 et 8,0) après un *single-feeding* avec des crevettes radiomarquées. Données issues de l'**Article 8**.

Les cinétiques de dépuration du ^{54}Mn et du ^{65}Zn chez les juvéniles de poisson-clown *A. ocellaris* à deux pH (7,5 et 8,0) ont été ajustées par un modèle exponentiel à deux composantes dans le cas du Zn ou incluant une constante dans le cas du Mn (R^2= 0,59-0,87). Les analyses statistiques effectuées sur les activités restantes au cours de la dépuration ont révélé que le pH n'a aucun effet significatif sur l'assimilation du Mn et du Zn chez cette espèce (p>0,05 ; **Fig. 13**).

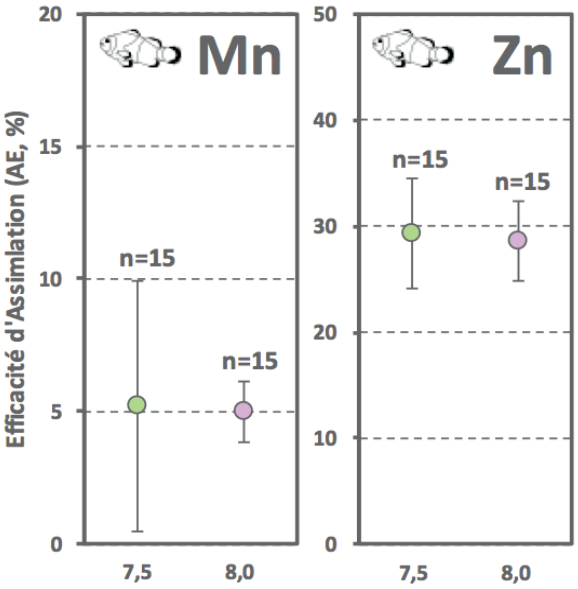


Figure 13. Comparaison des efficacités d'assimilation (AEs, moyennes ± écarts-types) calculées pour chaque individu du Mn et du Zn chez des juvéniles de poisson-clown *A. ocellaris* acclimatés à deux pH (7,5 et 8,0) après un *single-feeding* avec des granulés radiomarqués. Données issues de l'**Article 7**.

Dans des études antérieures où les effets de l'acidification des océans sur l'activité des enzymes digestives ont été étudiés chez des espèces benthiques tels la sole sénégalaise *S. senegalensis* et le requin chabot *C. punctatum* (Pimentel et al. 2015, Rosa et al. 2016), la valeur sélective et/ou la survie des individus ont été affectées négativement par l'acidification de l'océan. Cependant, d'autres études ont également mis en évidence que l'acidification n'avait que peu d'effet sur ces paramètres chez d'autres espèces (Munday et al. 2011). Ces exemples soulèvent les effets très contrastés de l'acidification sur les poissons. Les résultats combinés des **Articles 7** et **8** révèlent, grâce à l'approche intégrative adoptée, que l'acification n'affecte pas le transfert trophique des métaux chez les deux espèces de poissons étudiées. S'agissant des premiers travaux à ce sujet, aucun point de comparaison n'est disponible dans la littérature. Par ailleurs, l'utilisation de deux modèles biologiques très différents avec un poisson benthique, d'eaux tempérées et carnivore, le turbot *S. maximus* d'une part et d'autre part un poisson récifal, démersal et omnivore, le poisson-clown *A. ocellaris*, semblent indiquer que de tels résultats peuvent être généralisés à des espèces dont le régime alimentaire et l'écologie diffèrent largement. Par ailleurs, certains auteurs ont émis l'hypothèse que le taux métabolique pourrait expliquer les différences de réponses entre espèces face à l'acidification. En effet, Melzner et al. (2009) ont suggéré que les animaux marins avec des taux métaboliques plus élevés pourraient être moins affectés par l'acidification des océans en raison de pCO_2 extracellulaires plus élevées et d'un métabolisme avancé pour l'élimination du CO_2 la régulation acide-base. Or ici, le poisson-clown possède un métabolisme relativement élevé comparé au turbot qui est considéré comme une espèce dont le taux métabolique est bas (Norin & Clark 2016). Malgré cela, les résultats indiquent une tolérance de ces espèces aux pCO_2. Toutefois, il convient de tenir compte du fait que seuls des juvéniles ont été utilisés dans ces études. En effet, plusieurs travaux ont mis en évidence que les plus jeunes stades de vie et notamment le stade larvaire sont plus sensibles aux changements de pCO_2, soit parce leurs mécanismes physiologiques de compensation ne sont pas entièrement développés, ou parce que leur petite taille augmente le coût de l'homéostasie et les rend ainsi plus sensibles aux fluctuations environnementales (Brauner 2008, Munday et al. 2009). Ainsi, il convient de répéter ces expériences chez les larves de ces espèces afin de valider les résultats obtenus tout au long du cycle de vie.

4.3. LA SALINITÉ, DES EFFETS CONSTRASTÉS SUR L'ASSIMILATION DES MÉTAUX

4.3.1. Etat de l'art

De nombreuses conditions environnementales peuvent affecter l'accumulation des métaux chez les poissons (voir les revues de Dallinger et al. 1987, Phillips & Rainbow 1998). Parmi ces facteurs, la salinité affecte l'accumulation des métaux en provoquant directement des changements dans la spéciation de ces éléments et en produisant des modifications dans la physiologie des organismes notamment au niveau de l'osmorégulation. Ainsi, les paramètres cinétiques tels que l'efficacité d'assimilation ou encore le taux de perte peuvent être à leur tour impactés (Wang 2002). Néanmois, bien que l'importance de la voie trophique dans la bioaccumulation des métaux chez les poisons soit bien reconnue (e.g. Xu & Wang 2002, Mathews & Fisher 2009), la plupart des travaux expérimentaux réalisés jusqu'à présent en écotoxicologie des métaux chez les poissons n'ont considéré les potentiels effets de la salinité que lors d'expositions par voie dissoute (e.g. Zhao et al. 2001). Ainsi, l'information disponible quant aux effets de la salinité sur le transfert trophique des métaux est encore très limitée avec, à notre connaissance, seulement une étude utilisant pour modèle biologique le périophthalme *Periophthalmus modestus* (Ni et al. 2005).

4.3.2. Objectifs et hypothèse de recherche

Dans ce contexte, les effets de la salinité sur l'efficacité d'assimilation de deux métaux essentiels (Mn et Zn) ont été étudiés chez le turbot *S. maximus*. Cette espèce est euryhaline (Gaumet et al. 1994) et a fait l'objet de nombreuses études sur les effets de la salinité sur sa physiologie, notamment dans un contexte d'aquaculture (Gaumet et al. 1995, Imsland et al. 2001, 2003, 2007). Ainsi, les juvéniles de turbot ont été acclimatés durant trois semaines à trois salinités (10, 25 et 38 psu) avant d'être exposés lors d'un s*ingle-feeding* avec des granulés radiomarqués préalablement (^{54}Mn et ^{65}Zn). Ce protocole a été établi afin de tester si les changements dans les processus d'osmorégulation adoptés par cette espèce pour vivre dans une large gamme de salinité ont des conséqueces sur sa capacité à assimiler les métaux.

4.3.3. Résultats et Discussion

Les cinétiques de dépuration du ^{54}Mn et du ^{65}Zn chez les juvéniles de turbot S. maximus acclimatés à trois salinités (10, 25 et 38 psu) ont été ajustées par un modèle à deux composantes exponentielles (R^2= 0,88-0,98). Les analyses statistiques effectuées sur les AEs individuelles ont révélé que la salinité affecte significativement le transfert trophique du Mn avec une AE plus faible à la salinité la plus élevée ($p<0,05$; **Fig. 14**) tandis qu'aucun effet significatif sur l'AE du Zn n'a été observée chez cette espèce ($p>0,05$; **Fig. 14**).

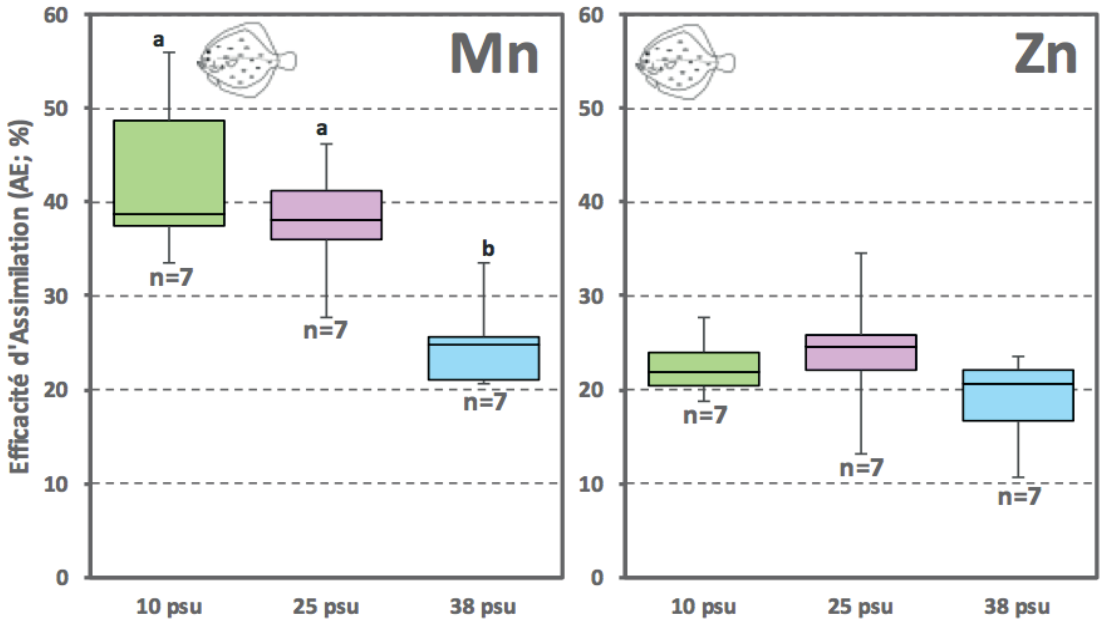


Figure 14. Comparaison des efficacités d'assimilation (AEs, moyennes ± écarts-types) calculées pour chaque individu du Mn et du Zn chez des juvéniles de turbot *S. maximus* acclimatés à trois salinités différents (10, 25 et 38 psu) après un *single-feeding* avec des granulés radiomarqués. Les lettres désignent les différences significatives ($p<0,05$) Données issues de l'**Article 9**.

Peu d'études ont été menées sur l'influence de la salinité sur le transfert trophique des métaux chez les poissons. Seuls Ni et al. (2005) ont mis en évidence, chez le périothalme *Periophthalmus modestus*, que les AEs du Co, du Se et du Zn ont été comparables ($p>0,05$) quelque soit les conditions de salinité (10, 20 et 30 psu) auxquelles les poissons ont été exposés. Dans l'étude présentée ici, les résultats obtenus sont plus contrastés. En effet, comme ces auteurs l'ont déjà démontré chez le périophthalme, aucun effet significatif de la salinité n'a été observé sur l'assimilation du Zn chez le turbot. Par contre, pour le Mn, une AE significativement plus basse a été mesurée chez les turbots exposés à la salinité la plus haute (38 psu).

La variabilité des effets de la salinité entre le Mn et le Zn peut être liée à une absorption différente des deux élements étudiés. En effet, puisque les tendances observées dans la seconde phase de la dépuration sont similaires entre les poissons exposés aux différentes conditions de salinité (**Article 9**), il est très probable que les différences d'AE observées soient une conséquence d'effets de la salinité lors de la première phase de la dépuration (i.e. quand les processus d'absorption prennent place ; Wang & Fisher 1999). Ainsi, il est intéressant, pour expliquer ces résultats, de se focaliser sur les mécanismes de transport de ces métaux de la lumière du tube digestif vers le compartiment interne des poissons.

En effet, le transport cellulaire du Zn de la lumière intestinale vers le compartiment interne est, semble-t-il, principalement gouverné par des processus actifs impliquant des transporteurs spécifiques (Bury et al. 2003). Ce métal est, entre autres, accumulé dans des cellules par des canaux spécifiques (canaux ZIP ; Bury et al. 2003, Hogstrand 2011). Cependant, comme les concentrations élevées sont toxiques, la concentration cytosolique en Zn est régulée par d'autres transporteurs de la famille ZnT qui permettent d'évacuer le Zn présent dans le cytosol à l'extérieur des cellules (Bury et al. 2003, Hogstrand 2011). Le Zn est donc sujet à une forte homéostasie qui pourrait expliquer l'absence de différence d'AE observée dans cette étude et ce malgré les variations de salinité.

Le transport du Mn semble être différent. En effet, bien que les mécanismes de transport et d'absorption du Mn à partir des aliments soient mal connus dans les poissons, par analogie avec d'autres taxons, le transport du Mn semble se faire majoritairement par voie passive. Ainsi, plusieurs études ont déjà mis en évidence que le Mn est capable de passer par les canaux Ca présent sur les membranes apicales des cellules (Fukuda & Kawa 1977, Anderson 1979, Fasolato et al. 1993). L'abaissement de la salinité et donc la concurrence des ions Ca^{2+} et Mg^{2+} pour les canaux Ca peuvent augmenter l'afflux et la bioaccumulation de métaux tels que le Mn (Langston & Bryan 1984). Ainsi, la combinaison entre l'importance de la voie passive dans le transport du Mn et une homéostase probablement moins forte que pour le Zn peut expliquer les différences significatives de Mn AE chez le turbot juvénile exposé à un gradient de salinité.

4.4. MISE EN PERSPECTIVE DES RÉSULTATS

L'ensemble des résultats obtenus dans les travaux conduits dans le contexte de cette thèse indique que parmi les facteurs environnementaux étudiés, les effets sont globalement contratsés. En effet, le pH ne semble pas affecter l'efficacité d'assimilation des métaux chez deux espèces de poissons côtiers d'environnements tempéré (le turbot) et tropical (le poisson-clown) chez des métaux essentiels (Co, Mn et Zn) et non-essentiel (Ag). Concernant la température, chez le turbot, un léger effet positif a été observé pour le Zn uniquement. Concernant la salinité, pour la même espèce, là aussi les effets sont contrastés avec une AE du Mn plus basse à la salinité la plus haute alors que ce facteur environnemental ne semble pas affecter l'AE du Zn chez la même espèce. L'ensemble de ces résultats indiquent que les facteurs environnementaux étudiés ont un effet limité sur le transfert trophique des métaux chez les poissons. L'approche utilisée dans ces travaux est basée, dans le cas de la température et du pH, sur une acclimatation des poissons à des valeurs moyennes observées de nos jours pour un facteur donné comparées aux valeurs prédites par les modèles pour les 200 ans à venir. Ces expériences n'ont donc pas permis d'explorer un plus large éventail de valeurs couvrant mieux les niches écologiques des espèces étudiées qui vivent en milieu côtier, un milieu changeant, et qui à ce titre subissent déjà des variations déjà importantes des facteurs étudiés. C'est pourquoi, une approche différente a été menée pour l'étude de la salinité où, cette fois-ci, une gamme de salinité plus importante a été considérée. Des expériences ultérieures devraient être menées afin de couvrir une plus large gamme de valeurs pour chacun des facteurs étudiés pour comprendre de façon plus exhaustive les effets des facteurs environnementaux sur l'AE des métaux chez les poissons. Pour cela l'approche, dites *tipping-point*, déjà utilisée en écophysiologie (e.g. Ventura et al. 2016) où l'idée est de déterminer, pour un facteur donné, le seuil où des effets apparaissent, pourrait s'avèrer pertinente. Par ailleurs, l'interprétation de certains résultats doit être confirmée notamment par une approche plus mécanistique visant à mieux comprendre les effets physiologiques des facteurs environnementaux (osmorégulation, activité métabolique, stress…) et ainsi établir un lien avec les résultats obtenus sur le transfert trophique des métaux.

Chapitre 5
Conclusions et perspectives

CHAPITRE 5
CONCLUSIONS ET PERSPECTIVES

5.1. CONCLUSIONS

5.1.1. Les résultats majeurs des travaux issus de cette thèse

Ce travail de doctorat fournit des nouvelles informations importantes quant aux effets des facteurs biologiques et environnementaux sur l'efficacité d'assimilation des métaux chez les poissons. Les différents travaux réalisés permettent une compréhension plus globale du transfert trophique des métaux chez les poissons. En effet, bien qu'il s'agisse de la voie majoritaire d'accumulation des métaux chez les poissons, un nombre limité de facteurs a été étudié expérimentalement dans le passé comme le met en évidence la revue de la littérature présentée dans l'**Article 1**. En utilisant différents modèles biologiques pertinents, cette thèse a montré que le transfert trophique des métaux :

- Semble être principalement dépendant des facteurs biologiques (type de nourriture, variations interspécifiques… **Articles 2-6**) avec une influence moindre des facteurs environnementaux (pH, température, salinité… **Articles 7-9**).
- Est fortement influencé par le type de nourriture ingéré avec des différences significatives observées notamment pour les métaux essentiels (Co, Mn et Zn ; **Articles 3** et **4**).
- Peut être variable entre deux espèces d'écologie trophique similaire (**Article 4**).
- Ne semble que peu influencé par le stade physiologique (juvéniles et adultes) ou la taille des individus en dehors des périodes de reproduction (**Article 5**).
- N'est pas affecté par l'ingestion de biotoxines (**Article 6**).
- N'est que peu ou pas impacté par les modifications de pH et de températures dues aux changements globaux déterminées par les modèles prédictifs pour les 200 ans à venir (**Articles 7** et **8**).
- Peut être dépendant de la salinité (cas du Mn ; **Article 9**).

Le travail de doctorat et la thèse ont principalement porté sur les métaux essentiels (Co, Mn et Zn) en raison de rôle vital dans la survie des organismes et du manque d'information relatif au transfert trophique de ces métaux chez les poissons (**Article 1**).

5.1.2. Une meilleure compréhension du transfert trophique de certains métaux

D'après la revue de la littérature effectué sur le transfert trophique et plus particulièrement sur l'efficacité d'assimilation des métaux chez les poissons (**Article 1**), il ressort que 12 métaux et métalloïdes différents ont été considérés dans des études expérimentales. Parmi ces métaux, ce sont les métaux non-essentiels (Ag, Am, Cd, Cs, Hg et Po) qui ont été les plus étudiés. A titre d'exemple, le transfert trophique du Cd a été étudié chez 15 espèces différentes de poissons. L'influence de plusieurs facteurs biologiques sur l'AE a été étudiée pour le Cd avec notamment une information conséquente relative aux effets du type de nourriture ingérée (e.g. Ni et al. 2000, Xu & Wang 2002) et dans une moindre mesure aux effets d'une pré-exposition en Cd par voie trophique (Zhang & Wang 2005) ou encore à la taille des organismes prédateurs (Zhang & Wang 2007). Parmi les travaux présentés dans cette thèse, les métaux non-essentiels n'ont été que peu considérés. Seul l'Ag a fait l'objet d'une étude plus approfondie (**Annexe 4**). En effet, la bioaccumulation de cet élément par les poissons a fait l'objet de recherches dans les années 1990-2000 (e.g. Baudin & Garnier-Laplace 1994, Ausseil et al. 2002, Bertram & Playle 2002) notamment en raison des potentielles conséquences du relargage de l'isotope ^{110m}Ag lors de l'accident de la centrale de Tchernobyl. L'intérêt pour ce métal dans la recherche en écotoxicologie s'est amoindri par la suite. Toutefois, à l'heure actuelle, de nouvelles utilisations de l'Ag ont émergé. En effet, ce métal est couramment utilisé en électronique et électricité car il présente une meilleure conductivité électrique que le cuivre et assure la protection des dispositifs électroniques utilisés en aéronautique. Par ailleurs, des sources de pollution plus récentes du milieu marin par l'Ag ont également été identifiées, liées notamment à l'utilisation des nanoparticules d'Ag (Savery et al. 2013) ou au traitement des eaux usées (Sanudo-Wilhelmy & Flegal 1992). Ainsi, il apparaît intéressant de poursuivre l'effort de recherche sur ce métal d'autant plus dans le contexte de changements globaux auxquels les écosystèmes aquatiques doivent faire face.

La revue de la littérature effectuée dans l' **Article 1** a permis de mettre en évidence le manque d'information relatif au transfert trophique des métaux essentiels chez les poissons. Parmi les métaux essentiels étudiés (Co, Cu, Cr, Mn et Zn), seul le Zn a été largement étudié. Tout comme pour le Cd, se sont principalement les effets de facteurs qui ont étudiés sur l'AE du Zn chez les poissons tels que le type de nourriture ou encore la pré-exposition au Zn stable contenus dans la nourriture (e.g. Ni et al. 2000, Baines et al. 2002, Xu & Wang 2002, Zhang & Wang 2005).

Le Zn est un des métaux essentiels les plus importants pour les poissons en raison de son rôle structurel et catalytique dans plus de 300 protéines. Il sert par ailleurs de cofacteur dans de nombreux systèmes enzymatiques jouant ainsi un rôle essentiel dans le métabolisme des lipides, des protéines et des glucides (Watanabe et al. 1997, Bury et al. 2003). Ce métal est ainsi directement impliqué dans la croissance, la reproduction, le développement et l'immunité chez les poissons (Tacon 1987, Watanabe et al. 1997). Par ailleurs, le Zn, plus présent dans les environnements côtiers peut s'avérer toxique à des concentrations plus élevées (e.g. Hogstrand 2011). L'ensemble de ces raisons peut expliquer l'engouement pour ce métal dans la recherche en écotoxicologie et en écophysiologie. L'information disponible dans la littérature concernant ce métal a permis de fournir des bases de comparaison intéressantes en vue de l'interprétation des résultats obtenus lors des différents travaux réalisés durant cette thèse. Par ailleurs, les connaissances mécanistiques plus approfondies relatives notamment au transport, à l'excrétion et au stockage du Zn chez les poissons ont permis une meilleure compréhension des mécanismes physiologiques à l'origine des résultats d'AE obtenus pour les différentes conditions expérimentales considérées.

Concernant les autres éléments essentiels et notamment le Co et le Mn, étudiés dans différents travaux présentés dans cette thèse, moins de données sont disponibles dans la littérature. Toutefois ces éléments essentiels ont des rôles conséquents pour la survie des organismes et notamment des poissons. En effet, le Co fait partie intégrante de la cyanocobalamine (vitamine B12) et, en tant que tel, est essentiel à la formation de globules rouges et au maintien du tissu nerveux (Tacon 1987, Watanabe et al. 1997). Chez le poisson, le Mn est indispensable au fonctionnement normal du cerveau et pour le métabolisme des lipides et des glucides. Cet élément a un rôle clé en tant que cofacteur pour certaines enzymes et en tant qu'élément structurel des métalloenzymes. En tant que cofacteur ou composant de plusieurs systèmes enzymatiques clés, le Mn est impliqué, entre autres, dans la formation des os et la régénération des globules rouges (Tacon 1987, Watanabe et al. 1997).

Les études antérieures concernant l'AE de ces métaux chez les poissons sont principalement limitées à des travaux visant à la reconstitution de chaines trophiques simplifiées en laboratoire (Mathews & Fisher 2008) ou la comparaison des AEs entre élasmobranches et téléostéens (Mathews et al. 2008).

L'ensemble de ce travail doctoral permet donc d'apporter une information nouvelle et plus exhaustive sur le transfert trophique et notamment sur l'AE de ces métaux chez les poissons (**Fig. 15**).

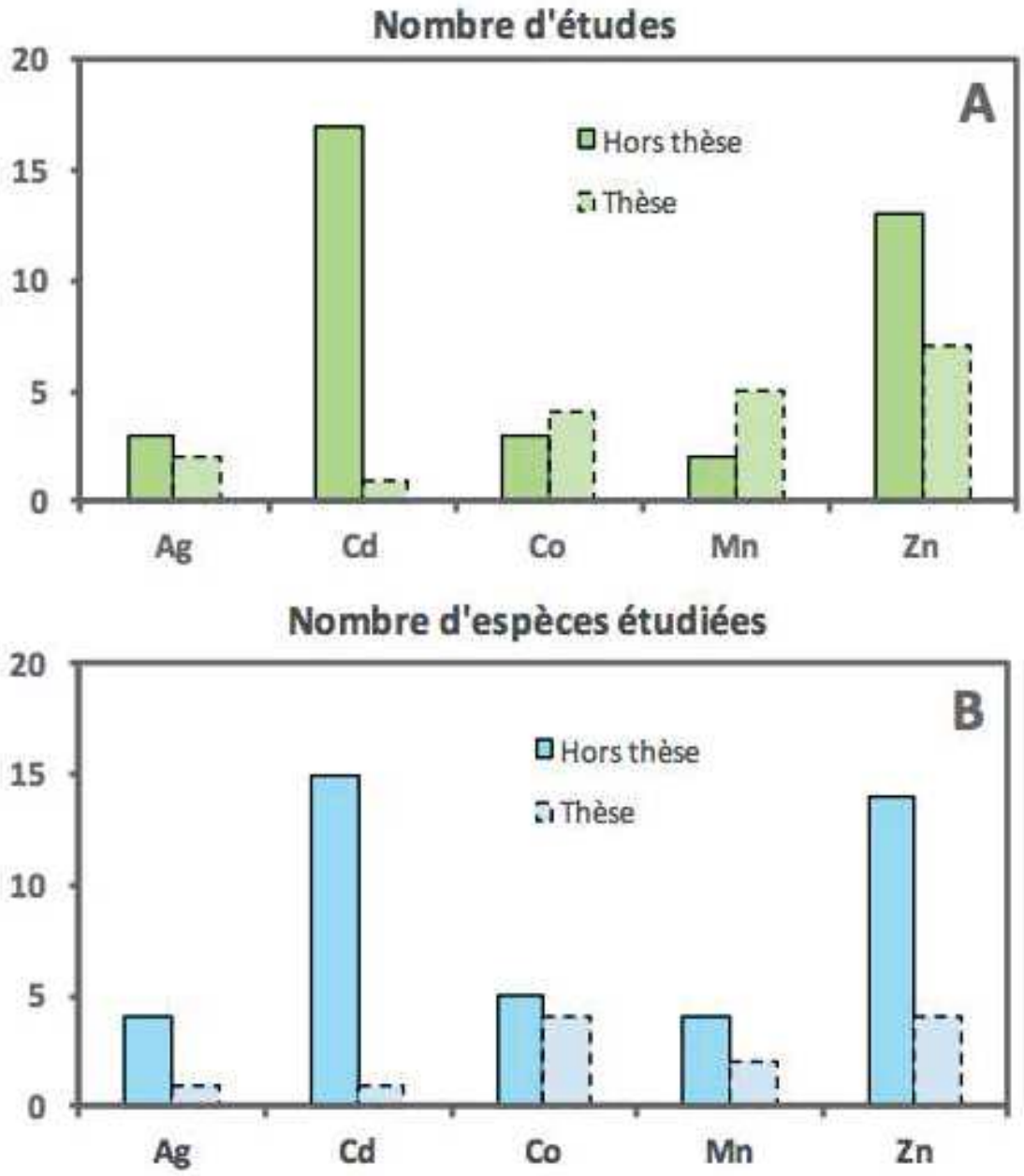


Figure 15. Bilan des recherche effectuées sur la mesure de l'efficacité d'assimilation de deux métaux non-essentiels (Ag et Cd) et de trois métaux essentiels (Co, Mn et Zn) et apport des travaux réalisés durant cette thèse exprimé (A) en nombre d'études publiées ou en voie de publication et (B) en nombre d'espèces étudiées.

5.1.3. Le rôle majeur des facteurs biologiques sur l'AE des métaux

Un des points les plus importants qui ressort de l'ensemble des travaux inclus dans cette thèse concerne le rôle majeur du type de nourriture ingéré sur le transfert trophique des métaux et tout particulièrement. En effet, d'après l'ensemble des résultats obtenus, il s'avère que les différences d'AE les plus importantes ont été mesurées lorsque les juvéniles turbots, modèle biologique retenu dans le cadre de cette étude pour son régime alimentaire varié (Sparrevohn & Støttrup 2008, Florin & Lavados 2010), ont été nourris avec différents types de nourriture (trois proies naturelles et du granulé utilisé en aquaculture ; **Article 3** et **4**). Il s'agit d'ailleurs du seul facteur, parmi tous ceux considérés dans cette thèse pour lequel des différences d'AEs ont été observées pour l'ensemble des métaux considérés à savoir trois métaux essentiels (Co, Mn et Zn) et un non-essentiel (Ag). D'autres études, réalisées avec d'autres espèces, ont déjà mis en évidence que le type de nourriture influence fortement l'AE des métaux. A titre d'exemple, Wang & Wong (2003) ont démontré chez le gaterin noir *Plectorhinchus gibbosus* que l'AE du MeHg variait de 66 à 98% en fonction du type de proies ingérées (crustacés et poissons). De la même façon Dang et al. (2009) ont mis en évidence que l'AE du Cu, un élément essentiel variait de 2 à 11% chez des pagres à tête noire *A. schlegeli* nourris avec des mollusques ou des crustacés.

L'influence des autres facteurs étudiés dans les travaux réalisés durant cette thèse est plus mitigée. En effet, deux cas de figure ont été observés : soit il n'y a pas eu d'influence significative du facteur en question sur l'AE des métaux étudiés (i.e. taille, pH, biotoxines) soit des effets significatifs ont été observés sur une partie seulement des métaux étudiés (i.e. variations interspécifiques, température, salinité). D'un point global, les facteurs biologiques semblent avoir une influence plus importante sur le transfert trophique des métaux. A l'inverse, les facteurs environnementaux (température, pH, salinité),, bien que comprenant des variables environnementales connues pour fortement affecter la physiologie digestive des poissons, ont eu moins d'effets sur l'AE des métaux.

Peu d'études sur le transfert trophique des métaux chez les poissons ont considéré à la fois des facteurs biologiques et environnementaux. Toutefois, partant du principe qu'un poisson prédateur dans un environnement dulçaquicole doit faire face à des fluctuations de températures de l'eau, des différences dans la concentration et la composition métallique des proies ainsi que dans leur disponibilité, Van Campenhout et al. (2007) ont étudié l'influence de l'ensemble de ces facteurs sur l'AE du Cd et du Zn chez la carpe commune *Cyprinus carpio*.

Les résultats obtenus dans cette étude sont très constratés pour les deux métaux considérés (Cd et Zn). En effet, la concentration en Cd des larves de moucherons utilisées comme proies n'a pas affecté l'AE du Cd chez la carpe. Par contre, l'AE du Zn était négativement reliée à la concentration de Zn dans les proies. Par ailleurs, la quantité de nourriture ingérée et l'état de jeûne n'ont pas affecté de manière significative l'AE du Cd mais une diminution significative de l'AE du Zn a été constatée lorsque les carpes ont été nourris *ad libitum*. Le type de proies ingérée a également influencé le l'AE du Cd mais pas celle du Zn. Concernant le seul facteur abiotique considéré dans cette étude, la température, une diminution de 25 °C à 15° C n'a pas influencé l'AE du Cd, tandis que pour le Zn, une diminution significative de l'AE a été mesurée. Cet exemple illustre le fait qu'il peut être complexe de conclure à une influence plus forte des facteurs biologiques et c'est pourquoi il est nécessaire de continuer à utiliser le turbot comme modèle biologique en écotoxicologie pour confirmer les tendances observées dans les travaux réalisés durant ce travail de thèse.

5.2. EVALUATION CRITIQUE ET PERSPECTIVES

5.2.1. Un apport de connaissances plus exhaustif mais de nouveaux facteurs à étudier

Contrairement à d'autres études réalisées indépendamment, l'ensemble des travaux de cette thèse a permis, par l'adoption d'un modèle biologique préférentiel : le turbot *S. maximus* d'explorer une gamme relativement large de paramètres biologiques et environnementaux susceptibles d'influencer l'AE des métaux (type de nourriture, taille, variations interpséci-fiques, température, pH, salinité, biotoxines, **Articles 2-9**). La réalisation de l'ensemble des travaux dans un environnement similaire, en conditions contrôlées est également un atout en vue de la comparaison des résultats. Il s'agit d'un point important, car la variabilité interspécifique dans l'AE des métaux chez les poissons peut être marquée. A titre d'exemple, Ni et al. (2000) ont mis en évidence une AE du Zn variable de 3,9 ± 1,2% chez le poisson de verre *Ambassis urotaenia* à 20,7 ± 5,1% chez le périophthalme *Periophthalmus modestus* nourris avec les mêmes copépodes. Il apparaît donc primordial d'explorer l'influence des différents facteurs chez une même espèce pour dresser un bilan représentatif quant à leurs effets sur l'AE des métaux. Or, dans la littérature il n'existe que peu d'espèces ayant été utilisées comme modèles dans de nombreux travaux. Le vivaneau des mangroves *L. argentimaculatus* et le térapon *T. jarbua* sont les seules espèces à avoir été considérées plus largement (e.g. Xu & Wang 2002, Long & Wang 2005a, Zhang & Wang 2006, Dang & Wang 2010, Pan & Wang 2016).

Cependant, bien que de nombreux facteurs aient été étudiées chez ces espèces (e.g. type de nourriture, pré-exposition à des métaux, taux d'ingestion...), il existe encore un manque de connaissances concernant l'effet de certains facteurs environnementaux et notamment de certaines variables environnementales (pH, température, salinité…) sur les AEs des métaux chez ces espèces. Les travaux présentés dans le cadre de cette thèse, réalisés chez le turbot apportent donc une vision plus exhaustive du transfert trophique des métaux au sein d'une même espèce. Toutefois, comme indiqué dans les précédents chapitres, une sélection a due être opérée dans le choix des facteurs étudiés. Il apparaît donc important de poursuivre les recherches afin d'élucider les effets de facteurs chez le turbot qui n'ont pu être considérés dans le contexte de cette thèse (voir **Tableau 3**). Parmi ces facteurs, il y en a notamment deux qui semblent particulièrement pertinents de considérer.

Le premier concerne la concentration métallique dans les proies et dans l'eau. En effet, dans leur environnement, les poissons sont continuellement exposés aux métaux présents dans l'eau et leur nourriture. Pour le Zn, un des métaux les plus étudiés, de nombreux travaux ont révélé que des changements dans l'absorption des métaux et les processus physiologiques se produisent après une pré-exposition à cet élément (e.g. Clearwater et al. 2002, Niyogi & Wood 2003). Par exemple, après une exposition chronique à du Zn dissous, l'affinité branchiale de cet élément diminuait (Alsop et al. 1999, Alsop & Wood 1999). En revanche, après une exposition chronique à du Zn par la nourriture, l'absorption branchiale du Zn a augmenté. Enfin une élévation de la concentration en métallothionéines (MT), qui sont une famille de protéines riches en cystéine et de faible poids moléculaire ayant une affinité élevée pour les métaux, est a été observée après des pré-exposition à du Zn chez plusieurs espèces de poissons marins (dont le turbot) et d'eau douce (Bradley et al. 1985, George et al. 1992, Zhang & Wang 2005). Par ailleurs, chez le pagre à tête noire *A. schlegeli* (Long & Wang 2005b) ont démontré que les AEs du Cd et du Ag augmentaient de façon linéaire en fonction de la concentration de MT ou encore de la concentration métallique dans les organismes.

Enfin, il existe un facteur environnemental (abiotique) qui occupe une place de plus en plus conséquente dans la recherche en aquaculture et en écophysiologie : la concentration en oxygène dissous et plus particulièrement les effets de l'hypoxie. En effet, l'oxygène dissous joue un rôle essentiel dans le métabolisme aérobie chez les poissons et est sujet à de grandes fluctuations dans le milieu aquatique.

Tableau 3. Liste des facteurs (biologiques et environnementaux) les moins étudiés dans la littérature concernant l'AE des métaux chez les poissons.

Facteur	Type	Nombre d'études*	Eléments traces
Concentration en dioxygène dissous de l'eau	Environnemental	0/30	-
Concentrations métalliques dans les proies	Biologique	2/30	Ag, Cd
Différences intergénérationnelles chez le consommateur	Biologique	0/30	-
Fréquence et taux d'ingestion	Biologique	2/30	Cd, Se, Zn
Etat physiologique du consommateur	Biologique	2/30	Cd, Cr, Zn
Sexe du consommateur	Biologique	0/30	-
Taille (âge) du consommateur	Biologique	2/30	Ag, Am, Cd, Se, Zn
Sélection des proies par le consommateur	Biologique	0/30	-
Concentrations métalliques dans l'eau	Environnemental	3/30	Ag, Cd
Répartition subcellulaire des métaux dans les proies	Biologique	3/30	Cd, Hg, Se, Zn

* D'après la revue de la littérature présentée dans l'**Article 1** en excluant les travaux issus de cette thèse.

Ainsi, de nombreuses zones côtières sont très sensibles à l'eutrophisation et les événements d'appauvrissement de l'oxygène (i.e. hypoxie ou dans les cas extrêmes anoxie) sont devenus de plus en plus fréquents au cours du siècle dernier (Johannesse & Dahl 1996). Parce que les poissons respirent dans l'eau, l'hypoxie influe sur de nombreuses variables écologiquement importantes (Poulin et al. 1987, Pihl et al. 1991, Pichavant et al. 2000) et est sans aucun doute un facteur critique influençant l'évolution et l'histoire de la vie de nombreuses espèces de poissons (Randall 1981).

Les effets de l'hypoxie chez les poissons sont étudiés depuis de nombreuses années (e.g. Cech et al. 1984) et il est maintenant connu que l'hypoxie peut influencer la digestion et la croissance des poissons (pour revue détaillée voir Wang et al. 2009). Les effets de l'hypoxie sur la croissance ont été caractérisés dans de nombreuses études sur différentes espèces de poissons, et les résultats de nombreuses études indiquent que l'hypoxie entrave inévitablement la croissance et que cette réduction de la croissance liée à l'hypoxie est principalement attribuable à la réduction de la consommation alimentaire (Davis 1975, Brett et al. 1979) et une réduction de l'efficacité de conversion alimentaire (Chiba 1983, Chabot & Dutil 1999). Dans ce contexte, il apparaît tout à fait pertinent d'étudier expérimentalement les effets de l'hypoxie sur l'AEs des métaux chez les poissons. Pour l'ensemble de ces raisons, de futures études sur l'AE des métaux chez le turbot devraient être menées en considérant les deux facteurs proposés ici.

5.2.2. Le transfert trophique des métaux : intérêt de l'organotropisme

L'AE est un paramètre physiologique qui présente l'avantage de pouvoir être comparé quantitativement entre les métaux, les espèces de poissons, les aliments utilisés et les conditions environnementales (Wang & Fisher 1999, Croteau et al. 2007). C'est pourquoi il s'agit de l'un des paramètres les plus pertinents et donc l'un des plus utilisés pour quantifier le transfert trophique des métaux chez les poissons. Toutefois, la détermination de l'AE, qui est approche intégrative, ne permet pas de mettre en évidence certains aspects du transfert trophique des métaux tels que notamment la dynamique interne de transport et de stockage dans les organismes. Ces informations s'avèrent pertinentes pour comprendre l'implication dans le temps des différents compartiments corporels dans l'absorption des métaux et leur excrétion. En effet, bien que certains mécanismes soient décrits dans la littérature pour quelques métaux et notamment du Zn (e.g. Bury et al. 2003, Hogstrand 2011), il s'avère qu'il peut exister des variations importantes en fonction des métaux.

La détermination de la dynamique du transfert inter-organes des métaux, grâce à des dissections régulières des différents organes et tissus pendant la phase de dépuration s'avère donc être une approche complémentaire pertinente. Bien qu'intéressante, cette approche n'est pas souvent rapportée dans la littérature. Elle a cependant toutefois été appliquée dans le cadre d'études toxicocinétiques chez des poissons (e.g. Hogstrand et al. 2003) ou encore chez d'autres prédateurs aquatiques actifs comme les céphalopodes (Bustamante et al. 2002, 2004).

Dans le cadre de cette thèse, bien qu'une telle approche mécanistique n'ait pas été systématique, elle a notamment été utilisée dans l'**Article 5**. Ainsi, des dissections ont été réalisées à différents temps durant la dépuration du Co et du Zn chez deux espèces de poissons tropicaux, il a été possible de mettre en évidence que le Co, les AE étaient très faibles et très similaires entre les deux espèces de poissons. Des différences, cependant, se sont produites en termes de concentration et de distribution entre les différents compartiments corporels. Les résultats soulignent le rôle majeur joué par le foie dans le stockage de Co dans le pavillon tacheté alors que chez le poisson-lune argenté la distribution est plus diffuse (**Fig. 16**). Par ailleurs, en réalisant des dissections à la fin de la dépuration de poissons exposés à différents facteurs (type de nourrissage, fréquence de nourrissage, pH et température de l'eau, **Article 10**), c'est à dire quand l'activité dans les turbots est stabilisée, nous avons démontré que l'organotropisme du Zn reste constant quelque soit les conditions expérimentales (**Fig. 17**). Ceci peut s'expliquer par la capacité des poissons à maintenir l'homéostasie de cet élément à des concentrations non-toxiques. Ces exemples montrent l'intérêt de la complémentarité entre l'étude de l'AE des métaux et de leur distribution chez les poissons.

5.2.3. Vers une nouvelle approche méthodologique : les expériences de « multi-stresseurs »

La plupart des travaux réalisés durant cette thèse ont consisté à exposer expérimentalement des organismes, en l'occurrence des poissons à différentes valeurs d'un facteur donné (biologique ou environnemental) et de suivre les effets de ces changements sur le transfert trophique et plus particulièrement l'AE des métaux chez ces poissons. Il s'agit d'une approche où l'influence de chaque facteur est étudiée de façon indépendante (i.e. dans des expériences séparées). Toutefois, dans leurs environnements naturels, les poissons sont soumis à des variations simultanément de très nombreux facteurs biologiques et environnementaux.

Il est donc nécessaire de considérer cet aspect pour accroître notre compréhension du transfert trophique des métaux chez les poissons.

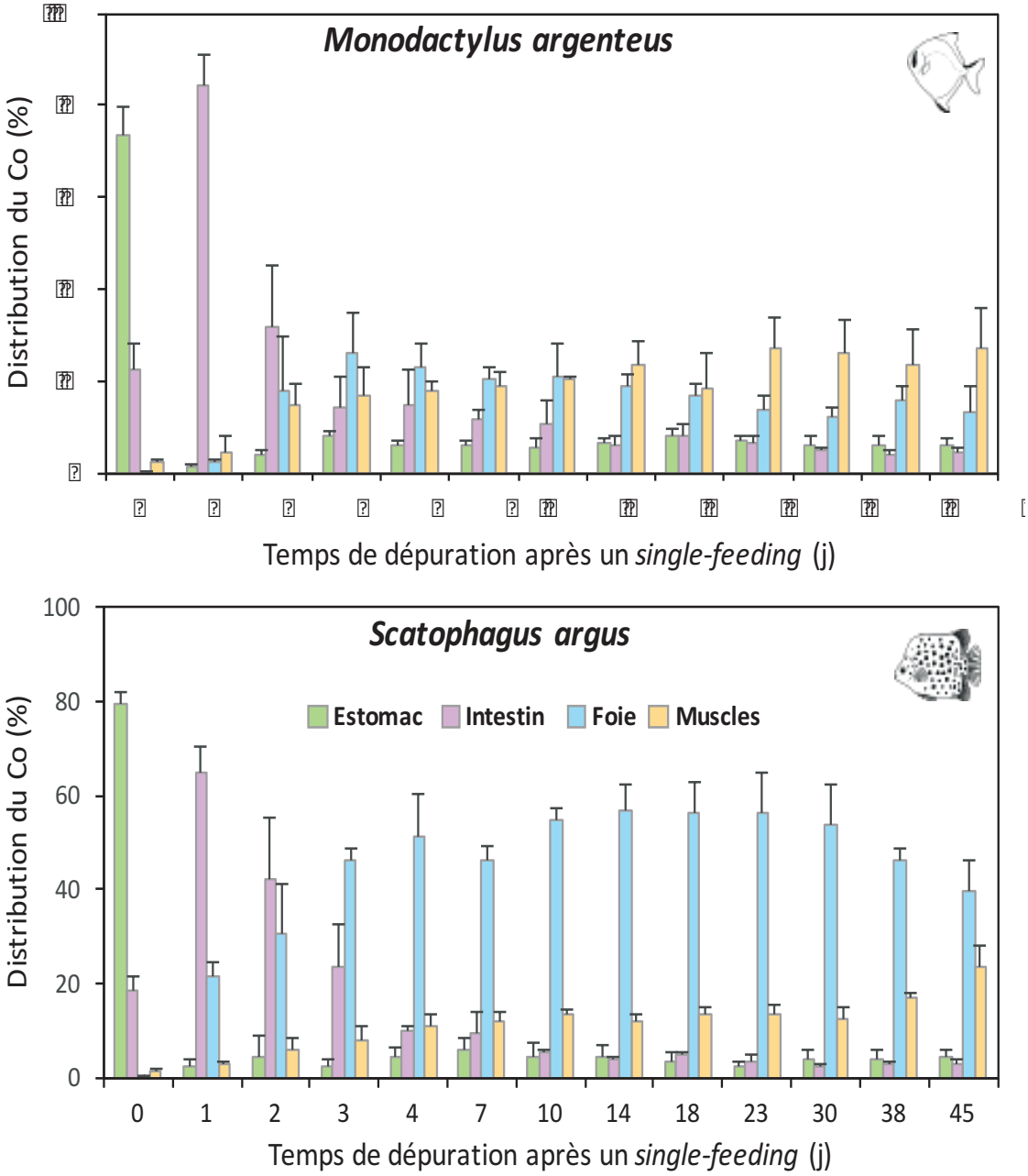


Figure 16. Distribution du Co dans 4 compartiments corporels (intestin, estomac, foie et muscles) chez des juvéniles de poisson-lune argenté (*M. argenteus*) et de pavillon tacheté (*S. argus*) en cours de dépuration après un *single-feeding* avec des artémies radiomarquées (^{57}Co). À chaque temps, trois poissons ont été disséqués. Données issues de l'**Article 5**.

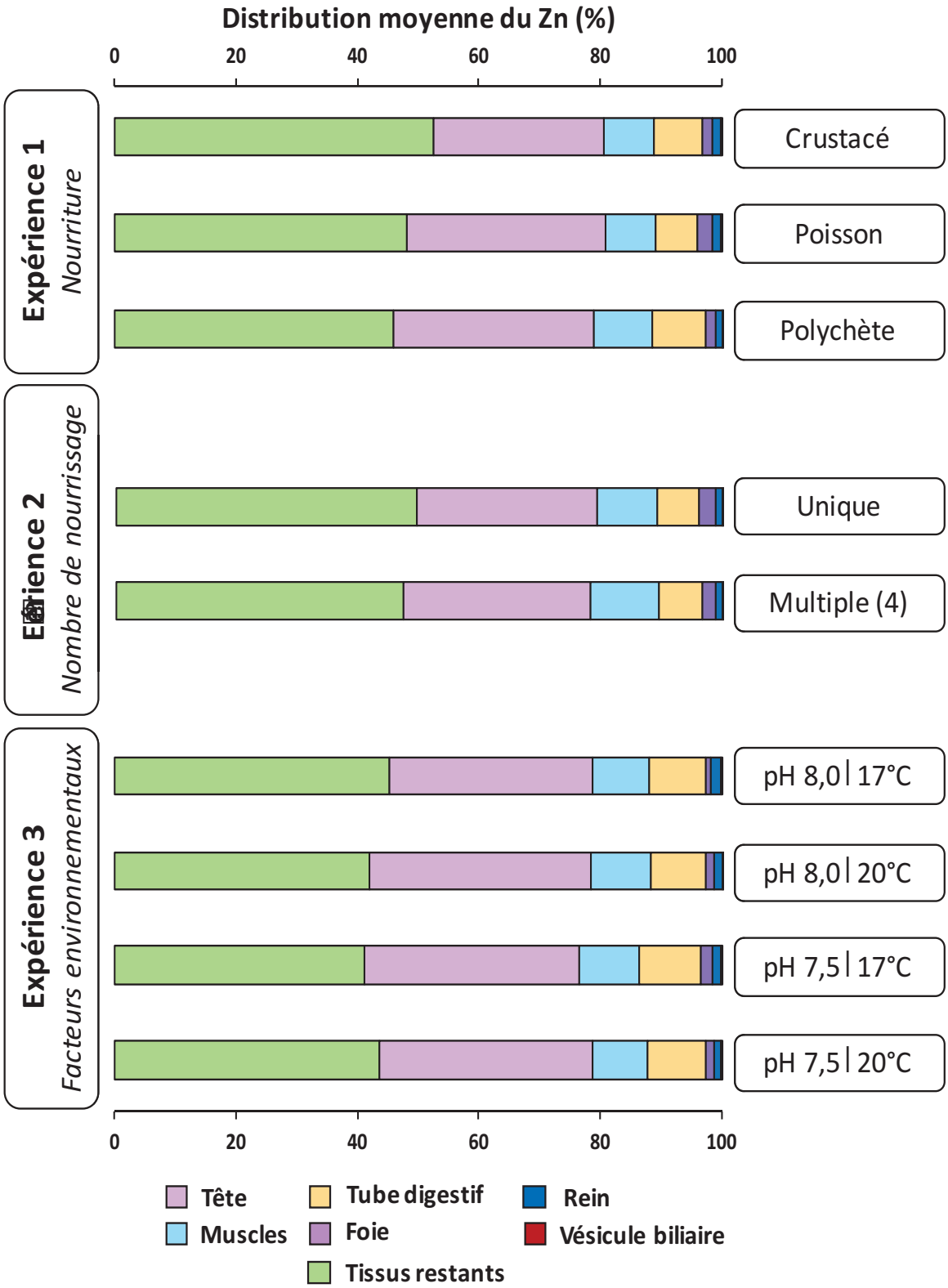
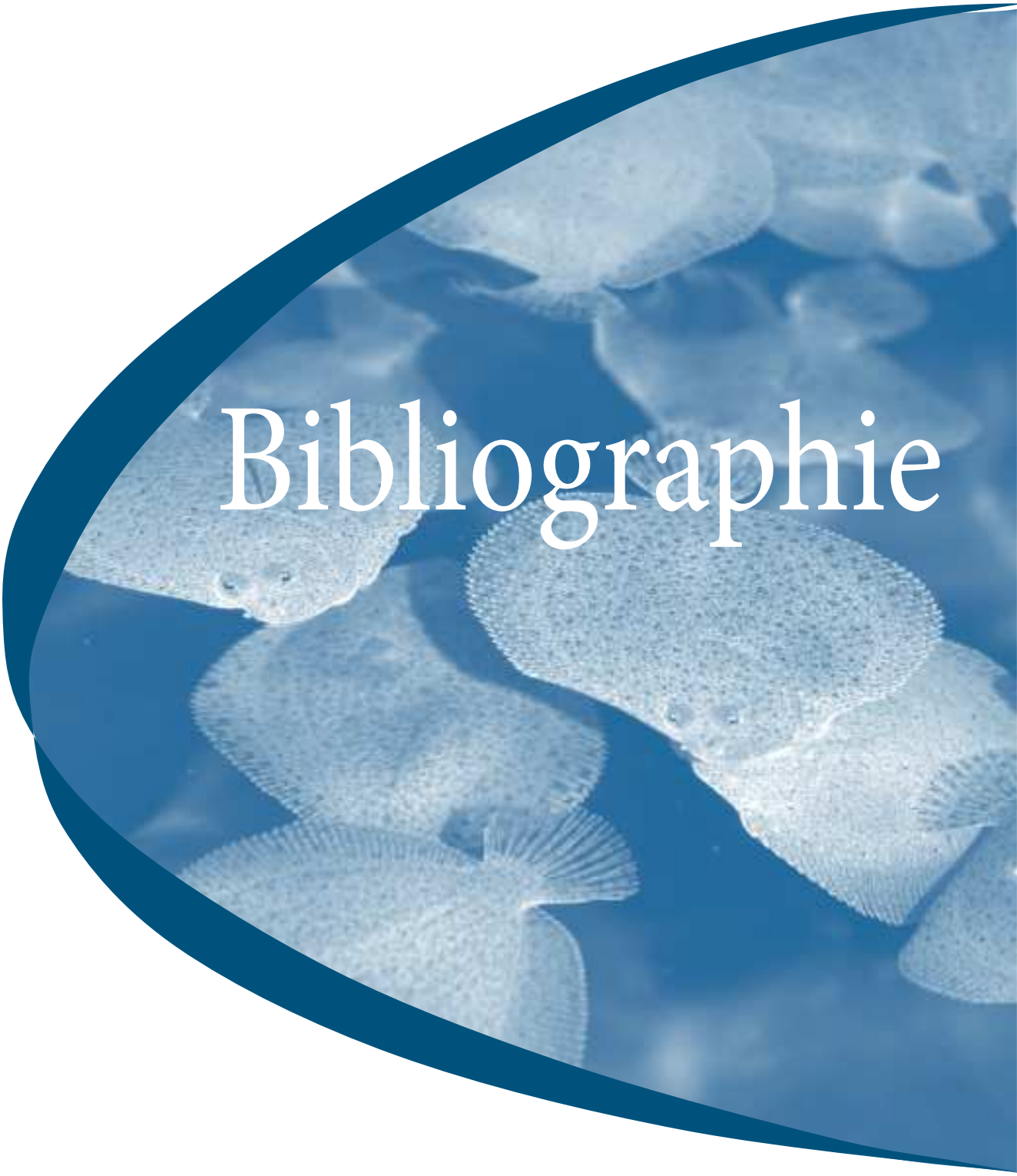


Figure 17. Distribution moyenne du Zn (%) dans 7 compartiments corporels de juvéniles de turbot nourris en *single-feeding* avec différentes proies radiomarquées (Expérience 1, n=5), nourris une ou plusieurs fois avec des granulés radiomarqués (Expérience 2, n=5) ou maintenus dans différentes conditions de pH et de température de l'eau (Expérience 3, n=4). Les tissus restants incluent les résidus de peau et de muscles, le squeletee, les nageoires et le cœur. Données issues de l'**Article 10**.

Ainsi, maintenant qu'un certain nombre de facteurs ont été étudiés, la tendance actuelle en recherche en écotoxicologie est d'accroître la complexité des systèmes expérimentaux en étudiant les influences de la combinaison de plusieurs facteurs au sein d'une même expérience. En ce qui concerne les effets physiologiques, la combinaison de deux facteurs pourrait être simplement la somme de chaque changement individuel (effet additif). Cependant, des interactions plus compliquées peuvent se produire, par exemple lorsque les effets combinés sont moindres (effets antagonistes) ou supérieurs à leur somme (effets synergiques) (e.g. Flynn et al. 2015) ce qui rend plus difficile l'interprétation des résultats. Pour ces raisons, et également dues aux difficultés méthodologiques liées au contrôle simultané de plusieurs facteurs en expérience, il n'existe encore que peu d'études en écotoxicologie des métaux adoptant ce type d'approches. Toutefois, dans le contexte des changements globaux, certaines études ont considéré pour étudier les effets combinés d'une élévation de température de l'eau et d'une diminution de pH sur la bioaccumulation des métaux chez des mollusques (e.g. Lacoue-Labarthe et al. 2009, 2012, Belivermiş et al. 2015). Dans le contexte de cette thèse, certaines expériences ont été réalisées dans ce sens avec notamment l'étude combinée de l'influence de la température de l'eau et du pH sur l'AE de l'Ag, du Co et du Zn chez le turbot *S. maximus* (**Article 8**) ou encore l'ingestion de biotoxines associée à une réduction du pH sur l'AE du Zn chez la même espèce (**Supplementary Data** de l'**Article 6**). Grâce notamment aux différents travaux réalisés durant cette thèse, l'influence d'un plus grand nombre de facteurs sur le transfert trophique des métaux chez les poissons et notamment le turbot est maintenant mieux connue ce qui permet de fournir les bases de connaisances préalables à la réalisation d'expériences de « multi-stresseurs » offrant une meilleure représentation des mécanismes pouvant intervenir en milieu naturel.



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Annexes

ANNEXE 1

Overview of trace elements trophic transfer in fish through the concept of assimilation efficiency

Pouil S[1,2], Bustamante P[2], Warnau M[1,3], Metian M[1] (soumis) Overview of trace elements trophic transfer in fish through the experimental concept of assimilation efficiency. *Marine biology*.

ABSTRACT: Among the different accumulation pathways of trace elements, water has initially retained the attention of the scientific studies on fish but the trophic transfer gradually gained considerations for now being generally identified as the major contribution pathway for trace element intake. The experimental approach is currently the most appropriate way to precisely quantify the trophic transfer of trace elements in fish. Thus, the assimilation efficiency (AE) of trace elements from ingested food is a commonly-determined parameter. However, there are still some discrepancies in the literature regarding the definition and the determination of AE in aquatic organisms and especially in fish. In this context, this review gathers the information about this concept as well as a description of the methods and protocols used to quantify the AE of trace elements thanks to experimental studies. It also looks over the main results concerning trace element AE in fish from the available literature. Most studies reporting AE considered the effects of biotic factors, especially the influence of the quality of the food. Abiotic factors have received less attention although they affect fish physiology and by extension potentially affect AE of trace elements. The need for further investigations is thus rising from the review, especially looking at the influence of abiotic factors such as temperature, salinity or pH on trace element AE or in the context of multiple stressors co-occurrence; this will help the better understanding trophic transfer of trace elements in fish and thus the overall their bioaccumulation in fish.

Keywords: Fish, Metals, Radioecology, Trophic transfer, Assimilation efficiency

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1. INTRODUCTION

In the field of ecotoxicology, the first use of fish in scientific studies originated in the 1930s with the purpose of testing the effect of various chemicals on them, including toxic trace elements usually released in aquatic environments by anthropogenic activities (Valavanidis and Vlachogianni 2010). Since then, fish have proved their suitability for ecotoxicological studies (Braunbeck et al. 1998) given their broad species diversity, the wide range of diets (from algae to other fish) and their broad geographical distribution in various environments. Furthermore, the relevance of fish in ecotoxicology is also connected to their ecological and economic importance (Holmlund and Hammer 1999; Tidwell and Allan 2001). Fish accumulate trace elements through both the dissolved and particulate pathways but the diet appears to be the predominant source for a series of elements (Xu and Wang 2002; Mathews and Fisher 2009). Therefore, understanding the trophic transfer of trace elements is a key aspect to assess the accumulation capacities in fish and their exposure to contaminants. Since distinction of the food contribution to the global bioaccumulation is complex to perform on individuals collected in the field, experimental approach appears to be the best option to assess unambiguously the trophic transfer of trace elements in fish (Wang and Fisher 1999).

One of the most relevant parameters to quantify trophic transfer of a contaminant its the assimilation efficiency (AE) from ingested food. AE is a first-order physiological parameter that can be quantitatively compared among trace elements, fish species, diets and environmental conditions (Wang and Fisher 1996; Croteau et al. 2007). Because dietary trace elements bioaccumulation is directly related to AE, this parameter is important to understand and predict global trace elements uptake (Wang and Fisher 1996; Luoma and Rainbow 2005; Croteau et al. 2007). This parameter is thus widely used in modern ecotoxicological studies. However, the concept of AE appears sometimes unclear in the literature due to some discordances in the way it is defined.

This review provides a general definition of the concept of AE, critically examines the methodologies used for AE measurements in fish to date and discusses the recent improvements made on the different methods. It also extensively analyzes results of trace elements' AE in fish reported in the literature. This review finally presents a summary of perspectives for guiding future studies on the subjecting and in complements the review made 18 years ago on AE in invertebrates (Wang and Fisher 1999).

2. THE NEED TO CLEARLY DEFINE THE CONCEPT OF ASSIMILATION EFFICIENCY

Assimilation efficiency (AE) is a physiological parameter determined to understand the trophic transfer of chemicals in organisms. However, as Wang and Fisher (1999) have already pointed out in their review, there are still discrepancies in experimental studies regarding the definition of the AE. According to these authors, "In bioenergetic studies, absorption of an element or compound equals total ingestion of the substance minus its quantity in faecal matter and is the sum of assimilation and post-digestive soluble excretion (i.e., loss of material into the dissolved phase after post-ingestive metabolism)". According to this definition, AE is the fraction of ingested element or compound that is incorporated into biological tissue, whereas absorption efficiency is the fraction of the ingested element or compound that passes through the gut epithelium by passive and active transports (Brett and Groves 1979; Penry 1998). Assimilation thus equals absorption minus defecation and excretion. This AE definition is in line with Warnau et al. (1996), which, in essence, indicates that the AE could be defined as the fraction of the ingested material that is tightly bound (i.e. incorporated) in the organs and tissues of a given organism. From a theoretical point of view, the difference between absorption and assimilation is obvious, but in practice, it is difficult to delineate quantitatively these two mechanisms at the whole-body level because during the gut transit, these physiological processes can occur at the same time. Thus, another physiological parameter is used to determine the required time to assess AE (e.g. Ni et al. 2000; Xu and Wang 2002): the gut transit time (GTT; i.e. duration that a food ration spends in the digestive tract between its ingestion and its defecation). Indeed, it is during that phase that the absorption of chemicals takes place. This method has some limitations which must be taken into account. Indeed, during the GTT, it is difficult to guaranty that only absorption of the ingested compounds takes place since excretion can also already intervene; hence a part of the absorbed fraction can be already excreted. Indeed, after intestinal absorption compounds such as trace elements are conveyed through the bloodstream to the liver and can be directly excreted via the biliary secretions discharged in the intestine or, latter through the gills and the urine (Wood 2011). Furthermore, there are some assumptions that egestion directly from the gut can occur through compounds secreted with digestive juices or sloughed inside detached enterocytes and then evacuated via the faeces or rectal fluid (Wood 2011).

In addition, we assume that part of the non-assimilated fraction might remain a bit longer in the digestive tract, associated to the intestinal mucus that can play a regulatory role in the absorption of ingested elements such as trace elements (Warnau et al. 1996, Bury et al. 2003). This situation might thus impact the accuracy for determining AE. This fact raises the crucial importance of the design and duration of experiments (i.e. the duration of the feeding period and the time during which depuration will be followed after ingestion of food) in order to accurately determine AE (see also section 3.2.3).

3. DETERMINATION OF THE ASSIMILATION EFFICIENCY IN FISH

3.1. Assimilation efficiency of macromolecules by fish

Since AE of a given element or compound is defined as its absorption minus its excretion, it could be calculated as the difference between its quantity ingested (quantity presents in the food) minus quantity egested (quantity in the faeces). This method, called mass-balance, has been used to study the AE of nutrients such as proteins and lipids in farmed fish. Using this method, AE can be calculated as follow:

$$AE\ (\%) = ((ingested\text{-}fecal)/ingested)\ \ x\ 100$$

However, urinary and branchial excretions are not taken into account in this calculation, which limits its accuracy. Furthermore, to be efficient, the mass-balance approach requires to be able to get an accurate quantification of the studied compound in the food and faeces. Some challenges may appear at this stage such as the ability to collect the faeces before their complete or partial dissolution in the water, that could lead to the loss or partial loss of the studied element (Choubert 1999).

Another method, based on the same mass-balance principle, uses an inert tracer, such as chrome oxide Cr_2O3 (Austreng 1978; Austreng et al. 2000), titanium oxide TiO2 (Weatherup and McCracken 1998; Vandenberg and De La Noüe 2001; Richter et al. 2003) or acid-insoluble ash (Sarker et al. 2016).

Incorporated in the compounded feed or ingredients/constituents of the food matrix (Tacon and Rodrigues 1984; Morales et al. 1999), the inert tracer allows correcting AE measurement for possible post egestion loss. In this case, AE can be calculated using the following equation (Maynard and Loosli 1969):

$$AE\ (\%) = ((\%\ \text{inert marker in the food}) / (\%\ \text{inert marker in the feces}) \times (\%\ \text{element in the feces}) / (\%\ \text{element in the food})) \times 100$$

This ratio is widely used in aquaculture nutrition since it does not require a complete recovery of faeces, as it is the case for the original approach. Its use is nevertheless limited nowadays given the fact that the selected inert tracer/marker must fulfil several characteristics, which are not easily met. Indeed, the inert marker, in principle, should: (1) be absolutely inert, without physiological effect on fish; (2) not be absorbed or metabolized; (3) not influence absorption and/or digestion; (4) be easily and quickly measurable (Choubert 1999). To the best of our knowledge, no marker perfectly fits all these conditions at once. Furthermore, this method does not take into account urinary and branchial excretion. Despite some disadvantages, this method is however still used in aquaculture studies to determine the assimilation efficiency of macromolecules such as proteins and lipids in fish (e.g. Zhang et al. 2015; Sarker et al. 2016). With an increasing research interest in the trophic transfer of trace elements in fish, other methods for AE determination, developed specifically for these elements, have emerged.

3.2. Assimilation efficiency of trace elements in fish

3.2.1. Use of radiotracers

One of the most efficient method to determine AE of trace elements in fish is the use of radiotracers. As isotopes of a given share the same properties among each other, radioactive isotope of a trace element can be used as tracer of that element. Thus, the two approaches described in the previous section (mass-balance and ratio) can be applied in the determination of AE for trace elements, using radiotracers in aquatic organisms such as fish. e determination of AE for trace elements, using radiotracers in aquatic organisms such as fish.

For example, Ni et al. (2000) have already compared AE of Cd, Cr and Zn in the mudskipper *Periophthalmus modestus* and the glassy *Ambassis urotaenia* obtained, using radiotracers with mass-balance and ratio approaches. In that study, the authors concluded that to two approaches give similar results. Since then, the ratio approach has not been tested again in the determination of trace element AE in fish.

In addition to the two previous methods, the use of radiotracers and particularly gamma-emitting radiotracers allowed developing a new approach in the determination of the AE of trace elements: the pulse-chase feeding mathod. This technique has many advantages that explain its widespread use in the literature (e.g. Xu and Wang 2002; Wang et al. 2012; Pouil et al. 2016). The use of gamma-emitting radioisotopes allows radiocounting fish alive, thus limiting the number of individuals to sacrifice and generating data with reduced biological variability (Warnau and Bustamante 2007). In the pulse-chase feeding method, fish are fed with radiolabelled food (natural prey or compounded feeds) are radiocounted just after the radiolabelled feeding. Then, fish are regularly counted alive in order to describe the depuration kinetic of the radiotracers and, thereby, to determine the AE (see details in section 3.2.3). The determination of AE based on a kinetic approach is done from a unique feeding with a radiolabelled food item. The fish are allowed to feed on radiolabelled food for a short period of time (shorter than their GTT; usually from 5 min to 2 h) to ensure that the radioactivity ingested can be accurately quantified without any possible radiotracer recycling from seawater due to leaching from the radiolabelled food, leading to an overestimation of AE. Recently, Pouil et al. (2017) provided an experimental validation of the single-feeding approach for the determination of Co, Cd, Mn and Zn AEs in the turbot *Scophthalmus maximus* fed with radiolabeled compounded food.

3.2.2. Improvements in the AE calculation

Two methods are commonly used to calculate trace element AE using gamma-emitters. For both methods, the proportion of trace elements retained in the fish during the depuration period is followed using regular gamma-countings of live organisms. In the first method, AE is determined at a given time and expressed as a percentage of trace element retained after the GTT from the total ingested fraction (e.g. Xu and Wang 2002; Van Campenhout et al. 2007; Goto and Wallace 2009).

Usually, in this method, the depuration is followed over a short time (i.e. few hours or few days; Table 1); it provides therefore a rapid insight on the transfer of trace elements in fish from their food. The second method is based on the actual determination of the trace element depuration kinetics. This technique has been extensively used in radioecological studies and improved by the use of multi exponential models, which parameters are solved by iterative adjustment. Depuration of trace elements are typically expressed as the percentage of remaining radioactivity (radioactivity at time t divided by the initial radioactivity measured in the organism at the beginning of the depuration period*100). Depuration kinetics are genrally best fitted by a two-component exponential model:

$$A_t = A_{0s} x\ e^{-kes\ x\ t} + A_{0l} x\ e^{-kel\ x\ t}$$

where A_t and A_0 are the remaining activities (%) at time t (d) and 0, respectively; k_e is the depuration rate constant (d^{-1}). “s” and “l” subscripts are related to the short- and long-lived component, respectively. The “s” component represents the depuration of the radiotracer fraction that is weakly associated with the organisms and rapidly eliminated (i.e. proportion associated with the faeces). The “l” component describes the depuration of the radiotracer fraction that is actually absorbed by the organism and eliminated slowly (Hubbell et al. 1965; Reichle 1967; Reichle et al. 1970; Whicker and Schultz 1982; Warnau et al. 1996). The long-lived component allows estimating the assimilation efficiency (AE), by calculating the y-axis intercept of the “l” component, of the radiotracer ingested with food (AE = A0l; Reichle 1967; Fowler and Guary 1977; Miramand et al. 1982). In some studies, the depuration of the assimilated fraction of trace elements was shown to be very slow (e.g. Pouil et al. 2015; Pouil et al. 2016). When the long-term depuration rate constant (kel) is not significantly different from 0, the “l” component of the exponential model can therefore be simplified and replaced by a constant (e.g. Pouil et al. 2015; 2016) and the equation becomes:

$$A_t = A_{0s} x\ e^{-kes\ x\ t} + A_{0l}$$

with $A_{0l} = AE$

This method requires that the fish be depurated for a sufficiently long period of time to get an accurate determination of the slope of the slowest depurating compartment. Usually, the depuration of the fish is followed for several weeks (Table 1). Because all the excretion processes (urinary, branchial and biliary) are taken into account, it is the most robust method to accurately determine AE (see section 3.2.3).

3.2.3. How the duration of depuration influences AE determination

In fish, the depuration can be usually described in three different phases. The first phase,
In fish, the depuration can be usually described in three different phases. The first phase,
usually few hours after the feeding, very rapid, corresponds to the passage of the ingested food from the stomach to the intestine where absorption process occurred (Baines et al. 2002; Dutton and Fisher 2011). The second phase, usually in the first week of depuration, is dominated by the occurrence of the absorption and rapid excretion processes (Baines et al. 2002; Dutton and Fisher 2011; Pouil et al. 2016). During phases 1 and 2, as shown by Pouil et al. (2017), almost all the trace elements ingested were distributed in the stomach and the intestine. Then, the third phase reflects the physiological turn-over from the slowest depurating compartment after absorption and excretion (Wang and Fisher 1999). The loss of trace elements during this phase is reduced and the body burden of trace elements is stabilizing. From a practical point of view, the duration of follow-up period of the depuration, defined by the experimenters, is therefore decisive to catch these biological processes. As explained in Section 2, GTT can be used to estimate the duration of the experiments in order to determine AE accurately. Some authors estimate the GTT by the frequent collection and the radiocounting of faeces subsequently to single-feeding and thus GTT ends at the moment when the last radioactive faeces have been collected (e.g. Ni et al. 2000). Thus, the duration of depuration is chosen to cover the GTT (i.e. in general from 24 to 72 hours in fish; e.g. Xu and Wang 2002; Van Campenhout et al. 2007; Goto and Wallace 2009). When depuration follow-up is made over a period close to GTT ("short-term" approach), AE is determined at a given time and expressed as a percentage of trace element retained. This approach does not allow taking into consideration the third phase depuration kinetics (i.e. when physiological turn-over occurs after absorption and excretion) as it is usually achievable when the duration of depuration extends over several weeks (i.e. "long-term" approach).

In order to compare AEs obtained using both "short-term" and "long-term" approaches, from data provided by Pouil et al. (2017, supplementary material), statistical comparison (i.e. Wilcoxon-Mann-Whitney non-parametric test) was done on 54Mn remaining activities at different times throughout a 21-d depuration in turbot *Scophthalmus maximus* fed with compounded pellets (Fig. 1). Remaining activities were stable from day 2 (i.e. less than 24h after the GTT) up to day 21 after the beginning of the depuration ($p>0.05$). Nevertheless, statistical comparison between individual AE estimated as the percentage of remaining activity after 2 days ("short-term" approach) and individual AE obtained by fitting a model (i.e. "long-term" approach) indicated a significant overestimation of AE by "short-term" approach ($p=0.04$).

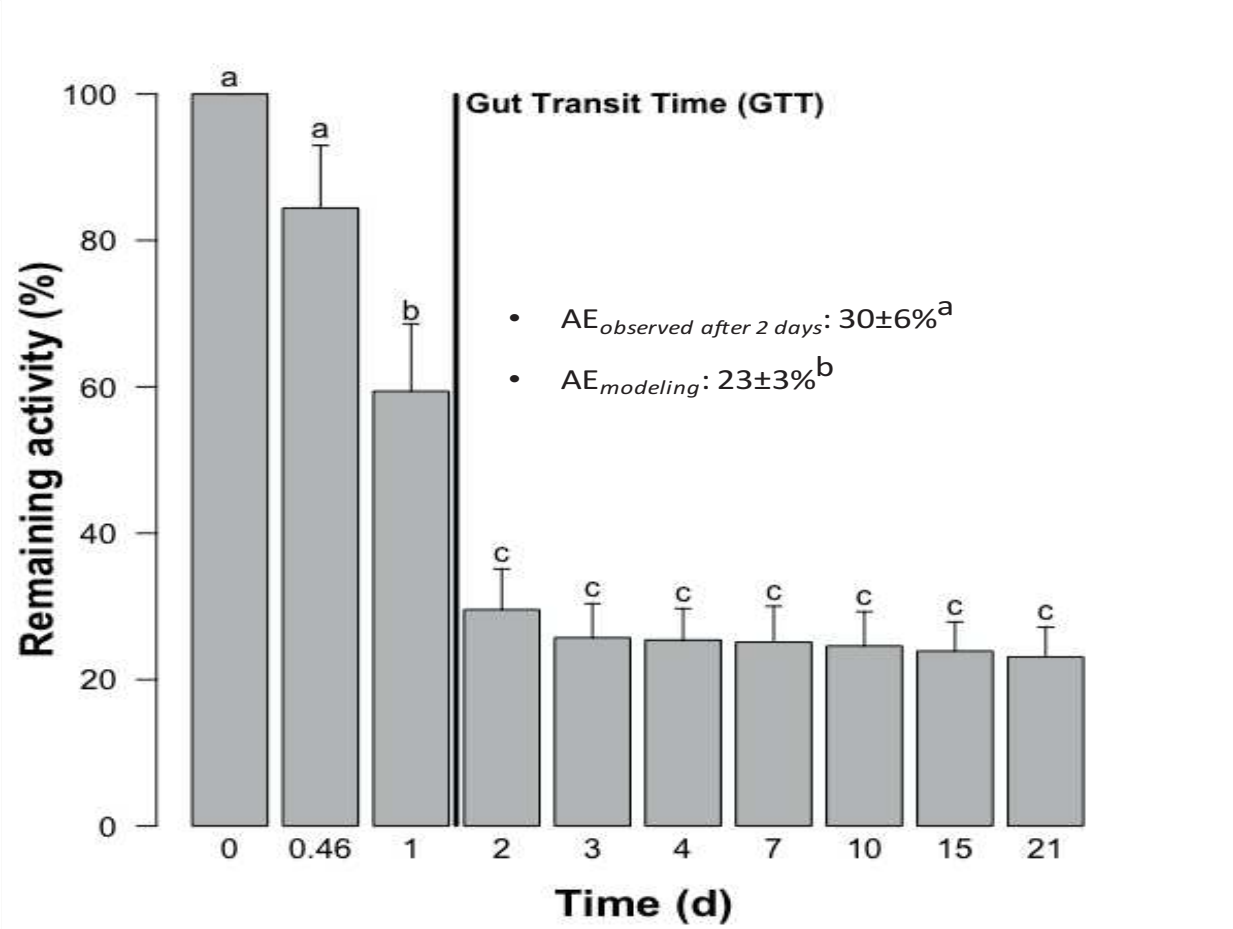

Figure 1. Remaining activities of ^{54}Mn during a 21-d depuration in turbot (n=12) fed radiolabelled with pellets. Data from Pouil et al. (2017, supplementary material). For comparison, AE observed after 2 days of depuration ("short-term" approach) and AEs estimated using kinetic modeling ("long-term" approach) are indicated as bullet-points. Letters indicated significant differences ($p<0.05$).

This example shows that, on a given dataset, “short-term” and “long-term” approaches may lead to different AE estimations. Such bias can be avoided using a sufficiently long period of depuration that encompasses both the absorption and the excretion processes and allows an accurate delineation of the AE. In the “short-term depuration” approach, a part of the excretion processes occurring during the last phase of the depuration are assumed negligible, which is obviously not correct. Therefore, this approach can only be considered after a careful investigation of the depuration processes in given experimental conditions.

4. REVIEW OF TRACE ELEMENT ASSIMILATION EFFICIENCY IN FISH

4.1. Results of AE related to trace elements and depuration duration

Figure 2 shows reported range of AEs of different essential (i.e. metabolically required) and non-essential (no biological role) elements in fish. This overview of results from 35 experimental studies reveals that the findings regarding trace element AE are overall similar regardless of the method of determination (i.e. “short-term” and “long-term” approaches, Fig 2A and 2B). However, using Zn, one of the most studied elements, an analysis of the coefficients of variation (i.e. allowing to estimate the dispersion of the values from the average) for AE values reveals that the “short-term” approach leads to a higher AE variability than the «long-term» approach. This analysis provides an overall picture of AE variability according to the approach adopted for its determination. These findings, however, must be nuanced by the fact that other experimental factors that can also affect the AE variability (e.g. objectives of the study, number of organisms, etc.) are not taken account.

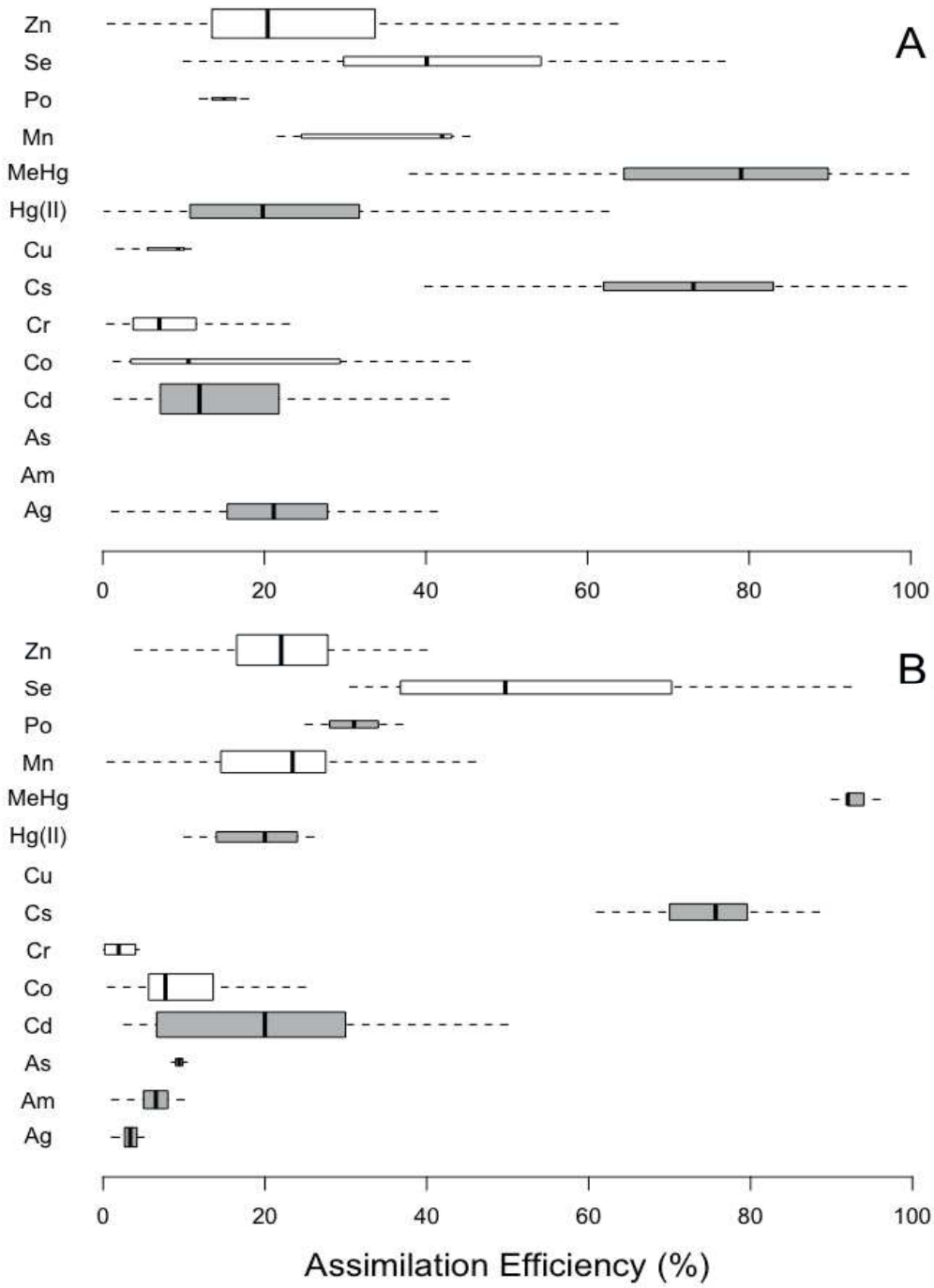

Figure 2. Comparison of AEs values of essential (white bars) and non-essential (grey bars) elements assessed in fish. Duration of experiments are either :(A) "short-term depuration" and (B) "long-term depuration" approaches. The width of the boxes is proportional to the number of observations. Extreme values are not represented. Data extracted from the literature are detailed in Table 1.

Table 1. Assimilation efficiencies of trace elements in freshwater and marine fish reported in experimental studies

Species	Objectives	Metal	Food	Depuration (d)	AE (%)	References
Acanthopagrus schlegeli	Allometry	Ag	Crustaceans	2	10-41	(Long and Wang 2005b)
	Food composition	Cd	Crustaceans - Fish - Molluscs - Pellets	1.5-2	2-38	(Wang et al. 2012)
	Trace element pre-exposure	Cu	Crustaceans - Molluscs	2	2-11	(Zhang and Wang 2005)
		Hg(II)	Pellet	2	3-55	(Zhang and Wang 2007)
		Z n	Crustaceans - Pellets	1.5-2	2-50	
Ambassis urotaenia	Interspecific comparison	Cd	Crustaceans	1	10-43	(Ni et al. 2000)
	Food composition	Cr	Crustaceans	1	1-10	
		Zn	Crustaceans	1-2.1	2-32	
Amphiprion ocellaris	Water pH	Mn	Pellets	20	1-10	(Jacob et al. in press)
		Zn	Pellets	20	24-35	
Cyprinodon variegatus variegatus	Pharmacokinetic model	MeHg	Phytoplankton - Pellets	0.1-35	38-100	(Leaner and Mason 2002)
Cyprinus carpio	Food composition and quantity	Cd	Insects - Oligochaetes - Molluscs	2	9-80	(Van Campenhout et al. 2007)
	Water temperature	Zn	Insects - Oligochaetes - Molluscs	2	20-97	
Danio rerio	Food composition	Ag	Polychaetes	3	1-7	(Boyle et al. 2011)
	Trace element pre-exposure	Cd	Crustaceans - Polychaetes	2.5-3	3-18	(Liu et al. 2002)
		Cr	Crustaceans	2.5	2-47	
		Zn	Crustaceans	2.5	12-54	
Dicentrarchus labrax	Food chain	Am	Fish	24	4-8	(Mathews and Fisher 2008)
		Cd	Fish	24	14-31	
		Co	Fish	24	13-28	
		Cs	Fish	24	76-82	
		Mn	Fish	24	24-42	
		Se	Fish	24	52-76	
		Zn	Fish	24	28-48	
Fundulus heteroclitus heteroclitus	Food chain	As	Crustaceans	9	9-10	(Dutton and Fisher 2011)
	Food composition	Cd	Crustaceans - Fish - Insects - Polychaetes	1-13	3-70	(Seebaugh et al. 2005)
	Subcellular control	Cr	Crustaceans - Polychaetes	9	0-4	(Goto and Wallace 2009)
		Hg(II)	Crustaceans - Polychaetes	9	10-26	(Mathews and Fisher 2008)
		MeHg	Crustaceans - Fish - Insects - Polychaetes	1-13	47-96	
		Po	Crustaceans	13	25-37	
Gambusia affinis	Food chain	Hg(II)	Crustaceans	6	25-78	(Pickhardt et al. 2006)6)
	Trace element pre-exposure	MeHg		6	81-98	
Ictalurus punctatus	In-vitro digestion	MeHg	Polychaetes	1.5	52-65	(Leaner and Mason 2002)
Lepomis microlophus	Food chain	Hg(II)	Crustaceans	6	0-18	(Pickhardt et al. 2006)
	Trace element pre-exposure	MeHg		6	84-94	
Lutjanus argentimaculatus	Food composition	Cd	Crustaceans - Molluscs	3	4-33	(Xu and Wang 2002)
	Ingestion rate	Cs	Crustaceans - Fish - Molluscs	3	82-99	(Zhao et al. 2001)
		Se	Crustaceans - Molluscs	3	27-60	
		Zn	Crustaceans - Molluscs	3	13-53	
Menidia sp	Food composition	Cd	Crustaceans	0.8	2-4	(Reinfelder and Fisher 1994)
		Co	Crustaceans	0.8	1-3	
		Se	Crustaceans	0.8	25-33	
		Zn	Crustaceans	0.8	4-8	

Table 1. To be continued

Species	Objectives	Element	Food	Depuration (d)	AE (%)	References
Monodactylus argenteus	Interspecific comparison	Co	Crustaceans	45	4-5	(Pouil et al. 2017)
		Zn	Crustaceans	45	14-16	
Morone saxatilis	Allometry	Ag	Crustaceans	13	17-20	(Baines et al. 2002)
	Food chain	Am	Crustaceans	13	5-7	(Mathews and Fisher 2008)
		Cd	Crustaceans - Fish	2-14	19-51	
		MeHg	Fish	2	82-94	
		Po	Fish	2	12-18	
		Se	Crustaceans	13-14	31-47	
		Zn	Crustaceans	13-14	21-45	
Periophthalmus modestus	Food composition	Cd	Crustaceans - Polychaetes	1-2	2-31	(Ni et al. 2000)
	Interspecific comparison	Cr	Crustaceans	1-2	1-26	(Ni et al. 2005)
	Salinity	Se	Crustaceans - Polychaetes	1-2	32-40	
		Zn	Crustaceans - Polychaetes	1-2	1-36	
Plectorhinchus gibbosus	Food composition	Hg(II)	Crustaceans - Fish	1	6-32	(Wang and Wong 2003)
		MeHg	Crustaceans - Fish	1	45-98	
Scatophagus argus	Interspecific comparison	Co	Crustaceans	45	5-6	(Pouil et al. 2017)
		Zn	Crustaceans	45	23-25	
Scophthalmus maximus	Allometry	Ag	Pellet - Polychaetes	21	0-4	(Mathews et al. 2008)
	Food chain	Am	Fish	21	6-10	(Pouil et al. 2015)
	Food composition	Cd	Fish - Pellets	21	6-42	(Pouil et al. 2016)
	Interspecific comparison	Co	Crustaceans - Fish - Pellets - Polychaetes	21	1-45	(Pouil et al. in press)
	Subcellular control	Cs	Fish	21	61-65	(Pouil et al. in press)
	Water pH	Mn	Crustaceans - Fish - Pellets - Polychaetes	21	22-46	
	Water temperature	Zn	Crustaceans - Fish - Pellets - Polychaetes	21	13-33	
Scyliorhinus canicula	Interspecific comparison	Am	Fish	21	5-7	(Mathews et al. 2008)
		Cd	Fish	21	25-33	
		Co	Fish	21	8-14	
		Cs	Fish	21	69-77	
		Mn	Fish	21	24-30	
		Zn	Fish	21	16-18	
Sebastiscus marmoratus	Food composition	Cs	Molluscs	2.75	62-83	(Pan and Wang, 2016)
Siganus canaliculatus	Food composition	Cd	Macroalgae	2	2-47	(Chan et al. 2003)
	Starvation	Cr	Macroalgae	2	3-24	
		Zn	Macroalgae	2	4-42	
Siganus fuscescens	Food composition	Cs	Macroalgae - Molluscs	2.75	40-67	(Pan and Wang, 2016)
Sparus aurata	Food chain	Am	Crustaceans - Fish	15-21	1-9	(Mathews and Fisher 2008)
	Interspecific comparison	Cd	Crustaceans - Fish	15-21	6-50	(Mathews et al. 2008)
		Co	Crustaceans - Fish	15-21	7-23	
		Cs	Crustaceans - Fish	15-21	71-89	
		Mn	Crustaceans - Fish	15-21	11-28	
		Se	Crustaceans	15	61-92	
		Zn	Crustaceans - Fish	15-21	4-25	

Table 1. To be continued

Species	Objectives	Element	Food	Depuration (d)	AE (%)	References
Terapon jarbua	Food composition Trace element pre-exposure Subcellular control	Ag Cd Cs Hg(II) MeHg Se Zn	Crustaceans Crustaceans - Fish - Molluscs Molluscs Fish - Molluscs Fish - Molluscs Crustaceans - Molluscs Crustaceans - Fish - Molluscs	2 1.5-2 2-2.75 2 2 1 .5 1.5	12-41 2-41 65-83 12-100 52-97 10-63 1-67	(Dang and Wang 2010) (Long and Wang 2005a) (Pan and Wang, 2016) (Zhang and Wang 2006)

Non-essential elements, such as Ag, Am, Cd, Cs, Hg(II), MeHg and Po, are the most studied trace elements with, in particular, Cd AE values available for 15 species of fish (Fig. 2, Table 1). Among the 7 studied essential elements: As, Co, Cu, Cr, Mn, Se, and Zn, the latter element is the one with the most AEs values available (more than 180 data expressed as Means ± SD). The analysis of the AEs for the different trace elements shows that there is no obvious relation between the essential character of a trace element and its assimilation by the fish, in constrat to what has been observed in invertebrates (Wang and Fisher 1999). Interestingly, the trace elements with the highest AEs are MeHg and Cs, which are both non-essential elements very efficiently assimilated by fish. These high AEs values explain for a large part why Hg and Cs biomagnify in aquatic food webs in both freshwater and marine ecosystems (e.g., Garnier-Laplace et al. 2000; Zhao et al. 2001; Harmelin-Vivien et al. 2012; Lavoie et al. 2013; Pan and Wang 2016). Among the most efficiently assimilated elements, Se is an essential trace element known to have an antagonistic action with Hg in aquatic organisms (Belzile et al. 2006). Thus, field investigations have shown that high Se concentrations may force a preferential assimilation of this element over Hg through a competitive adsorption on binding sites. The occurrence of Se at high concentrations may also restricts the solubility and bioavailability of Hg to aquatic organisms or reduce it methylation in freshwater ecosystems (Cuvin-Aralar and Furness 1991; Belzile et al. 2006; Yang et al. 2008). To the best of our knowledge, no experimental study has investigated such effect in fish.

4.2. Factors influencing trace element AEs in fish

In theory, AE can be influenced by both abiotic and biotic factors because the latter factors potentially affect fish physiology and bioavailability of, or bioaccessibility to trace elements. The biotic factors have been the most studied in the literature (Fig. 3). The AE of trace elements in fish depends on the relation between the prey and their predators (Fig .3). Thus, it is possible to distinguish two types of biotic factors: those related to prey and those related to predators. Numerous studies have investigated the influence of food quality (type of natural prey and compounded food) on AE in fish. Several studies have shown that, in the same predator species, AEs can be very different depending on the type of food ingested (e.g. Dutton and Fisher 2011; Wang et al. 2012; Pouil et al. 2016). By a mechanistic approach, some authors have studied the factors related to the prey (i.e. bivalves and oligochaetes) that could explain these differences.

In particular, based on studies initiated with invertebrates (i.e. crustaceans, Wallace and Lopez 1996; Wallace and Luoma 2003), the relationship between subcellular fraction of trace elements in food and AE observed in predators has been investigated in several species (Dang and Wang 2010, Zhang and Wang 2006).

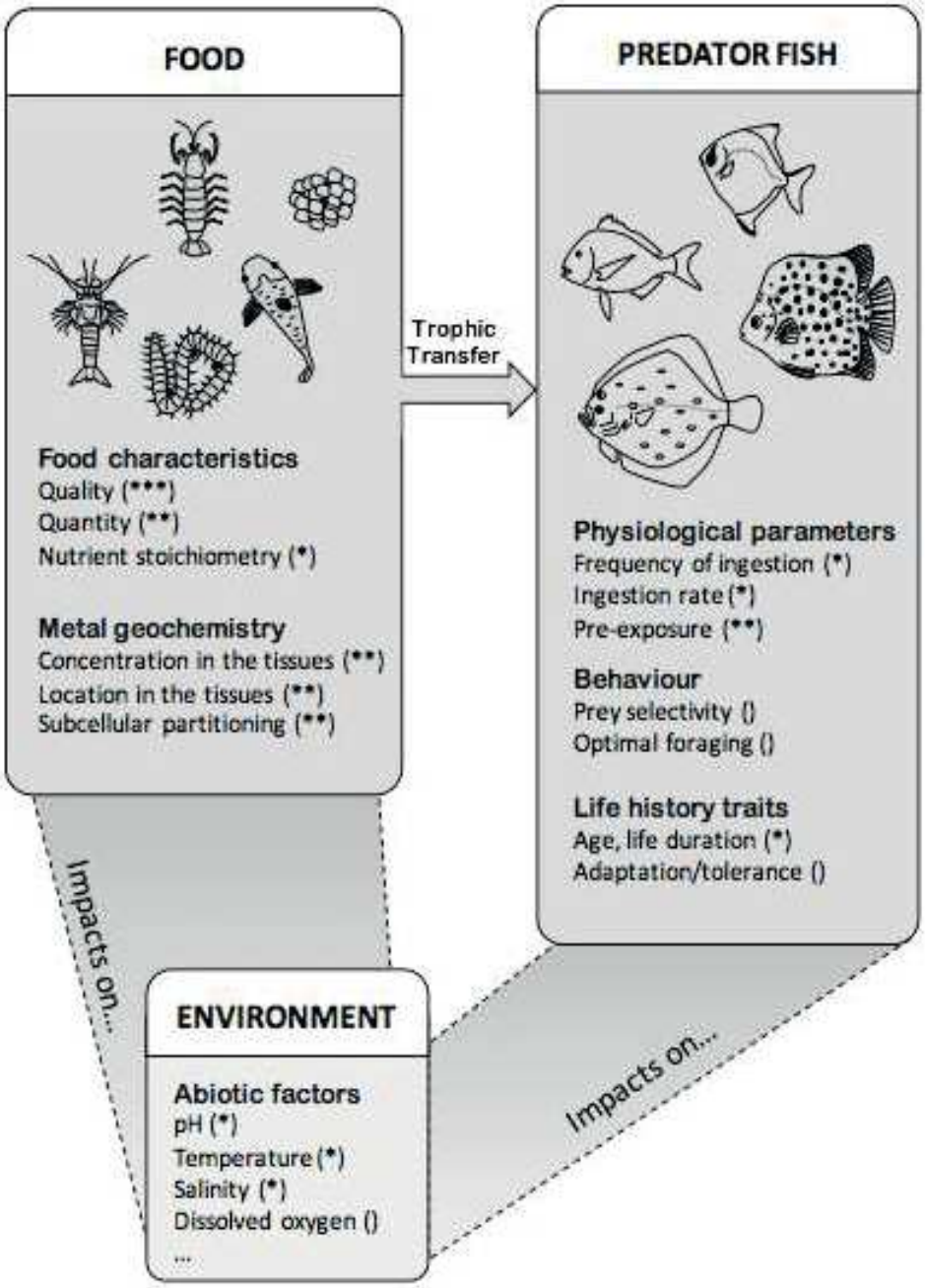

Figure 3. Schematic view of processes controlling the assimilation efficiency of metals in predator fish. The stars under brackets indicate the process already studied in the literature. Le number of stars is proportional to the quantity of information available in the literature. The absence of star indicates that the process is not yet investigated.

However, the results of these studies are contrasted. Some studies highlighted a positive relation between the cytosolic fractionation of Cd, MeHg, Se, and Zn in the prey and the AEs of these elements in different species of fish (Seebaugh et al. 2005; Zhang and Wang 2006; Dang and Wang 2010). However, more recently, Pouil et al. (2016) have shown that no obvious relationship was observed for essential elements (Co, Mn, and Zn) in juvenile *Scophthalmus maximus* fed more complex food matrices (complex pluricellular natural prey). Interspecific comparisons of trace element AEs have also been made (e.g. Ni et al. 2000, Pouil et al. 2017). The differences observed were often related to the trophic ecology of the organisms or their phylogeny. The influence of the predator size (i.e. allometry) on their AE was also investigated in black seabream *Acanthopagrus schlegeli* (Zhang and Wang 2007). In this study, Cd AE was independent of body size, whereas Se and Zn AE increased with the predator size. Regarding the feeding behavior of predators, although this parameter appears to be important in the understanding of trace element assimilation, there are still only few studies that have tackled this aspect. Among these, Van Campenhout et al. (2007) demonstrated in common carp *Cyprinus carpio* that frequency and rate of ingestion have a significant impact on the AE for Cd and Zn. In the same study, the influence of water temperature on AE has also been investigated. Authors observed that decreasing the temperature from 25°C to 15°C did not influence Cd AE, while a significant decrease of Zn AE was measured. The influence of trace element concentrations in the environment on AE in fish has been considered in few studies. It was for instance shown that Ag AEs were higher in waters highly contaminated by this element (Long and Wang 2005b; Boyle et al. 2011). However, no effect was observed for Cd or Zn (Zhang and Wang 2005; Boyle et al. 2011). Besides temperature or element concentrations, there is still a lack of knowledge regarding the possible effects of other abiotic factors on AE in fish.

Salinity, which is a key parameter in brackish and marine environments that influences both bioavailability of trace elements and fish physiology, has been investigated only in fish by Ni et al. (2005). These authors found no significant differences in Cd, Se, and Zn AEs in Periophthalmus modestus acclimated from 10 to 30 psu. Recently, environmental pH, known to influence the digestive physiology of fish (Zhang and Wang 2006; Dang and Wang 2010), was considered to explore the possible effects of ocean acidification on stomach pH and the assimilation of essential elements (Co, Mn, and Zn) in the clownfish *Amphiprion ocellaris* (Jacob et al. 2017).

Another study investigated the influence of pH and temperature on the AE of Ag, Co, and Zn in turbot *Scophthalmus maximus* (Pouil et al. 2017). These studies showed no significant effect of environmental pH.

5. CONCLUSION

AE is a key parameter in the trophic transfer of trace elements in fish and is therefore widely investigated in ecotoxicology and aquaculture research. Despite it is intensively used, there are still divergences in the definition of the AE concept, as highlighted in this review, which may affect its experimental determination. Thus, we provided a critical analysis of the methods used to determine AE in fish in order to provide guidance for further studies. In complement, the emphasis on trace element AE in fish reveals that among the 35 experimental studies identified from the available open literature, the influence of environmental variables in the trophic transfer of these elements has received little attention. This research topic continues to offer exciting and challenging scientific questions for ecotoxicology and fish nutrition research.

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ANNEXE 2

Comparing single-feeding and multi-feeding approach for experimentally assessing trophic transfer of metals in fish

Pouil S[1,2], Warnau M[1], Oberhänsli F[1], Teyssié J-L[1], Bustamante P[2], Metian M[1] (2017)
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ABSTRACT: Diet is an important pathway for metal uptake in marine organisms, and assimilation efficiency (AE) is one of the most relevant parameter to quantify trophic transfer of metals along aquatic food webs. The most commonly used method to estimate this parameter is pulse-chase feeding using radiolabeled food. This approach is, however, based on several assumptions that are not always tested in experimental context. The present work aimed at validating the approach by assessing single-feeding and multiple-feeding approaches, using a model species (the turbot *Scophthalmus maximus*). Using the kinetic data obtained from the single-feeding experiment, we tested whether the reconstruction of a multi-feeding was consistent with data provided by an actual multi-feeding performed under the same experimental conditions. Our results validated the single-feeding approach.

Keywords: Fish, Metals, Radioecology, Trophic transfer, Assimilation efficiency

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1. INTRODUCTION

Fish are exposed to various sources of metals, including water and food. It has become increasingly clear that diet represents the main contribution to global accumulation of metals (such as Mn, Cd, and Zn) in marine fish (e.g. [1–3]). Understanding the trophic transfer of metals in fish is therefore key to properly qualify and quantify their accumulation capacities [4]. Bioaccumulation though trophic transfer in fish has been studied in natural environments (e.g. [5–7]), but the relationship between metal concentration in prey and bioaccumulation in consumers/predators is difficult to establish under these conditions. Indeed, metal concentrations in whole-body prey, identified by stomach content analysis, are often compared with those in predator specific tissues without questioning bioaccumulation and biotransformation processes, feeding relationships, and trophic status [7–10]. Experimental approach in controlled conditions is an excellent option to unambiguously assess the transfer of a metal from an organism to another [9], in particular by using radiotracer techniques because of their high sensitivity [11,12].

One of the most relevant parameters to quantify trophic transfer is the assimilation efficiency (AE), i.e. the proportion of metal in the prey that is assimilated by the consumer (e.g. [13–15]). AE can be compared quantitatively among different elements, biological models, food items and environmental conditions. To estimate this parameter, one valuable method used since the 1980s is the pulse-chase feeding (e.g. [16–18]). Briefly, this technique consists of feeding organisms with radiolabeled food (live prey or compounded feed) for a short period of time (typically shorter than their gut transit time) and then to follow the depuration kinetics of the radioisotopes [18,19]. The limited period of feeding ensures that the ingested fraction can be accurately quantified by counting the whole organism and allowing to limit the confounding influence of elimination thus avoids error in the assessment of AE [18-19]. This technique has the advantage to not requiring complete recovery of egested feces, and of allowing easier estimation of AE than other methods. For example, the mass balance requires quantification of total ingestion, excretion, and egestion (i.e., loss of material in feces after absorption or post-ingestive metabolism) [12,19]. In principle, any radioisotope can be used. However, gamma-emitting radioisotopes are generally preferred as they allow radiocounting the predator alive, which allows limiting the number of individuals to sacrifice and generating data with reduced biological variability [12].

Experimental determination of AE using pulse-chase feeding approach assumes that each food ration is processed in the same way by the organism which is actually the prerequisite of this method as only one ration is followed, and then extrapolated to the entire digestion process. Some evidences indicate that this assumption may not always be satisfied. Indeed, some studies have suggested that concentration of Cd in food can impact its assimilation in sea urchins [18]. Furthermore, methods for determining the AE are variable in the literature [19, 20]. As explained by Wang and Fisher [19], two approaches are commonly used: the short-term and the long-term approaches. In the "short-term approach", the depuration phase is limited to a short period of time (i.e. gut purge phase), usually a few hours; [1,21,22]. Conversely, some authors recommend a "longer-term approach" (i.e. allowing to describe the loss of the fraction that is actually absorbed by the organism and slowly eliminated), for several days, weeks or months [15,23,24]. Using one method or the other may lead to variable AE measurements [19] with, in particular, a higher estimation of AE in the "short-term" experiments.

In this context, the present study aimed at validating the pulse-chase feeding approach (i.e. single-feeding) in the turbot *Scophthalmus maximus* fed with radiolabeled compounded pellets using gamma-emitters (^{109}Cd, ^{57}Co, ^{54}Mn and ^{65}Zn) through a single-feeding vs. a four-feeding experiment.

2. MATERIALS AND METHODS

2.1. Origin and acclimation of organisms

In January 2014, one hundred juvenile turbot *S. maximus* were purchased from a fish farm (France Turbot) and shipped to the IAEA-EL premises in the Principality of Monaco. Fish were acclimated to laboratory conditions for 21 days (open circuit, 500-L aquarium; water renewal: 100 L h^{-1}; 0.45-µm filtered seawater; salinity: 38 p.s.u.; temperature: 15 ± 0.5°C; pH: 8.1 ± 0.1; light/dark: 12h/12h). During the acclimation period, the fish were fed a daily ration of 2% of their estimated biomass with 1.1-mm pellets (Le Gouessant).

2.2. Experimental procedure

2.2.1. Radiolabeling of pellets

In order to compare metal AE estimates in *S. maximus* fed by single- or multi-feedings, 1.1-mm manufactured pellets (Le Gouessant) were radiolabeled. Radiotracers of high specific activity were purchased from Isotope Product Lab (^{109}Cd as $CdCl_2$ in 0.5 M HCl, [$T_{1/2}$] = 463.9 days; ^{57}Co as $CoCl_2$ in 0.1 M HCl, [$T_{1/2}$] = 271.8 days; ^{54}Mn as $MnCl_2$ in 0.5 M HCl, [$T_{1/2}$] = 312.2 days; ^{65}Zn as ZnCl2 in 0.1M HCl, [$T_{1/2}$] = 243.9 days). Seventeen grams of dry pellets were dipped for 1 h in 22 mL of seawater previously spiked with 2 kBq mL^{-1} of ^{57}Co, ^{54}Mn and ^{65}Zn, and 4 kBq mL^{-1} of ^{109}Cd. Pellets were then dried for 48 h at 50°C and kept in a dry environment in order to prevent mold growth. The pellets used were radioanalysed (1g dry wet per measurement, i.e. ≈ 650 pellets) prior to each feeding. Activities were 4908 ± 155 Bq ^{109}Cd g^{-1} dry wt, 2305 ± 63 Bq ^{57}Co g^{-1} dry wt, 2215 ± 75 Bq ^{54}Mn g^{-1} dry wt and 2344 ± 79 Bq ^{65}Zn g^{-1} dry wt. In terms of stable metal concentration, these activities were negligible compared to those found in the pellets: they corresponded to 0.15 ng.g^{-1} for Cd, 0.3 ng g^{-1} for Co, 55 pg g^{-1} for Mn and 6 ng g^{-1} for Zn, which represent concentrations at least 3 orders of magnitude lower than those measured in non-radiolabelled pellets (see the details of the methodology in Supplemental Data; 0.6 ± 0.0 µg Cd g^{-1} dry wt, 0.3 ± 0.0 µg Co g^{-1} dry wt, 66.4 ± 0.2 µg Mn g^{-1} dry wt and 139 ± 4 µg Zn g^{-1} dry wt).

Although the acclimated fish consumed pellets in less than 1 min during regular feeding events, preliminary tests were performed to assess the possible leakage of radioisotopes from the pellets when placed in seawater. These tests consisted of pouring dry radiolabeled pellets (100 mg per treatment) for 1 to 10 min in 50 mL seawater and to measure any radioactivity in the seawater. This ratio pellets/seawater was intentionally high (approx. 10 times higher than in experimental conditions; worst case scenario). The radiotracer leakage from pellets in seawater never exceeded 0.8% and 16% of the initial activity after 1 min and 10 min, respectively. Although these tests confirmed a sole contamination pathway (viz. food) of the fish, two additional turbot were used in each treatment, as controls to take into account the possibility of radiotracer recycling through seawater (see section 2.2.2).

2.2.2. Single-feeding experiment

Single-feeding (protocol detailed in Figure 1A) with radiolabeled pellets was carried out using 5 juvenile turbot (22.5 ± 5.8 g wet wt) randomly picked and transferred into an aerated, open circuit 70-L aquarium (water renewal: 100 L h^{-1}; 0.45-µm filtered seawater; salinity: 38 p.s.u.; temperature: 15 ± 0.5°C; pH: 8.1 ± 0.1; light/dark: 12h/12h). Slits cut into the fins were used to facilitate individual recognition. Turbot were constantly fed with radiolabeled pellets over a 30-min period. During this feeding period, care was taken to ensure an instant ingestion of the pellet provided to the fish and if few pellets were uneaten they were rapidly removed to avoid radioactive leaching from the radiolabeled pellets. Two additional, non-exposed (non-fed) turbot were placed within a net in the same aquarium to check any radiotracer recycling from seawater due to possible radiotracer leaching from the contaminated food or from fish depuration. After the feeding period, all turbot were whole-body γ-counted alive and then replaced in clean, flowing seawater conditions (parameters as previously described). All the individuals (including control individuals) were then γ-counted alive for the first time 2h after the radiolabeled feeding and then at different time intervals over a 21-d period to follow the depuration kinetics of the radiotracers. The aquarium was cleaned during the counting to avoid contamination from radiotracers contained in feces. Fish were fed non-labeled pellets one time per day (2% of their biomass).Since body size (i.e. age) is known to affect metal bioconcentration in marine organisms [25], the single-feeding experiment was repeated in the same conditions, using smaller size juvenile turbot (8.05 ± 2.14 g, wet wt). Detailed methods and results are available in the Supplemental Data (out of the main scope of the present study). No difference of AE was observed between the 2 fish sizes for any of the elements tested (see Figure S1).

2.2.3. Multi-feeding experiment

Multi-feeding exposure (see Figure 1B) to radiolabeled pellets was carried out using 5 juvenile turbot (20.2 ± 2.9 g wet wt) kept in the same conditions as described in section Single-feeding experiment. One week before the exposure, turbot were individually identified as described previously. In this experiment, 4 feedings were carried out (each time, turbot were fed for 30 min; the uneaten food was then removed) during a 12-day period (one-labeled pellet feeding every 4 days; Figure 1B). Between each labeled-pellet feeding, fish were fed daily with non-labeled pellets.

The duration (4 d) between two labeled feedings was chosen to match with the beginning of the "slowest" part of the depuration phase as determined in the single-feeding experiment, i.e. when the activity in the turbot tended to stabilize. Each fish was whole-body γ-counted alive 2 h before and 2 h after each feeding exposure and then replaced in clean, flowing seawater conditions (parameters previously described). The 2-h period between the feeding and the counting was adjusted to guarantee the minimum digestion process on the ingested radiolabelled pellets and at the same time to avoid their potential regurgitation during handling. Control individuals were placed in the aquarium as previously described and depuration was followed in each individual by daily whole-body γ-counting over 21 days. No mortality was recorded during all the experiments.

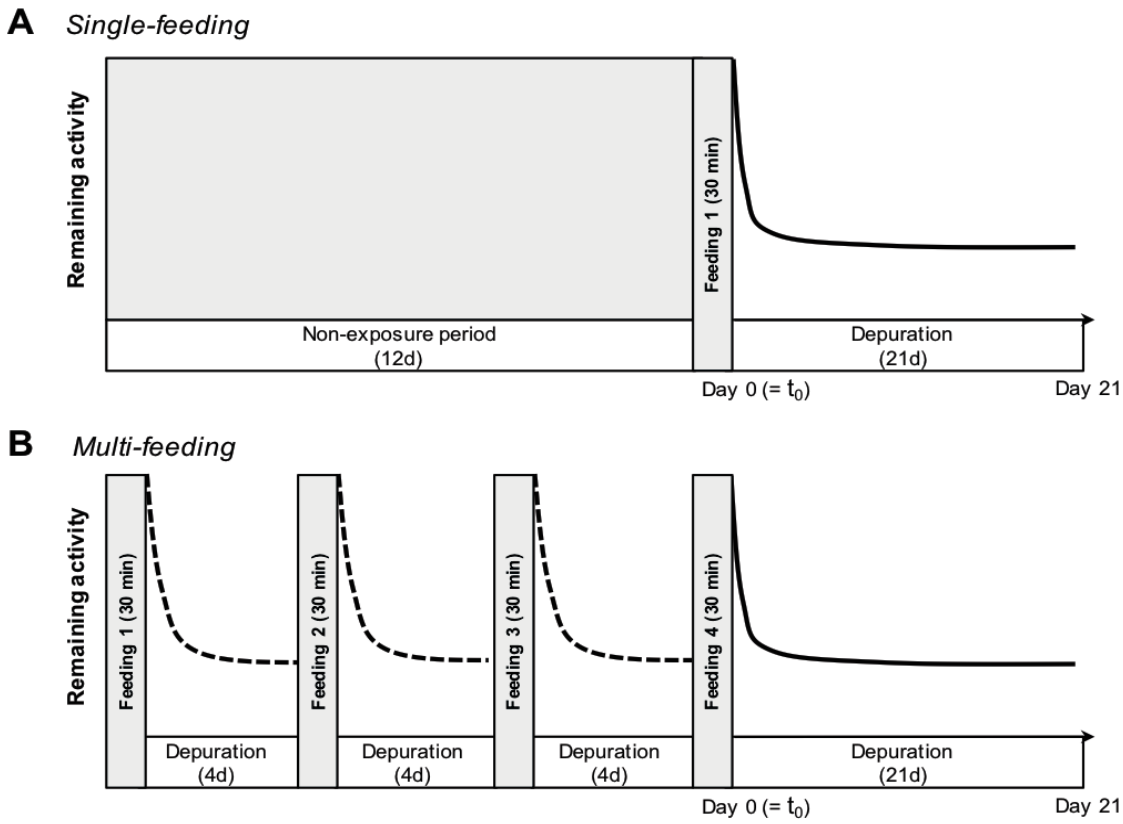

Figure 1. Protocols of (A) single-feeding and (B) multi-feeding experiments. For each radiolabeled pellet feeding, turbot were fed *ad libitum* for 30 min and the uneaten food was then removed. For comparison between single-feeding and multi-feeding experiments, we considered the Day 0 and the Day 21 respectively as the beginning and the end of the depuration period after the last feeding.

2.3. Radioanalyses

The radioactivity of the tracers was measured using a high-resolution γ-spectrometer system composed of 5 Germanium – N or P type – detectors (EGNC 33-195-R, Canberra and Eurysis) connected to a multi-channel analyzer and a computer equipped with spectra analysis software (Interwinner 6, Intertechnique). Radioactivity was determined by comparison with standards of known activity and of appropriate geometry (calibration and counting). Measurements were corrected for background and physical radioactive decay. Organisms were counted in plastic containers (diameter: 80 mm, height: 50 mm) filled with 150 ml of clean seawater during the counting period. The counting time was adjusted to obtain a propagated counting error less than 5% [26,27]. The counting time varied between 25 and 60 min in order to maintain fish health and ensure normal behaviour.

2.4. Data treatment and statistical analyses

2.4.1. Validation of the single-feeding approach

In order to validate the single-feeding approach, we tested whether the reconstruction of a multi-feeding (using the kinetic data obtained from the single-feeding experiment) was consistent with data provided by an actual multi-feeding performed under the same experimental conditions. Depuration of radiotracers was expressed as the percentage of remaining radioactivity (radioactivity at time t divided by the initial radioactivity measured in the organism at the beginning of the depuration period*100 [18]). These kinetics were best fitted using a two-component exponential model:

$$A_t = A_{0s} x\ e^{-kes\ x\ t} + A_{0l} x\ e^{-kel\ x\ t}$$

where A_t and A_0 are the remaining activities (%) at time t (d) and 0, respectively; ke is the depuration rate constant (d-1). “s” and “l” subscripts are related to the short- and long-lived component, respectively. The “s” component represents the depuration of the radiotracer fraction that is weakly associated with the organisms and rapidly eliminated (i.e. proportion associated with the feces). The “l” component describes the depuration of the radiotracer fraction that is actually absorbed by the organism and eliminated slowly [18]. The long-lived component allows estimating the assimilation efficiency (AE) of the radiotracer ingested with food (AE = A_{0l}).

Thus, AE could be defined as the fraction of the radiotracer pool that is incorporated (tightly bound or not) into the tissues of the organism [18]. In the present study, the depuration of the assimilated fraction of all elements was very slow. The long-term depuration rate constant (k_{el}) was not significant different from 0 and the "l" component of the model could therefore be simplified and replaced by a constant [28] and the equation becomes:

$$A_t = A_{0s} x\, e^{-k_{es} x\, t} + A_{0l}$$
$$\text{with } A_{0l} = AE$$

A short-term biological half-life can be calculated ($T_{b1/2}$) from the depuration rate constant according to the relation $T_{b1/2s} = \ln 2/k_{es}$. Model constants were estimated by iterative adjustment of the model using the Quasi-Newton method in the Statistica software 7.0.

2.4.2. Reconstructed vs. actual multi feeding

A theoretical multi-feeding was built using kinetics parameters obtained from the single-feeding and compared with data measured during our multi-feeding experiment. The expected values of our model (i.e. assuming that the experience of multi-feeding is like a succession of independent single-feedings) were calculated for each individual. At this end, the ingested quantities during each feeding were estimated by subtracting the activity measured 2 hours before feeding to that measured 2 hours after. From these values and the kinetic parameters obtained in the single-feeding experiment (see the section Validation of the single-feeding approach), it was possible to calculate the remaining activities after 4 days of depuration for each the feedings. Taking into account the residual activities from the previous feedings, we reconstructed the evolution of the theoretical whole-body activity in the multi-fed turbot (n = 5). Then, reconstructed activities were compared to the activities measured at the same times in the actual multi-feeding experiment using non-parametric Mann-Whitney U test [29]. The level of significance for statistical analyses was always set at $\alpha = 0.05$. All the statistical analyses were performed using the R software 3.0.1 [30].

2.4.3. Complementary data from the multi-feeding experiment

As indicated earlier in section 2.2.3, γ-countings were performed on live turbot before and after each of the 4 feeding events, allowing an individual determination of the gain and loss of the radiotracers between the feedings of the turbot with radiolabeled pellets. Calculation of each new input of pellet-borne tracers transferred to the fish was then possible considering the whole-body activity measured 2h after the ingestion of radiolabelled pellet minus the background activity (measurement done before the new feeding; Figure 1B). This can be compared with the whole-body activity after 4 d without new inputs (measurement before the next feeding with radiolabelled pellets) for which the "background" activity can also be subtracted to allow comparing each feeding as an independent single-feeding.

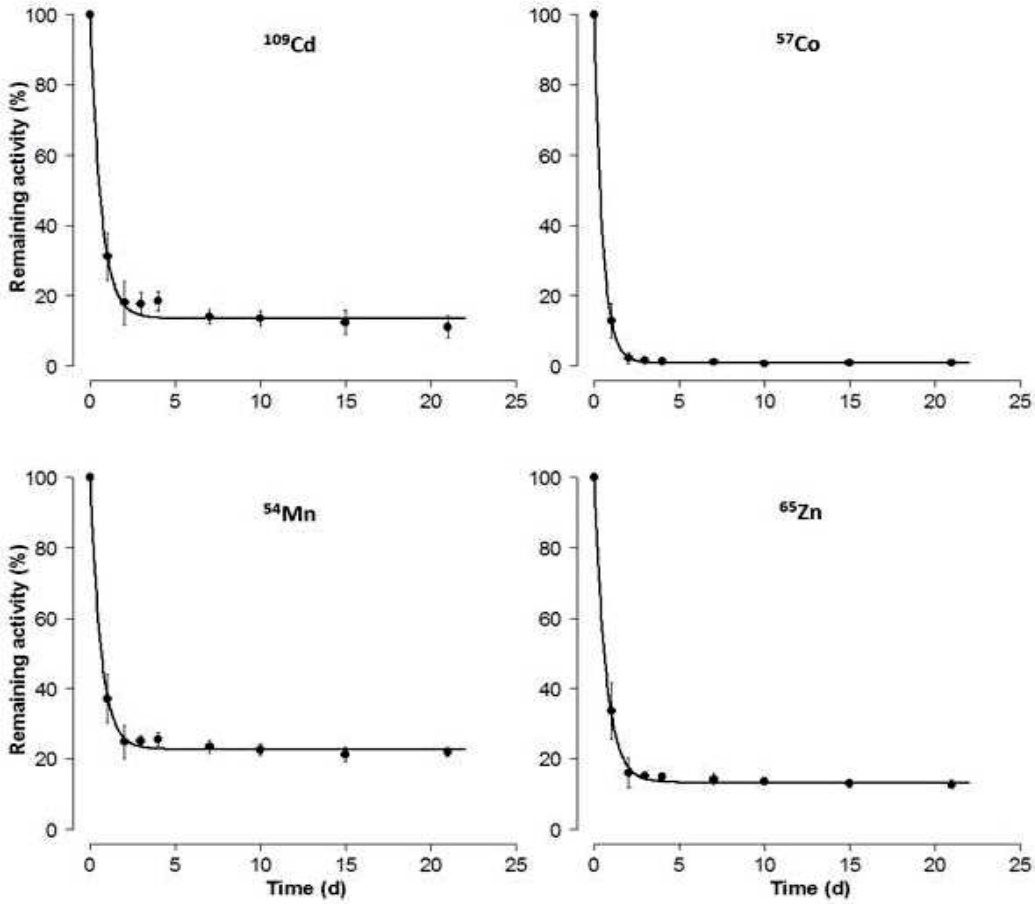

Figure 2. Comparison of activities measured at different times in multi-feeding experiment and those expected using the kinetic parameters obtained from single-feeding experiment (i.e. assuming that the experience of multi-feeding is similar to a succession of independent single-feedings; see "Materials and Methods" section for more details). Values (Bq) are means ± SD; n=5.

3. RESULTS

3.1. Depuration kinetics after the single-feeding exposure

Whole-body depuration kinetics of ^{109}Cd, ^{57}Co, ^{54}Mn, and ^{65}Zn in single-fed turbot were always best fitted by a two-phase model (one exponential component model and a constant; Table 1 and Figure 3; R^2: 0.98-0.99). The assimilation efficiency (AE) depended on the investigated metal, with average values ranging from 1% for Co to 23% for Mn (Table 1). As indicated in the Supplemental Data, no difference of AE was observed between the 2 fish sizes for all elements tested.

Table 1. Estimated depuration kinetic parameters of ^{109}Cd, ^{57}Co, ^{54}Mn and ^{65}Zn in turbot exposed to the radiotracers by single-feeding with labeled pellets (n = 5 per treatment) and then maintained for 21d in clean seawater. kes: depuration rate constant (d^{-1}); $T_{b½s}$: biological half-life (d); AE: assimilation efficiency (%); ASE: asymptotic standard error; R^2: determination coefficient.

Tracer	Short-term		Long term	R^2
	k_{es} ± ASE	$T_{b½s}$ ± ASE	AE ± ASE	
^{109}Cd	1.59 ± 0.10***	0.44 ± 0.03	13.8 ± 0.7***	0.98
^{57}Co	2.12 ± 0.06***	0.33 ± 0.01	1.0 ± 0.3***	0.99
^{54}Mn	1.62 ± 0.09***	0.43 ± 0.02	22.9 ± 0.5***	0.99
^{65}Zn	1.50 ± 0.08***	0.46 ± 0.02	13.4 ± 0.6***	0.99

Probability of the model adjustment:
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

3.2. Reconstructed vs. actual multi-feeding

Figure 2 displays the activities of the reconstructed multi-feeding (using model parameters from the single-feeding experiment) as well as those actually measured in the multi-feeding experiment. The comparison of the reconstructed vs. actual data did not reveal any significant difference ($p>0.05$) in terms of whole-body activities, for all the 4 feedings, whatever the considered metal.

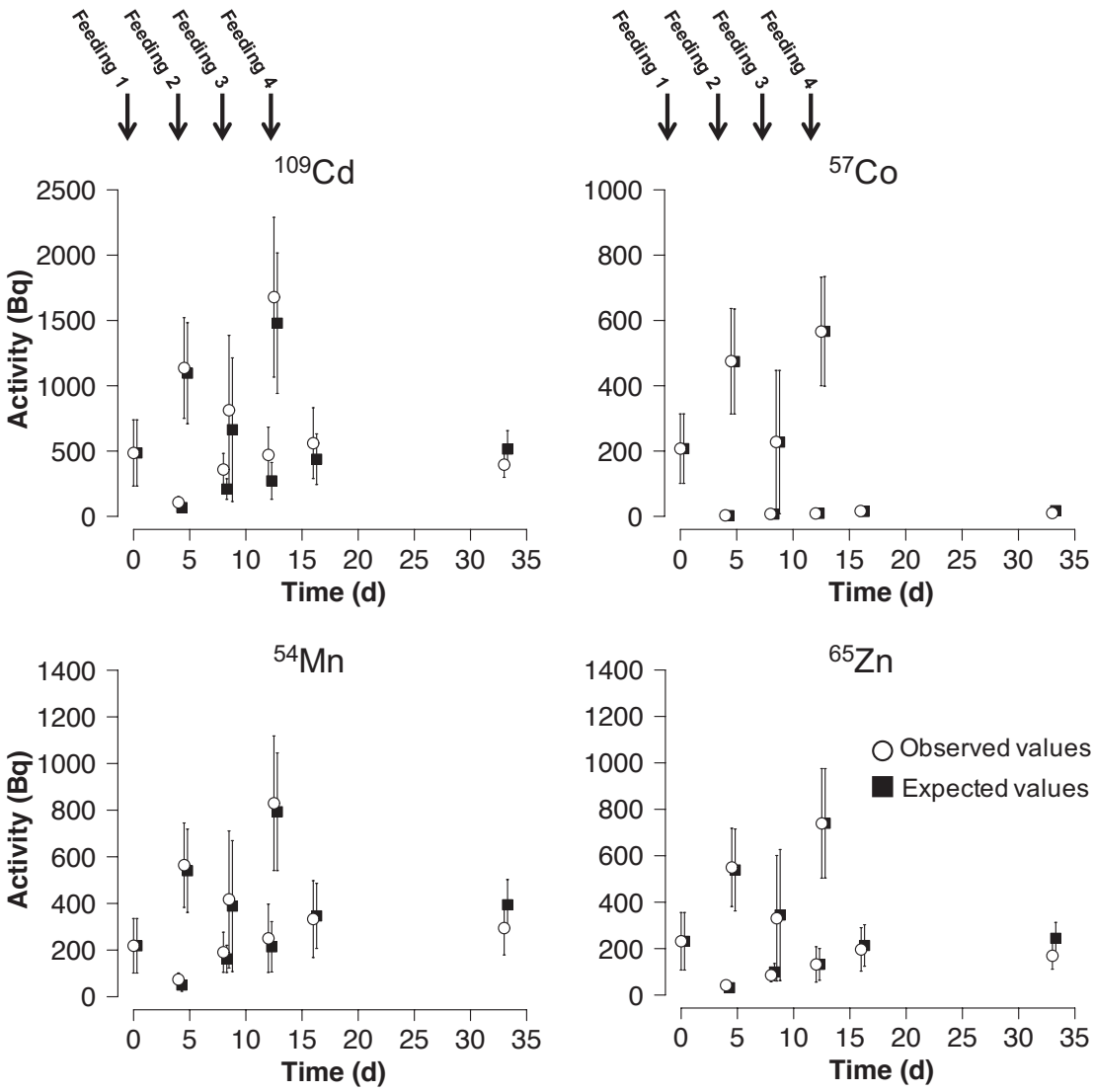

Figure 3. Kinetics of the whole-body depuration of ^{109}Cd, ^{57}Co, ^{54}Mn and ^{65}Zn in single-fed turbot (% remaining activities, means ± SD, n=5). Parameters and statistics of depuration kinetics are given in Table 1.

3.3. Variability among the successive feedings

The multi-feeding experiment allowed following how the whole-body activity in the multi-fed turbot changed at each feeding (4 feedings in 12 d with radiolabeled pellets) and during the depuration period (Figure 4). The whole-body activity after 4 days and 21 days of depuration were not significantly different ($p>0.05$; Figure 4, Table 2). At the end of the depuration period (21 d), total retained activity represented less than 1% of the total ingested activity for Co and up to 23% for Cd.

Table 2. Different ratios calculated between ingested and retained activity during the multi-feeding and the single-feeding exposures. Only the three individuals having eaten during the 4 radiolabeled pellet feedings are included in the calculations.

Experiment			^{109}Cd	^{57}Co	^{54}Mn	^{65}Zn
Multi-feeding experiment	Ingested activity (Bq, Mean ± SD)		2975 ± 1301	1338 ± 640	1405 ± 581	1048 ± 448
	Remaining activity after the 4th feeding (Bq, Mean ± SD)	Depuration (4 d)	643 ± 264	19 ± 3	380 ± 166	225 ± 92
		Depuration (21 d)	391 ± 119	12 ± 1	322 ± 125	183 ± 62
	Ratio between ingested and remaining activity after 4 days (less unit, Mean ± SD)	Feeding 1	4.64 ± 1.38	55.47 ± 11.81	2.75 ± 0.56	5.29 ± 2.23
		Feeding 2	3.61 ± 1.76	65.89 ± 15.71	3.14 ± 1.00	6.50 ± 1.20
		Feeding 3	1.09 ± 0.36	26.41 ± 13.09	1.08 ± 0.27	2.14 ± 0.75
		Feeding 4	2.10 ± 0.34	30.86 ± 6.28	1.70 ± 0.51	3.06 ± 1.10
	Ratio between cumulated activity in the 4 feedings and remaining activity at the end of the depuration period (less unit, Mean ± SD)		9.31 ± 2.92	138.40 ± 42.58	5.27 ± 0.50	9.70 ± 1.35
Single-feeding experiment	Total ingested activity (Bq, Mean ± SD)		1410 ± 440	614 ± 202	637 ± 219	663 ± 224
	Remaining activity (Bq, Mean ± SD)	Depuration (4 d)	265 ± 99	8 ± 3	164 ± 60	101 ± 37
		Depuration (21 d)	160 ± 77	6 ± 3	142 ± 51	86 ± 35
	Ratio between ingested and remaining activity at the end of the depuration period (less unit, Mean ± SD)		9.64 ± 2.58	116.93 ± 41.82	4.56 ± 0.27	7.91 ± 0.80

For the individuals (n=3) that had successively and significantly eaten four times, the multi-feeding exposure led to a linear increase of the whole-body activity in turbot for the four studied elements (Figure 4).

In order to get a better understanding of the whole-body metal retention after each feeding, a ratio between ingested and remaining activities was calculated (after subtraction of the "background" activity). Our results indicate that, for each element, this ratio decreased throughout the multi-feeding experiment with values ranking from 3 (Mn) to 56 (Co) for the first feeding against less than 2 (Mn) to 31 (Co) for the 4th feeding (Figure 4, Table 3).

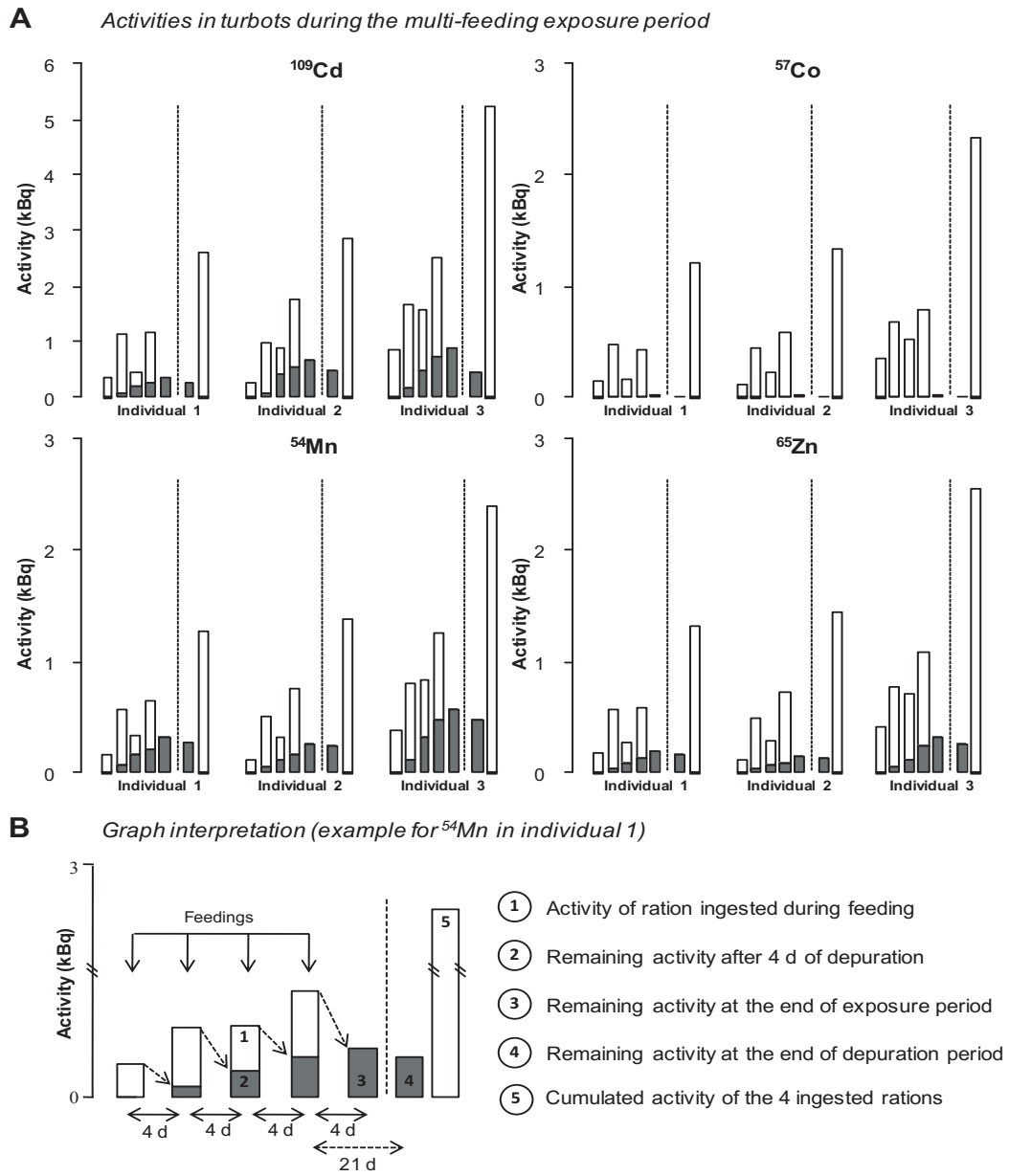

Figure 4. Uptake of ^{109}Cd, ^{57}Co, ^{54}Mn and ^{65}Zn by the turbot during the multi-feeding exposure (12d). Only the three individuals having eaten during the 4 radiolabeled pellet feedings are represented. Values (kBq) are means ± SD; n=3.

4. DISCUSSION

The present study aimed at testing the validity of the single-feeding approach commonly used to assess trophic transfer of contaminants in marine organisms [31,32]. The protocol consisted of conducting in parallel two experiments where turbot, kept in controlled laboratory conditions, were exposed to the metals 1 single time or 4 successive times, using radiolabeled pellets. Kinetic data from the single-feeding experiment then allowed building a reconstructed multi-feeding situation and comparing it with the observations made under actual multi-feeding conditions.

The depuration of metal transfer to the turbot through the food was always characterized by a biphasic process. Depuration kinetics from the single-feeding experiment were always best described by a model including exponential component and a constant for all the studied elements. Such biphasic depuration kinetics for metals are commonly observed in aquatic organisms (e.g. [14,18,33]). The constant indicated that the assimilated fraction of metal was strongly bound after 4 days. Indeed, the whole-body activity was not significantly different between 4 and 21 d after of depuration. This finding corroborates the results from a previous study on the trophic transfer of essential elements in the same fish, using natural prey [15, 28]. Our results also indicate that assimilation efficiency (AE) is metal dependent. AE of Mn was higher than one of the other three elements: AEs of Cd and Zn were very close (respectively 14% and 13%) whereas, in contrast, Co was poorly assimilated (AE ≈ 1%). According to the literature available on this fish, AEs observed in the present study appeared to be relatively low (Table 3; [23]). However, some authors (e.g. [34, 35]) have already highlighted that AEs of metals in fish are highly dependent on the diet composition and on metal speciation and, to date, investigations using commercial food as we did here are still limited to a small number of species and few elements (Table 3; [15,12,34]).

Our results displayed in Figure 2 indicate that the reconstruction of a multi-feeding (using the data from a single-feeding, then repeated over time) is consistent with the data provided by an actual multi-feeding performed under the same conditions. These results providing an experimental validation of the single-feeding approach widely reported in the literature since the 1980s [16-18]. It would however be of interest to study the influence of repeated dietary exposures over a longer period of time in order to confirm the trends observed in the present study over 4 feedings.

Table 3. Comparison of metal dietary assimilation efficiencies (AEs; Means in %) in marine and brackish water fish species.

Species	Food	Metal (AE)				References
		Cd	Co	Mn	Zn	
Acanthopagrus schlegelii (Blackhead seabream)	Artificial diets	8-14			15-26	[22]
	Mullet muscle	41			42	[22]
	Mussel tissue	20			25	[22]
	Squid viscera	40			14	[22]
	Brine shrimp	5-10			12-34	[36]
Ambassis urotaenia (Banded-tail glassy perchlet)	Brine shrimp nauplii	27-33			15-17	[37]
	Copepods	14-15			5-9	[37]
Dicentrarchus labrax (European seabass)	Seabream juveniles	23	21	33	38	[24]
Lutjanus argentimaculatus (Mangrove red snapper)	Brine shrimp	10			15	[1]
	Clam tissue	9			30	[1]
	Copepods	6			20	[1]
	Manilla clam	7			19	[1]
Menidia sp. (Silversides)	Copepods	3	2		6	[38]
Periophthalmus cantonensis (New Guinea mudskipper)	Brine shrimp larvae	15-26			11-21	[37]
	Copepods	10-22			21-31	[37]
Scophthalmus maximus (Turbot)	Seabream juveniles		27		22	[23]
	Ragworms		5	44	17	[33]
	Seabream juveniles		44	23	23	[33]
	Shrimp		16	42	32	[33]
Sparus aurata (Gilthead seabream)	Brine shrimp nauplii	45	21	25	18	[23]
Terapon jarbua (Jarbua terapon)	Barnacles	3			2	[34]
	Copepods	6			23	[34]
	Clams	9			36	[34]
	Fish viscera	6			52	[34]
	Mussels	4			22	[34]

The single-feeding approach has been used to determine AE in various aquatic organisms such as crustaceans, echinoderms, molluscs and fish. In order to get an exhaustive validation of this approach it would also be appropriate to expand the protocol applied in the present study to other biological models exposed to different food items.

In addition to provide the experimental validation of the single-feeding approach, the experimental protocol used in the present study allows better understanding the mechanisms involved in the storage and depuration of trace elements during a multiple trophic exposure. In multi-fed turbot, the whole-body activity increased linearly for all the metals after each of the 4 radiolabeled feedings. Despite the increase in metal concentrations in turbot in response to the multiple-exposure, the percentage of retained activity from each ration was constant during the entire multi-feeding experiment. Some authors have already highlighted that pre-exposure to contaminated food had no effect on the assimilation [35]. Indeed, in the black sea bream *Acanthopagrus schlegeli* and the grunt *Terapon jarbua* AE of Cd and Zn was not influenced significantly following Zn dietary pre-exposure for 1 or 3 wk. Our results indicate that metal storage capacities of turbot are not limited over the 3-week period of exposure. In this context, we can assume that there are neither major changes in metal regulatory mechanisms in this species nor toxic effects of the metal on the assimilation process.

SUPPLEMENTAL DATA

Supplemental Data provide information regarding on the methodology used for stable metal analysis in pellets including the comparison between measured and certificated values in a reference material (Table S1). Thus, we provided kinetics of depuration for two batches of turbots of two different sizes (Figure S1).

ACKNOWLEDGMENTS

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ANNEXE 2

SUPPLEMENTARY MATERIAL

Comparing single-feeding and multi-feeding approach for experimentally assessing trophic transfer of metals in fish

Stable metals in pellets

For essential element analyses, samples (n = 3) of 250 mg were digested using 5 mL of 65% HNO_3 and 2 mL of H_2O_2. Acidic digestion was performed overnight at ambient temperature and then heated in a microwave for 40 min, with a temperature increase to 190°C for 20 min, followed by 20 min at 190°C (1600W). After the mineralization process, each sample was diluted to 50 mL with milli-Q quality water and an extra 1:5 dilution was prepared. Cd, Co and Mn were analysed by ICP-MS (iCAP Q ICP-MS, Thermo Scientific®) and Zn by flame atomic absorption spectrometry (SpectrAA 220, Varian®). A certified reference material (fish muscle, IAEA 407) was treated and analysed in the same way as the samples. Results were in good agreement with the certified values (Table 1). For each set of analyses, blanks were included in analytical batch. The detection limits were (µg g^{-1} dwt): 0.025 (Cd, Co, Mn) and 2 (Zn). All metal concentrations are given on a dry weight basis (µg g^{-1} dwt).

Table S1. Comparison of metal concentration (mean ± SD, n = 3) in reference material (fish muscle, IAEA 407) measured by ICP-MS (Cd, Co and Mn) and by flame atomic absorption spectrometry (Zn) with certified values. All the values are expressed in µg.g^{-1} dwt.

Element	Measured	Certified
Cd	0.133 ± 0.002	0.189 ± 0.019
Co	0.08 ± 0.01	0.10 ± 0.02
Mn	2.50 ± 0.07	3.52 ± 0.32
Zn	65.4 ± 0.7	67.1 ± 3.8

Single-feeding experiments and size-effect

The first experiment was a single exposure to radiolabelled pellets (single feeding method) carried out using juvenile turbots (n=15, 8.05 ± 2.14 g, wet weight) randomly picked and transferred in an aerated, open circuit, 70-L aquarium. The same protocol was repeated in the same conditions using larger juvenile turbot (n=5, 22.5 ± 4.6 g, wet weight). Whole-body depuration kinetics of ^{109}Cd, ^{57}Co, ^{54}Mn, and ^{65}Zn in turbots exposed by single-feeding were best fitted by a two-phase model (simple-exponential model and a constant; Fig. 2 & Table 2; R^2: 0.94-0.99). The retention of the four radiotracers depended on the studied metal. The major fraction (77-99%; Fig. 2) of the four elements is very rapidly lost ($T_{b½s}$ < 2.2 d; Table 1).

We observed no significant different (p>0.05) between the AE of turbots exposed to the radiotracers by single-feedings during the experiment 1 (smaller individuals: 8.05 ± 2.14 g) and experiment 2 (larger individuals: 22.5 ± 4.6 g). However, comparison of kes between the two single-feedings indicates that the values of this parameter were significantly lower (p<0.01) in the smaller turbots (Fig. S1).

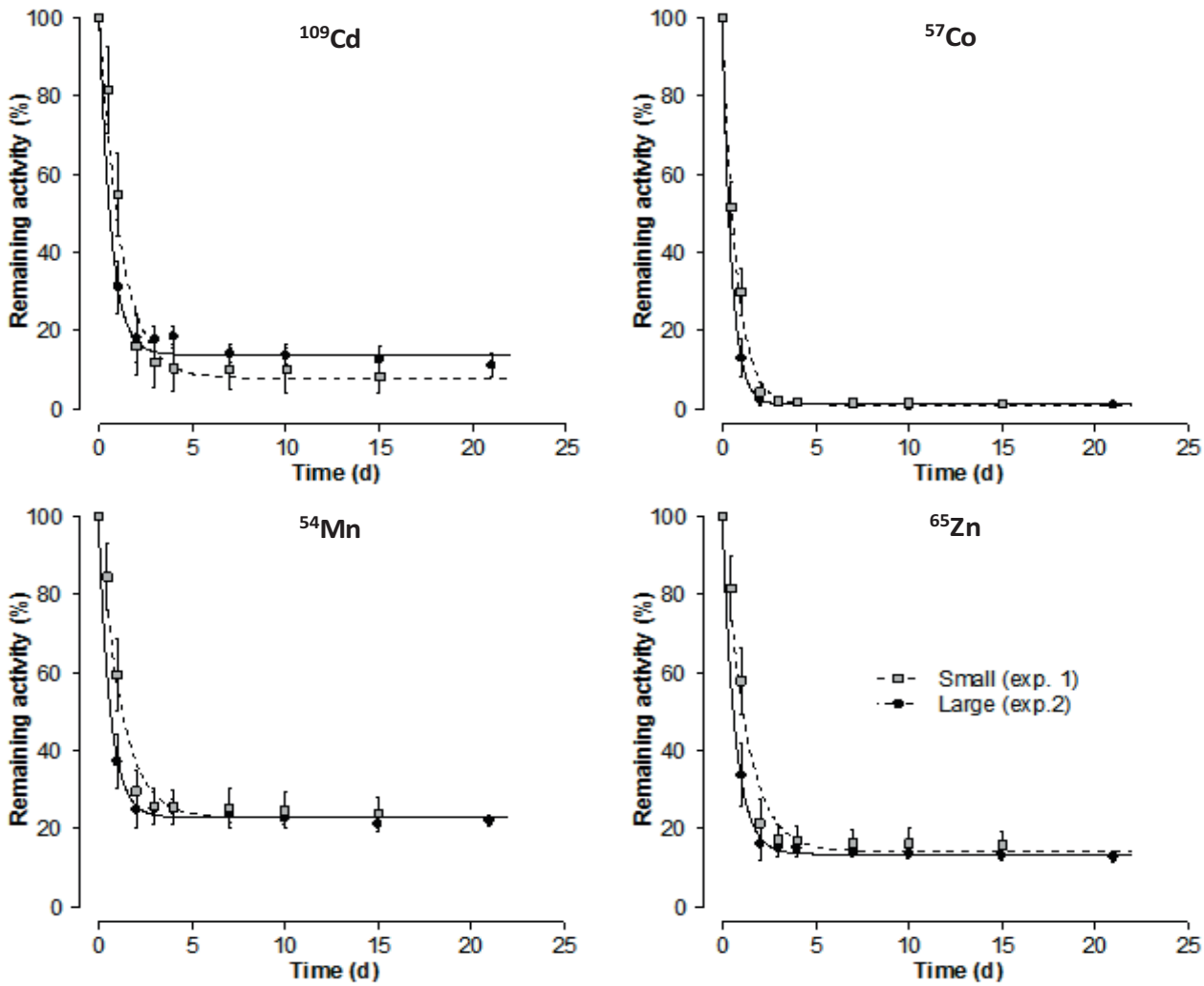

Figure S1. Kinetics of the whole-body depuration of ^{109}Cd, ^{57}Co, ^{54}Mn and ^{65}Zn in juvenile turbot (% remaining activities, means ± SD, n=5-15) of two different sizes after a single-feeding with radiolabeled pellets.

ANNEXE 3

Influence of food on the assimilation of essential elements (Co, Mn & Zn) by turbot (*Scophthalmus maximus*)

Pouil S[1,2], Warnau M[1], Oberhänsli F[1], Teyssié J-L[1], Bustamante P[2], Metian M[1] (2016)
Influence of food on the assimilation of essential elements (Co, Mn, and Zn) by turbot *Scophthalmus maximus*. *Marine Ecology Progress Series* 550: 207-218.

ABSTRACT: Food is an important route of metal uptake in marine organisms and assimilation efficiency (AE) is a key physiological parameter that can be used to systematically compare the bioavailability of different metals from food. This parameter may be influenced by various factors, including diet. The present study aimed at examining the influence of diet on AEs of three essential metals (Co, Mn and Zn) in the turbot, *Scophthalmus maximus*. The pulse-chase feeding method was used with three radiolabelled natural prey: fish, shrimp and ragworm. The results showed that AE was strongly influenced by the prey and the metal considered. However, the influence of these parameters on AE was variable and no general trend was observed. The AEs ranged between 5-43% for Co, 23-44% for Mn and 17-32% for Zn. Results suggest that relationships between metal distribution in the prey (at tissue and subcellular levels) and bioavailability to predator fish is not obvious as previously assumed based on marine organisms feeding on unicellular or simple pluricellular organisms. Finally, we modelled how *S. maximus* is accessing foodborne essential elements using experimentally-derived parameters, the concentration of these elements in prey, and different data on stomach contents from wild turbot. Results emphasize the importance of crustaceans in the nutrition of turbot showed that this taxon is generally the most important source of essential metals for turbot although in some cases polychaetes can make a high contribution to dietary Co and Mn uptake.

Keywords: Marine fish, Assimilation efficiencies, Natural prey, Depuration, Metals, Nutrition

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1. INTRODUCTION

Fish accumulate metals through different pathways (e.g. Warnau & Bustamante 2007; Dutton & Fisher 2011). Over the last decade, food has been increasingly identified as a pathway of major importance for metal intake in fish (Xu & Wang 2002; Mathews & Fisher 2009). However, despite a growing understanding of trophic transfer mechanisms, few studies have focused on the influence of the diet on the assimilation of essential metals in these organisms (Baudin & Fritsch 1989; Garnier-Laplace et al. 2000; Bury et al. 2003).

Essential metals such as Co, Mn and Zn, are metabolically required; they are part of the functional groups of various enzymes, play a structural role in respiratory pigments and metalloenzymes, and can act as activating co-factors for various proteins (see e.g. Simkiss 1979; Williams 1981). Fish health can be optimal if essential metals are present in sufficient amounts in their tissues: depletion in these elements can provoke pathological impairments and/or physiological alterations and excess of essential elements can provoke toxic effects (e.g. Förstner & Wittmann 1983).

One critical parameter for understanding metal trophic transfer in fish is the assimilation efficiency (AE) of the metal from ingested food. If derived under controlled experimental conditions, AE is a first-order physiological parameter that can be compared quantitatively among different metals, organisms, food types, or environmental conditions (Wang & Fisher 1999).

The main objective of the present study was to investigate the influence of the diet on essential metal assimilation by a marine predatory fish. We compare the AE of three essential metals (Co, Mn, and Zn) in the turbot *Scophthalmus maximus*, fed on three different natural prey (fish, shrimp and ragworm) using radiotracer techniques. In order to better understand assimilation processes for the aforementioned essential metals, depuration kinetics were determined and AEs estimated after a single feeding with radiolabelled prey (pulse-chase feeding methodology; e.g. Warnau et al. 1996; Metian et al. 2010). Relationships between metal fractioning in prey (tissue and subcellular levels) and metal AEs in their predators have been shown for invertebrates and planktivorous fish (Reinfelder & Fisher, 1994; Wallace & Lopez, 1996) but not yet for fish fed with complex pluricellular prey. Therefore, tissue and subcellular distribution of essential elements was characterized in order to assess possible influence on AEs in turbot.

Finally, AE results were combined with stable isotope analyses in the selected prey and with the natural diet of turbot to develop a model that was used to estimate the relative contribution of each prey in the dietary intake of metals.

2. MATERIALS AND METHODS

2.1. Origin and acclimation of organisms

In January 2014, one hundred juvenile turbot *Scophthalmus maximus* were purchased from a fish farm (France Turbot, France) and shipped to the International Atomic Energy Agency premises in the Principality of Monaco. Fish were acclimated to laboratory conditions for 21 days (open circuit, 500-L aquarium; water renewal: 100 L h^{-1}; 0.45µm filtered seawater; salinity: 38 p.s.u.; temperature: 15 ± 0.5°C; pH: 8.0 ± 0.1; light/dark: 12h/12h). During the acclimation period, the fish were fed a daily ration of 2% of their biomass with 1.1-mm pellets (proteins: 55% and lipids: 12%; Le Gouessant, France).

In order to investigate the influence of the diet on essential metal assimilation by *S. maximus*, three different natural prey were used: fish (seabream *Sparus aurata*), shrimp (common prawn *Palaemon serratus*) and ragworm (estuary ragworm *Hediste diversicolor*). Fish were obtained from the hatchery "Poissons du Soleil", France, shrimp were purchased from "Poissons Vivants", France, and ragworms were purchased from fishing bait seller "Normandie Appâts", France. All prey were acclimated to the same laboratory conditions as the turbot for a minimum of two weeks prior to experiments. Shrimp and worms were fed a mix of fish feed and crushed mussels whereas juvenile fish were fed 300-µm pellets (Biomar, France). Since body size (weight) is known to affect metal bioaccumulation in marine organisms (Boyden, 1974; Warnau et al., 1995; Hédouin et al. 2006), only prey individuals with homogeneous size were used for the experiments (*S. aurata*; 60-day-old hatchlings, approx. 1.5 to 2 cm in total length, 0.06 ± 0.01g wet weight –wwt–), *P. serratus*, 0.58 ± 0.11g wwt and *H. diversicolor*, 0.82 ± 0.14g wwt).

2.2. Nutritional characteristics and stable metals in prey

Preliminary characterization of metal concentration and basic nutritional composition of the prey was carried out prior to radiolabelling. Protein content (using N content), percentage of dry matter (DM) and essential metal concentrations (Co, Mn and Zn) were measured.

To determine the amount of N, samples of food items (n = 3) were freeze-dried (Free-Zone 18L Console Freeze Dry System, Labconco®) before being manually crushed. Aliquots of 1 to 5 mg were analysed using a vario EL CHN analyser, Elementar®. For each food item, the protein content (expressed as % of dry matter) was estimated using conversion coefficients from N values (i.e. 5.58 for fish and 5.60 for the other prey; Tacon et al. 2009). Dry matter (DM) content was determined by drying the samples in a ventilated oven at 105°C for 24 h. For essential element analyses, samples (n = 3 for each prey) of 250 to 1000 mg were digested using 5 mL of 65% HNO_3 and 2 mL of H_2O_2. Acidic digestion was performed overnight at ambient temperature and then heated in a microwave for 40 min, with a temperature increase to 190°C for 20 min, followed by 20 min at 190°C (1600W). After the mineralization process, each sample was diluted to 50 mL with milli-Q quality water and an extra 1:5 dilution was prepared. Co and Mn were analysed by ICP-MS (iCAP Q ICP-MS, Thermo Scientific®) and Zn by flame atomic absorption spectrometry (SpectrAA 220, Varian®). A certified reference material (fish muscle, IAEA 407) was treated and analysed in the same way as the samples. Results were in good agreement with the certified values (Table 1). For each set of analyses, blanks were included in analytical batch. The detection limits were (µg g-1 dwt): 0.006 (Co, Mn) and 0.5 (Zn). All metal concentrations are given on a dry weight basis (µg g-1 dwt). For the shrimp, antennae, antennules, rostrum and telson were removed before analysis in accordance with experimental methodology (see section 2.3.2.).

Table 1. Comparison of metal concentration (mean ± SD, n = 3) in reference material (fish muscle, IAEA 407) measured by ICP-MS (Co and Mn) and by flame atomic absorption spectrometry (Zn) with certified values. All the values are expressed in µg g^{-1} dwt.

Element	Measured	Certified
Co	0.08 ± 0.01	0.10 ± 0.02
Mn	2.50 ± 0.07	3.52 ± 0.32
Zn	65.4 ± 0.7	67.1 ± 3.8

2.3. Experimental procedures

2.3.1. Radiolabelling of the prey

Preparation of the radiolabelled prey was carried out by exposing them for 7-21 days in aerated 20-L aquaria. Radiotracers of high specific activity were purchased from Isotope Product Lab., USA (^{57}Co as $CoCl_2$ in 0.1 M HCl, [$T_{1/2}$] = 271.8 days; ^{54}Mn as $MnCl_2$ in 0.5 M HCl, [$T_{1/2}$] =312.2 days; ^{65}Zn as $ZnCl_2$ in 0.1M HCl, [$T_{1/2}$] = 243.9 days).

Seawater was spiked with the radiotracers (nominal activity of 0.5 kBq L^{-1} per isotope for fish and shrimp exposures and 1 kBq L^{-1} per isotope in the case of ragworm). In terms of stable metal concentrations, these additions corresponded to 0.2-0.4 pmol L^{-1} for Co, 3.7-7.4 pmol L^{-1} for Mn and 220-440 pmol L^{-1} for Zn, i.e. concentrations that are lower than the background concentrations of these metals in open sea (Bruland 1983). Small volumes (10 µL) of the diluted radiotracer solution were added to the aquaria and no change in pH was detectable in the aquarium (close circuit) after tracer addition. Seawater was regularly renewed and spiked daily to keep the activity as constant as possible. Activity of the metal tracers in seawater was checked daily, before and after each seawater renewal, to determine time-integrated activities (Warnau et al. 1996; Rodriguez y Baena et al. 2006). Prey were fed after each seawater renewal. For shrimp exposure, each organism was kept individually during the whole duration of the experiment in a cylindrical plastic container (drilled to allow for free water circulation) in order to avoid cannibalism (e.g. during moulting) and to facilitate individual recognition. For the ragworm exposure, the walls of the aquarium were obscured and plastic tubes were added as artificial burrows.

2.3.2. Exposure of turbot via radiolabelled prey

Three sets of experiments were conducted for each prey. For each set, 8 to 15 juvenile turbot (11.17 ± 4.76 g) were transferred in an aerated, open circuit, 70-L aquarium. The number of turbot depended on the amount of contaminated prey available. Slits cut into the fins were used to facilitate individual recognition. One week before the exposure to radiolabelled diet, fish were fed daily with the non-labelled prey to acclimate them to this diet. Each experiment consisted of a single feeding of fish with radiolabelled diet (see e.g. Metian et al. 2010). Turbot were fed 30 min ad libitum with freshly killed prey; uneaten diets were removed after the 30-min feeding.

To facilitate ingestion, shrimp were cut into pieces and antennae, antennules, rostrum and telson removed. After the 30-min feeding, individual fish were whole-body γ-counted alive and then placed in a new aquarium with flowing seawater conditions (parameters as previously described) to follow subsequent metal depuration. During depuration, fish were fed daily with non-labelled pellets (2% of their biomass, Biomar, 2014) to keep consistent digestive physiology amongst all individuals. During and after the labelled feeding, an additional turbot was placed in each aquarium to assess any possible radiotracer recycling from seawater due to leaching from the radiolabelled food or, later on, from fish depuration. After radiolabelled feeding, all the fish (including control individuals) were regularly radioanalysed to follow the radiotracer depuration kinetics over 21 days. After each counting fish were moved to another 70-L aquarium with clean water.

2.3.3. Radiotracer compartmentalization in prey

Radiolabelled fish (n=3) and shrimp (n=3) were dissected to isolate the hard body parts (skeleton and cuticle) that are assumed less digestible for predators (Reinfelder & Fisher 1994). Samples were radioanalyzed to quantify the percentage of activity sequestered in these body parts (i.e. skeleton and cuticle).

Distribution of radioelements between the soluble and insoluble fractions was determined in four individuals of each species of prey according to a method adapted from Bustamante and Miramand (2005). This method allows quantification of metals associated with the soluble fraction of the prey (i.e. cytosol; Wallace & Lopez 1996; Bustamante & Miramand 2005). Briefly, 4 contaminated prey stored at -80°C were crushed and tissue was homogenized (T25 Ultra-Turrax Basic, IKA®) in around 10 volumes of TRIS-HCl buffer 0.02 M sucrose 0.25 M with 1 mM PMSF (phenylmethylsulfonylfluoride, as protease inhibitor) and 5 mM DTT (dithiothreitol, as reducing agent), at pH 8.06. The homogenates were centrifuged at 45 000 G for 2 h at 4°C (Sorvall Evolution RC Superspeed Centrifuge, Sorvall instruments®) to separate cytosol (i.e. the soluble fraction) from the cellular debris, the organelles and the metal-rich granules (i.e. the insoluble fraction; Fig. 1). Aliquots of each fraction obtained were radioanalyzed in order to determine the radiotracer's activities. The same procedure was repeated over time for each prey.

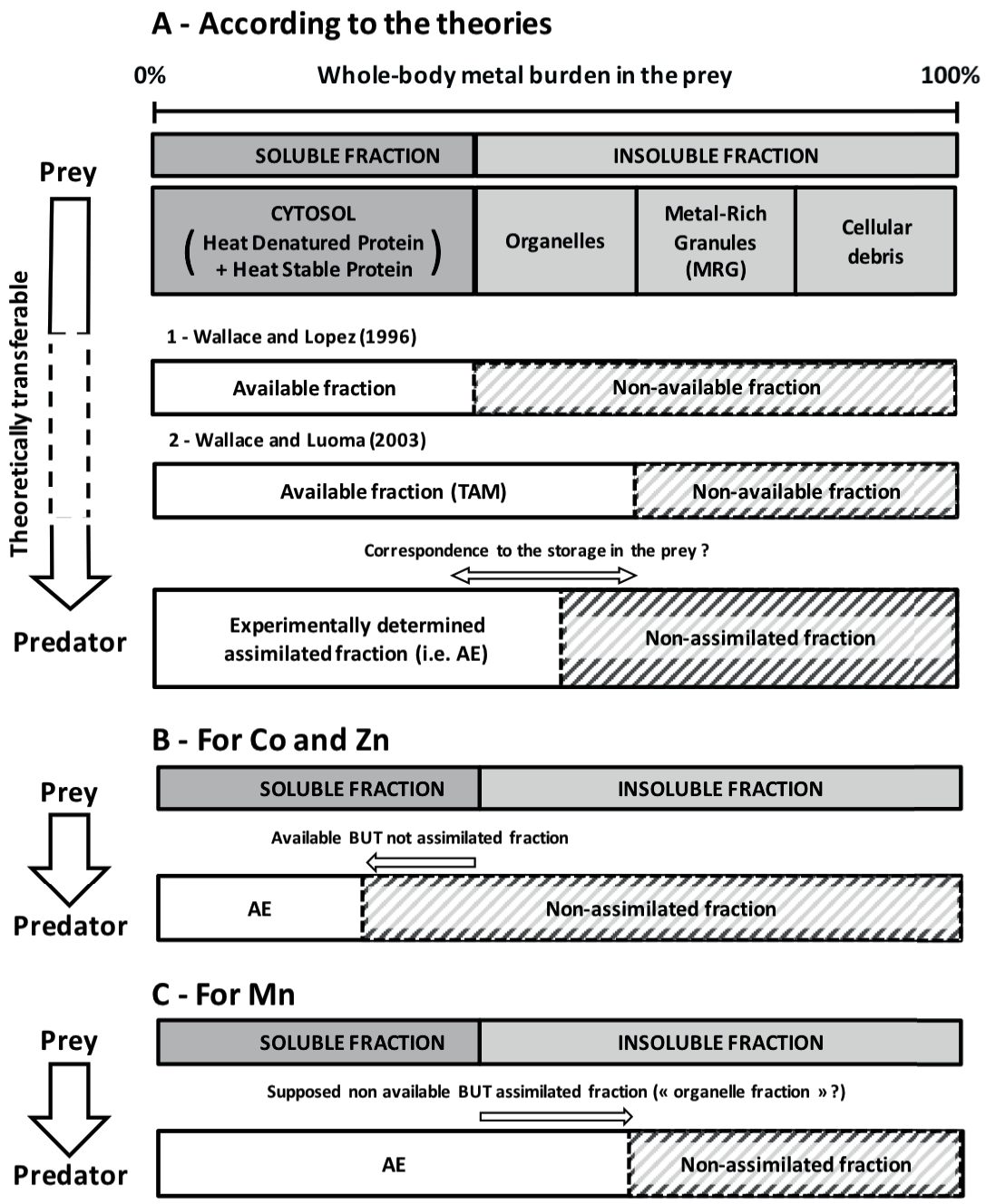

Figure 1. Definition of the different concepts used in metal's subcellular fractionation and relation between subcellular fractionation in the prey and assimilation efficiency measured in predator. (A) Description of theories developed by Wallace and Lopez (1996) and Wallace and Luoma (2003). (B) The present study where measured AE in the predator is lower than expected values on the basis of fraction of element present in the soluble fraction - part of metal available fraction in the prey is not assimilated by the predator. (C) The present study where measured AE in the predator is higher than expected values on the basis of fraction of element present in the soluble fraction - part of non-available metal fraction in the prey is assimilated by the predator.

2.4. Radioanalysis

The radioactivity of the tracers was measured using a high-resolution γ-spectrometer system composed of 5 Germanium - N or P type - detectors (EGNC 33-195-R, Canberra® and Eurysis®) connected to a multi-channel analyser and a computer equipped with a spectra analysis software (Interwinner 6, Intertechnique®). The radioactivity in living organisms and samples was determined by comparison with standards of known activity and of appropriate geometry (calibration and counting). Measurements were corrected for background and physical radioactive decay. Living organisms were placed in counting tubes filled with clean seawater during the counting period. The counting time was adjusted to obtain a propagated counting error less than 5% (e.g. Rodriguez y Baena et al. 2006). In the case of live turbot, the counting time varied between 25 and 60 min in order to maintain fish health and ensure normal behaviour.

2.5. Data treatment and statistical analysis

Depuration kinetics were fitted using non-linear model. Depuration of radiotracers was expressed as the percentage of remaining radioactivity (radioactivity at time t divided by the initial radioactivity measured in the organism at the beginning of the depuration period; Warnau et al. 1996). The depuration kinetics of the radiotracers were best fitted using a simple exponential model including a constant (1). Decision was based on F-test and examination of residuals:

$$A_t = A_{0s} x\, e^{-kes\, x\, t} + AE \quad (1)$$

where At and A0s are the remaining activities (%) at time t (d) and 0, respectively; kes is the depuration rate constant (d^{-1}) and AE is the assimilation efficiency (%). The first component represents the depuration kinetics of the radiotracer fraction that is weakly associated with the organisms and rapidly eliminated (the subscript s standing for short-lived), whereas the second component refers to the proportion of the radiotracer ingested with food that is actually assimilated by the organism (Warnau et al. 1996). For the short-lived component, a biological half-life can be calculated ($T_{b1/2}$) from the corresponding depuration rate constant according to the relation $T_{b1/2s} = \ln 2/k_{es}$.

Model constants and their statistics were estimated by iterative adjustment of the model and Hessian matrix computation, respectively, using the non-linear curve-fitting routines in the Statistica® software 7.0.

Statistical comparisons between the three different feeding experiments were conducted using individual depuration kinetics of each element: individual parameters (kes and AE) were obtained using the best fitting model at the global scale (eq. 1) to the data of each individual. Then differences between these parameters were tested using Kruskall-Wallis and Siegel & Castellan non-parametric tests. The same statistical tests were used to compare the bioavailability of metals in the different prey. The level of significance for statistical analyses was always set at $\alpha = 0.05$. All the statistical analyses were performed using R software 3.0.1 (R Development Core Team 2014).

A model was developed and used to estimate the relative contribution of each prey to the metal intake from food in wild turbot. The model assessing these contributions for each studied essential elements was determined using the following equations:

$$C_{pi} = \sum(AE_{pi} x\ Q_{pi}\ x\ IR\ x\ O_{pi} x\ BW) \quad (2)$$

$$C_{ri} = (C_{pi} / \sum C_p)\ x\ 100 \quad (3)$$

where Cp (µg d^{-1}; wwt) is the amount of metal from one prey retained by the turbot (eq. 2). This value was, then, expressed in percentage of total metal intakes from food (eq. 3). AE_p is the assimilation efficiency (%) estimated using (1); Q_p (µg g^{-1} wwt) is the stable metal concentration in prey; IR (% of body weight d^{-1}) is the ingestion rate for fish (range of values used in the literature: 0.1 to 10%; Xu & Wang 2002); O_p (%) is the occurrence of prey in natural diet estimated by stomach contents analysis (Sparrevohn et al. 2008; Florin & Lavados 2010) and BW (g wwt) is the average body weight of the turbot used in this study. All the values are expressed on a wet weight basis, using conversion from percentage of dry matter provided in Table 2.

In order to better capture a certain degree of variability for the trophic transfer of essential elements to the turbot that can occur in the field, 3 scenarios covering 3 different situations were created. Using the model previously described, these scenarios were implemented on the basis of 3 different diet compositions reported from field surveys (Florin & Lavados 2010; Sparrevohn et al. 2008). For each of these diets, 3 distinct values were assigned for the ingestion rate of the turbot, the concentration of essential elements in the prey and the turbot AE for the 3 elements studied (IR, Q and AE; details are provided in Table 2). Briefly, scenario "low" corresponded to the inclusion into the model of minimal values of these parameters found in the present paper (Q and AE) or in the literature (IR; Xu & Wang 2002) whereas maximum and average values of the same parameters were respectively used in "high" and "medium" scenarios.

Table 2. Food composition and nutritional values (mean ± SD). For the shrimps, antennae, antennules, rostrum and telson were removed.

Composition	Fish	Shrimp			Ragworm
		Cephalothorax	Abdomen	Whole (reconstituted)	
Dry matter (DM %)	22.2 ± 3.19	29.03± 0.43	27.1± 0.82	27.9 ± 0.63	20.6 ± 1.96
Stable metals					
Co (µg g^{-1} dwt)	0.11 ± 0.01	0.12 ± 0.03	0.04 ± 0.00	0.08 ± 0.01	2.21 ± 0.82
Mn (µg g^{-1} dwt)	15.8 ± 1.75	2.78 ± 0.28	1.34 ± 0.12	2.02 ± 0.09	43.06 ± 31.33
Zn (µg g^{-1} dwt)	110 ± 1	71 ± 3	43 ± 1	56 ± 1	127 ± 25
Nutritional values					
Nitrogen (N, % DM)	2.19 ± 0.07	2.72 ± 0.44	3.09 ± 0.30	2.93 ± 0.35	0.89 ± 0.59
Protein (% DM)*	12.2 ± 0.40	15.25 ± 2.44	17.32 ± 1.68	16.44 ± 1.94	4.99 ± 3.31

* Estimation based on nitrogen content

Table 3. Description of the 3 scenarios used in the model for estimating the relative contribution of prey in the essential metal's intakes of turbot and details on the values used for the variables. The variables in these scenarios are the assimilation efficiency of predator (AE), the ingestion rate of predator (IR) and stable metal concentration in the prey (Q).

Parameters	Scenario		
	Low	Medium	High
AE *(assimilation efficiency of predator)*	Mean - SD	Mean	Mean + SD
IR *(ingestion rate of predator)*	Min	Mean	Max
Q *(stable metal concentration in the prey)*	Mean - SD	Mean	Mean + SD

3. RESULTS

3.1. Nutritional characteristics and stable metal concentration in prey

Essential element concentrations and nutritional characteristics estimated of the different food items are given in Table 2. Although this corresponds to a rough estimate of these characteristics (n = 3 for each prey), ragworms were the prey with the highest levels for all studied essential elements (Co, Mn and Zn). For example, Co concentrations reached 2.21± 0.82 µg g^{-1} dwt in ragworms vs. 0.08 ± 0.01 µg g^{-1} dwt in shrimp and 0.11 ± 0.01 µg g^{-1} dwt in fish (Table 2). However, ragworms were less nutritious than fish and shrimp, with 5% of protein in dry matter compared to 12% and 16%, respectively (Table 2).

3.2. Compartmentalization of radiotracers in prey

3.2.1. Body distribution

After radiolabelling ^{57}Co and ^{65}Zn were mainly distributed in the soft parts of fish and shrimp (i.e. whole-body activity minus activities measured in skeleton or cuticle; respectively, 89 ± 3% and 60 ± 7% for Co and 78 ± 3% and 63 ± 6% for Zn; Fig. 2A). In contrast, storage of ^{54}Mn depended on the considered prey: for fish, this element was mainly concentrated in the soft parts of body (64 ± 8%) whereas soft parts of shrimp contained a smaller proportion of Mn (29 ± 8%).

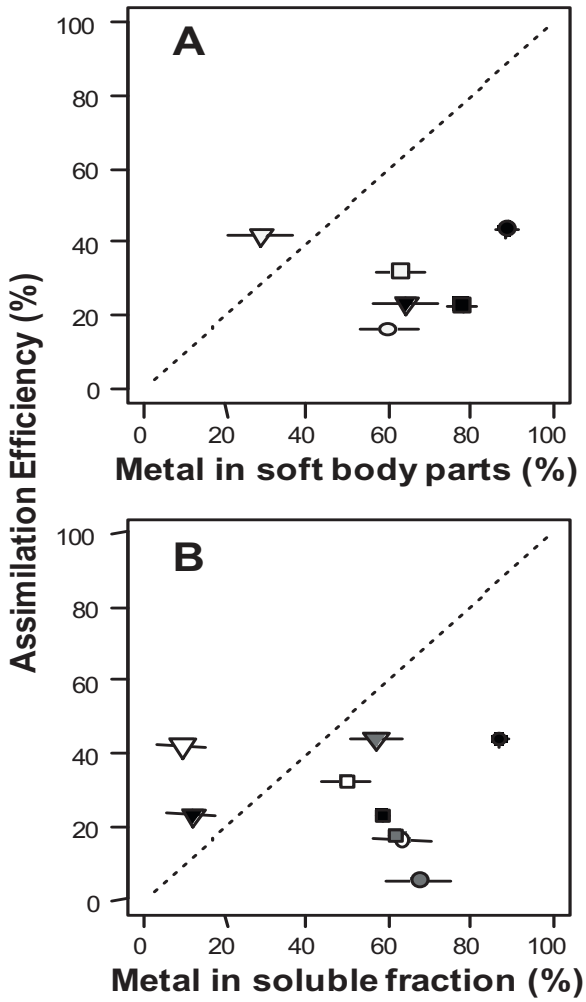

Figure 2. Relationship between metal fractioning in the prey (quantify by dissection and centrifugation) and Assimilation Efficiency (AE) in turbot. (A) Comparison between AE and metals: Mn (triangle), Co (circle) and Zn (square), included in soft body parts of fish in black and shrimp in white. (B) Comparison between AE in turbot and metals in soluble fractions of fish in black, shrimp in white and ragworm in grey.

3.2.2. Subcellular distribution

The majority of ^{57}Co taken up by the prey was located in the soluble fraction with a proportion ranging between 63 to 87%. The highest proportion (87 ± 2%) was measured in the soluble fraction of fish whereas, for shrimp, the soluble fraction contained 63 ± 7% of Co body burden (Fig. 2B). ^{54}Mn was mainly distributed in the insoluble fraction of shrimp and fish with respectively 91 ± 6% and 88 ± 6%.

On the other hand, for ragworms, ^{54}Mn was mainly (57 ± 7%) present in the soluble fraction (Fig. 2B). The subcellular compartmentalization of ^{65}Zn in the different prey was variable. In fish and ragworms, most of ^{65}Zn was located in the soluble fraction (~60%) whereas it was distributed equally between the soluble and insoluble fractions of shrimp.

3.3. Effects of diet on metal assimilation

To evaluate the influence of diet on metal assimilation in *S. maximus*, depuration kinetics of the three essential metals were followed after a pulse-chase feeding, using radiolabelled food items. The activity level of each element in each prey was measured prior to the feeding: the average activities were 50 Bq ^{57}Co g^{-1} wwt, 19 Bq ^{54}Mn g^{-1} wwt and 67 Bq ^{65}Zn g^{-1} wwt in fish; 22 Bq ^{57}Co g^{-1} wwt, 13 Bq ^{54}Mn g^{-1} wwt and 144 Bq ^{65}Zn g^{-1} wwt in shrimp without antenna, antennules, rostrum and telson, and 20 Bq ^{57}Co g^{-1} wwt, 7 Bq ^{54}Mn g^{-1} wwt and 250 Bq g^{-1} wwt of ^{65}Zn in ragworm.

Whole-body depuration kinetics of ^{57}Co, ^{54}Mn, and ^{65}Zn in turbot were always best fitted by a two-phase model (simple-exponential model and a constant; Fig. 3 and Table 4; R^2: 0.76-0.98). The assimilation efficiency (AE) and depuration rate of the three radiotracers depended both on the food and metal considered. The major fraction (53-95%) of the three elements was rapidly lost ($T_{b½s}$ < 1.4d) regardless of which prey had been ingested.

Estimated ^{57}Co AE varied significantly ($p < 0.05$) according to the prey type (Table 5). Indeed, ^{57}Co was poorly assimilated by turbot when fed with radiolabelled ragworms (AE = 5.1 ± 1.1%). Assimilation was elevated when fish were fed with juvenile fish (AE = 43.1 ± 12.0%) and an intermediate situation was observed when they were fed with shrimp (AE = 16.3 ± 4.0%). Estimated AE of ^{54}Mn also varied with the diet, though to a lesser extent. AE was significantly lower ($p < 0.001$, Table 5) when turbot were fed with fish (AE = 23.0 ± 7.7%) than when fed with shrimp and ragworms (42.0 ± 6.6% and 43.7 ± 2.3%, respectively; Fig. 3, Table 5). Variation of ^{65}Zn AE was less pronounced.

Table 4. Estimated depuration kinetic parameters of ^{57}Co, ^{54}Mn and ^{65}Zn in turbot exposed to the radiotracers by 3 different types of food (n = 8-12 per treatment) and then maintained for 21d in unspiked seawater. k_{es}: depuration rate constant (d^{-1}); $T_{b½s}$: biological half-life (d), AE: assimilation efficiency (%); ASE: asymptotic standard error; R^2: determination coefficient. Probability of the model adjustment: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Tracer	Feed	Short-term		Long-term	R^2
		k_{es} ± ASE	$T_{b½s}$ ± ASE	AE ± ASE	
^{57}Co	Fish	0.52 ± 0.10***	1.33 ± 0.24	43.46 ± 1.94***	0.76
	Shrimp	0.87 ± 0.05***	0.79 ± 0.05	16.30 ± 0.92***	0.97
	Ragworm	1.43 ± 0.07***	0.48 ± 0.02	5.04 ± 0.78***	0.98
^{54}Mn	Fish	0.63 ± 0.05***	1.10 ± 0.09	23.10 ± 1.47***	0.88
	Shrimp	0.61 ± 0.05***	1.14 ± 0.09	41.99 ± 1.20***	0.93
	Ragworm	0.71 ± 0.10***	0.98 ± 0.14	43.79 ± 1.62***	0.89
^{65}Zn	Fish	0.51 ± 0.04***	1.35 ± 0.12	22.80 ± 1.63***	0.94
	Shrimp	0.69 ± 0.04***	1.00 ± 0.03	32.20 ± 1.00***	0.96
	Ragworm	1.05 ± 0.06***	0.66 ± 0.04	17.39 ± 0.94***	0.97

The only significant difference occurred for AEs estimated when turbot were fed with shrimp and ragworm ($p < 0.05$, Table 5), ^{65}Zn being more efficiently assimilated from shrimp (AE = 32.2 ± 6.0%). Regarding depuration rate constants (k_{es}), values obtained for ^{57}Co and ^{65}Zn when fish were fed with ragworm were significantly higher ($p<0.05$) than when fed with the two other prey, indicating that their retention was shorter. For ^{54}Mn, no significant difference of k_{es} was observed ($p > 0.05$, Table 5).

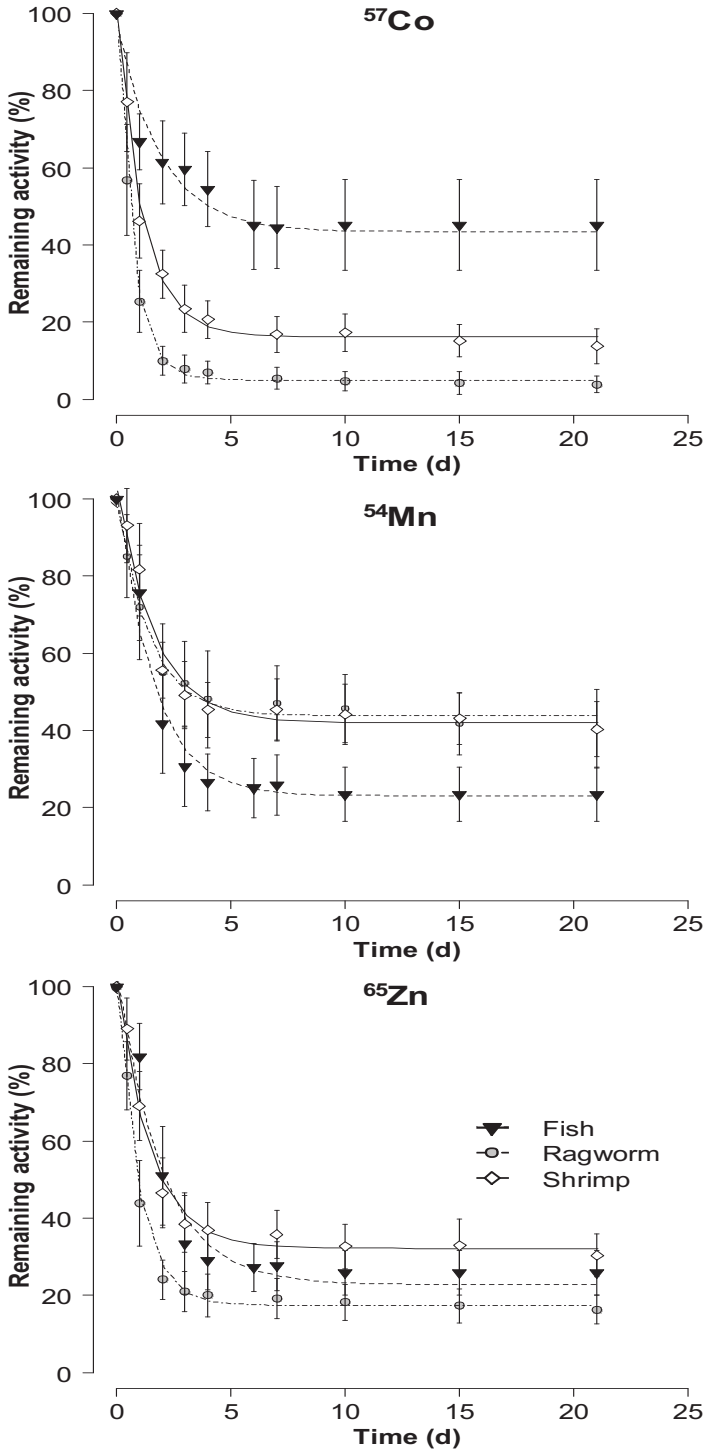

Figure 3. Influence of type of food (see Table 2) on whole-body depuration of ^{57}Co, ^{54}Mn and ^{65}Zn in turbot (% remaining activities, means ± SD). Parameters and statistics of depuration kinetics are given in Table 4.

Table 5. Comparison of assimilation efficiency (AE, %) and depuration rate constant (k_{es}, d^{-1}) of ^{57}Co, ^{54}Mn and ^{65}Zn in turbot exposed to the radiotracers by three different types of food (n = 8-12 per treatment) and then maintained for 21d in unspiked seawater. Underlines indicated that the values (means ± SD) are not significant different (p>0.05). Statistical comparisons between the three different feeding experiments were undertaken using individual depuration kinetics of each element: individual kinetic parameters (k_{es} and AE) were obtained using the best fitting model at the global scale (Table 4) to the data of each individual.

Parameter	Tracer	Prey		
		Fish	Shrimp	Ragworm
AE	^{57}Co	43.1 ± 12.0	16.28 ± 4.04	5.14 ± 1.14
	^{54}Mn	23.0 ± 7.73	41.99 ± 6.61	43.17 ± 2.34
	^{65}Zn	21.7 ± 6.85	32.19 ± 6.02	17.94 ± 1.92
k_{es}	^{57}Co	0.59 ± 0.24	0.93 ± 0.25	1.57 ± 0.10
	^{54}Mn	0.69 ± 0.21	0.63 ± 0.13	0.78 ± 0.16
	^{65}Zn	0.53 ± 0.10	0.71 ± 0.12	1.02 ± 0.12

3.4. Outputs of the model on metal intake

The relative contributions of the different prey in the daily trophic intake of stable metal in turbot under three natural diets are shown in Figure 4. When the diet of turbot was composed of fish and crustaceans, the latter taxon provided the highest essential element intake (Fig. 4A and 4B). However, when polychaetes were included in the diet (even in small proportion, viz. 28%), they contributed to the largest proportion of Co and Mn (38-58% and 40-78%, respectively, depending on the scenario; Fig. 4C).

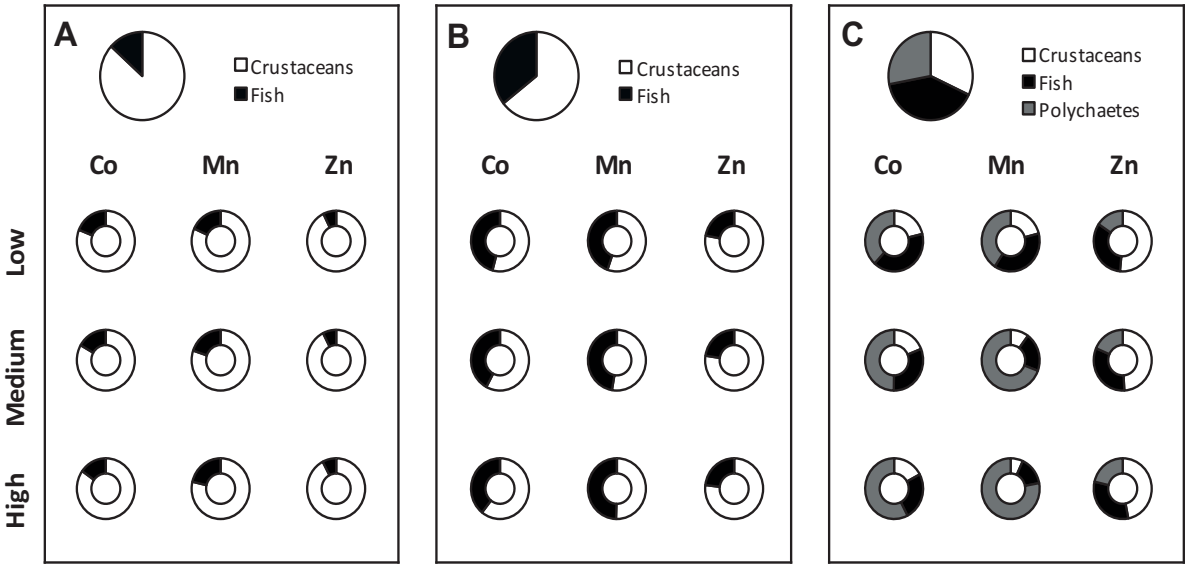

Figure 4. Relative contribution of the different prey in the daily intake of stable metal from food in turbot under three natural diets. Three different scenarios were considered to reflect the variability of parameter's values. Scenario "Low" takes into account lower concentration values of metals in prey with a low ingestion rate and reduced assimilation. Conversely, in the scenario "High", we considered the maximum values of these parameters. Finally, the average values were used in the "Medium" scenario.

4. DISCUSSION

Our results show that assimilation efficiencies (AEs) are metal-dependent and affected by the food items. Ranges of AE of ^{57}Co, ^{54}Mn and ^{65}Zn in turbot for the three different prey considered were respectively 5-43%, 23-44%, and 17-32%. Although, trophic transfer of Co and Mn is poorly documented in fish, information available show that AEs or remaining activities (multi-feeding experiments) reported for the carp *Cyprinus carpio* (Baudin & Fritsch 1989), the rainbow trout *Oncorhynchus mykiss* (Baudin et al. 2000), the silversides *Menidia* sp. (Reinfelder & Fisher 1994) and the turbot *S. maximus* (Mathews et al. 2008) are always lower than the ones determined in the present study. The values obtained for 65Zn are in accordance with the literature on marine and brackish fish fed with zooplankton (Ni et al. 2000; Xu & Wang 2002; Zhang & Wang 2005) or juvenile fish (Mathews et al. 2008) where reported AEs were between 5% and 31%. To the best of our knowledge, effect of food type on AE of Co and Mn has never been studied in fish. However, several studies have demonstrated that AE of Zn in a predator fish can be affected by the food composition.

For example, changes in AE were reported for the glassy *Ambassis urotaenia* (AE between 9 to 15%) and the mudskipper *Periophthalmus cantonensis* (AE between 11 to 31%) when respectively fed with *Artemia* sp. and *Acartia spinicauda* (Ni et al. 2000). According to these authors, differences in AE would be explained by metal storage in specific locations in the prey and would explain the tight correlation observed between AE and elemental distribution in the soft tissues of zooplankton prey.

It is well documented that storage forms and location of metals in prey determine the bioavailability of these elements for predators (e.g. Wallace and Lopez, 1996; Wallace and Luoma, 2003; Meyer et al. 2005) and impact the AE. In order to investigate the possible relationship between storage or location of Co, Mn and Zn in the prey and the AE of these elements in turbot, the measured AE were compared with metal distribution in the prey determined by the following methods (1) dissection (tissue distribution) and (2) ultracentrifugation (i.e. subcellular distribution, i.e. soluble vs. insoluble fraction). Several hypotheses examined to explain the relationship between the bioavailability of metals, their fractioning in prey and their assimilation in predators (Rainbow et al. 2011). Our results, obtained using complex pluricellular prey, were compared with the two main hypotheses often reported in the recent scientific literature for organisms fed with unicellular or simple pluricellular prey. The first hypothesis assumes that AE of the predator can be estimated from the percentage of metal in the non-exoskeleton fraction, or soft body parts of the prey (Reinfelder and Fisher 1994). Such a relationship has been reported for l09Cd, 57Co, 75Se, and 65Zn in the silversides *Menidia menidia* and *M. beryllina* fed with zooplankton. The second hypothesis assumes that the proportion of bioavailable metals for predators is related to the quantity of metal associated to the cytosolic fraction of the prey (Fig. 1; Wallace & Lopez 1996). Metal available fraction was further considered to be better reflected if the fraction of the metal associated with organelles was added to the cytosolic fraction (i.e. concept of Trophic Available Metal -TAM-; Wallace and Luoma, 2003). In the case of metal bioavailability to predatory fish, Zhang and Wang (2006) found a positive relationship between TAM fraction in a variety of prey organisms (barnacles, bivalves, fish viscera and zooplankton) and AE of Zn and Se in the grunt *Terapon jarbua*. Nevertheless, no strict equivalence between TAM in prey and AE in fish has been found yet, in contrast to invertebrates, while the determination of metal available fraction from one trophic level to another is still intensely studied or discussed in the scientific literature (e.g. Rainbow et al. 2011; Rainbow et al. 2015).

In the present study, no clear relationship was detected between AEs and metal fractioning in the prey either at a tissue (dissection) or subcellular (ultracentrifugation) level (Fig. 2A and B).. Therefore, our results do not support the hypotheses of Reinfelder and Fisher (1994) and of Wallace and Lopez (1996) in the case of turbot fed with complex pluricellular prey. Indeed, for Co and Zn, values of AE for predator were lower than those expected from both hypotheses, which advocate for its equivalence with metal fraction in the soft parts of the prey (Figs 1A and 2A) or metal soluble fraction in the prey (Fig. 2B). A fraction of Co and Zn contained in the supposed bioavailable compartments of prey (i.e. soft tissues and soluble fraction) was not assimilated by the turbot (Figs 1B, 2A and 2B). This overestimation of the bioavailable fraction of trace elements by measuring metals in the cytosolic fraction, indicates that the "TAM fraction" is not applicable to assess the trophically available fractions of Co and Mn in a natural prey of the turbot (Fig. 1A). One potential explanation could result from the ecology of turbot. Indeed, the optimal temperature of juvenile turbot (approx. 15°C as used in the present study) is lower than the examples mentioned previously (Menidia sp. and Terapon jarbua, respectively raised at 18 and 20°C). Acknowledging the positive relationship between temperature and the activity of the digestive enzymes in fish (Xiong et al. 2011), low temperature may lead to a low enzyme activity in turbot, resulting in a less efficient digestion of food and thus lower AEs than expected.

Interestingly, Mn was the only essential element for which AE was found to be greater than what was expected by theory (Fig. 1C and 2B), when the turbot were fed with shrimp or fish. In this specific case, the cytosolic fraction underestimated the fraction of the prey assimilated by the turbot (% soluble < AE). Therefore, the TAM theory (that adds the fraction in the organelles to the fraction present in the cytosol) may be relevant (Fig. 1) although our results cannot prove the equivalence of TAM and AE. Alternatively, our data suggests that other insoluble subcellular compartments of the prey found in the soft tissues (i.e., compare Fig. 2A and 2B) can be assimilated by the turbot (Fig. 1). For example, previous studies using invertebrate predators (i.e. two neogastropods fed with various species of molluscs and crustaceans) have shown that a part of the metal assimilated from the food was also associated to "metal rich-granules" and "cellular debris" of the prey (Cheung & Wang 2005; Rainbow et al. 2007).

Indeed, metals bound in metal-rich granules (MRG) appear to be more susceptible to the "assimilatory powers" of neogastropod molluscs than those of other invertebrates like decapod crustaceans (Wallace & Lopez 1997; Wallace & Luoma 2003; Rainbow et al. 2006; Rainbow et al. 2011). In this context, further studies are needed to assess which parts of insoluble fraction compartments (which include organelles, cellular debris and MRG; Fig. 1A) must be taken into account to assess accurately the trophically available fraction of Mn in a predator fish like the turbot.

Our results obtained in controlled conditions help understand the influence of diet on metal AEs. They also provide preliminary information on the contribution of each prey to the total intake of essential metals per ration in the fish, when taking into account the variation of natural diet assessed in the field. The natural diet of the juvenile turbot is mainly composed of crustaceans (in particular decapods), fish (mostly adults and larvae of small pelagic species; Fig. 4) and eventually polychaetes, although their relative proportion is variable and habitat- and season- dependent (Sparrevohn et al. 2008; Florin & Lavados 2010). When combining our results from radiotracer experiments and the level of stable elements measured in typical prey, we estimate that, although polychaetes (ragworms) represent only 28% of the stomach contents of turbot, they contribute 38-58% and 40-78% to the total intake of Co and Mn respectively in the "Low" and "High" scenarios (Table 2, Fig 4C). Ragworms tend to concentrate metals in the marine environment (Table 4). Our results confirmed other field investigations (see reviews of Eisler 2009a, b) and showed higher concentrations of Co, Mn and Zn in polychaetes than fish and crustaceans. In the case of Mn, the high contribution of polychaetes can be explained by the high Mn AE observed in turbot fed with ragworms. On the other hand, these turbot poorly assimilated Co and the contribution of polychaetes was related to the high concentration of stable Co in this species. Another aspect revealed by our assessment is the limited contribution of fish to the intake of Zn (always < 24%) despite the fact that this prey can represent up to 36% of stomach contents of juvenile turbot (Fig. 4B). Shrimp generally provide a major part of the essential elements from food and are also the prey that have the highest protein level (Table 3), highlighting the nutritional and ecological importance of crustaceans in the diet of the turbot.

5. CONCLUSION

This study provides new information on essential element assimilation in a marine fish. Our results suggest that diet composition plays a significant role in the assimilation of essential elements ingested with food in the turbot S. maximus. It also highlights that the supposed relationships between AE in predator and metal fractioning in prey are not necessarily confirmed when complex pluricellular food items are considered.

Our simple model, based on the relative contribution of the different prey to essential metal uptake, emphasizes the importance of crustaceans in the nutrition of turbot although when seasonally available polychaetes can make a disproportionately high contribution to dietary Co and Mn uptake by turbot.

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ANNEXE 4

Trophic transfer of ^{110m}Ag in the turbot *Scophthalmus maximus* through natural prey and compounded feed items

Pouil S[1,2], Warnau M[1], Oberhänsli F[1], Teyssié J-L[1], Metian M[1] (2015) Trophic transfer of 110mAg in turbot Scophthalmus maximus through natural and compounded feed items. *Journal of Environmental Radioactivity* 150: 189-194.

ABSTRACT: Industrial incidents can result in radionuclide release in the environment, among which ^{110m}Ag. Indeed, under particular circumstances, non-negligible amounts of ^{110m}Ag have been measured in the marine environment (as observed in Fukushima Dai-ichi incident). This element can be accumulated by aquatic organisms through different pathways including the trophic transfer. The present study aimed at examining the variation of ^{110m}Ag assimilation efficiency (AE) by turbots, *Scophthalmus maximus*, when exposed through different feeds. Pulse-chase feeding experiments were carried out in mesocosms, using radiolabelled feeds (natural prey and commercial pellets). Depuration kinetics of ^{110m}Ag over 21 days were generally fitted by a two-component exponential model; the ingested radioelement was poorly assimilated by turbots regardless of the food item that was used (AE always <3%). Concentration and subcellular distribution of ^{110m}Ag in prey did not seem to influence its assimilation by turbot. These results suggest that physiological mechanisms could occur in fish that would prevent the transfer of ^{110m}Ag from gut lumen to internal organs (e.g. ^{110m}Ag neutralization in the lumen of the stomach, detoxification mechanisms occurring in the gut).

Keywords: Silver, Accumulation, Aquaculture, Feeds, Radionuclides

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1. INTRODUCTION

The impact of artificial radionuclides on aquatic ecosystems, in which they act as micro-pollutants, is a topic of particular interest, both in terms of radiation protection and of environmental assessment. Under normal operational conditions of nuclear facilities, ^{110m}Ag is one of the radionuclides that can be emitted in small quantities (Adam et al., 2001). Under particular circumstances, non-negligible amounts of ^{110m}Ag have been measured in the marine environment, such as after the Chernobyl and Fukushima Dai-ichi Nuclear Power Plant accidents Buesseler et al., 2012; Aono et al., 2014).

Information on ^{110m}Ag bioaccumulation in fish is until now essentially confined to freshwater organisms (e.g. Baudin and Garnier-Laplace, 1994; Ausseil et al., 2002; Bertram and Playle, 2002). Although no biological role has been identified, these studies showed that the trophic transfer is an important pathway of accumulation in freshwater fish. In the few studies carried out on marine fish, the trophic transfer seemed to be a less important accumulation pathway of ^{110m}Ag (Pentreath, 1977; Long and Wang, 2005). However, only one type of food per species was investigated in these studies. There is thus currently a lack of knowledge on the possible influence of food quality on ^{110m}Ag assimilation by marine fish. Dissolved or particulate contaminations occur and contribute simultaneously in natural environment to the global metal bioaccumulation in organisms The delineation of the relative contribution of the different exposure pathways has to be assessed using models (see Thomann, 1981, Landrum et al., 1992; or Metian et al., 2008a), to really identify a main uptake route of contamination. These models that are using the kinetic parameters experimentally obtained are more and more considered in the literature but this is not systematic, and some papers argue the importance of one exposure pathway on another without mathematical validation. In the same time, it is key to determine within these models the variability of parameters that can affect the final determination of the main uptake pathway, such as for example the effect of food quality on metal transfer.

In this study, the turbot *Scophthalmus maximus* was chosen to examine the transfer of ^{110m}Ag from four different types of feeds: three different natural prey (seabream *Sparus aurata*, shrimp *Palaemon serratus*, and ragworm *Hediste diversicolor*) and manufactured pellets; this latter feed was selected since the turbot is a common species produced in aquaculture.

In order to better understand assimilation processes, two levels of biological organization were considered: (1) the whole individual (determination of the depuration kinetics and of assimilation efficiencies) and (2) the organs and tissues (dissections and determination of body distribution).

2. MATERIALS AND METHODS

2.1. Origin and acclimation of organisms

In January 2014, one hundred juvenile turbot *S. maximus* were purchased from a fish farm (France Turbot, France) and shipped to the International Atomic Energy Agency premises in the Principality of Monaco. Fish were acclimated to laboratory conditions for 21 days (open circuit, 500-L aquarium; water renewal: 100 L h^{-1}; 0.45 µm filtered seawater; salinity: 38 p.s.u.; temperature: 15 ± 0.5°C; pH: 8.0 ± 0.1; light/dark: 12h/12h). During the acclimation period, the fish were fed to a daily ration of 2% of their estimated biomass with 1.1-mm pellets (Le Gouessant, France). In order to investigate the influence of the diet on ^{110m}Ag assimilation by *S. maximus*,1.1-mm manufactured pellets (Le Gouessant, France) and three different natural prey of turbot were used (e.g. Sparrevohn et al., 2008; Florin and Lavados, 2010): juvenile fish (*S. aurata*; 60-day-old hatchlings, weight: 0.060 ± 0.013g), shrimp (*P. serratus*, weight : 0.58 ± 0.11g) and worms (*H. diversicolor*, weight: 0.82 ± 0.14g). Juvenile fish were obtained from a hatchery (Poissons du Soleil, France), shrimp were purchased from a fisherman (Poissons Vivants, France), and worms were purchased from fishing bait seller (Normandie Appats, France). All living feeds were acclimated to the same laboratory conditions than predator fish for a minimum of two weeks prior to experiments. Shrimp and worms were fed a mix of fish feed and crushed mussels whereas juvenile fish were fed 300-µm pellets (Biomar, France). Since body size is known to affect metal bioaccumulation in marine organisms (Boyden, 1974; Warnau et al., 1995), only individuals with homogeneous size of each prey species were used in the experiments.

2.2. Radiotracers and Counting

Experiments were carried out using a high-specific ^{110m}Ag radiotracer purchased from POLATOM, Poland (^{110m}Ag as $AgNO_3$ in 0.1 M HNO_3; [$T_{1/2}$] = 250 days; specific activity: 520 MBq mg^{-1}).

The radioactivity of the tracer was measured using a high-resolution γ-spectrometer system composed of 5 Germanium - N or P type - detectors (EGNC 33-195-R, Canberra® and Eurysis®) connected to a multi-channel analyser and a computer equipped with a spectra analysis software (Interwinner 6, Intertechnique®). The radioactivity was determined by comparison with standards of known activity and of appropriate geometry (calibration and counting). Measurements were corrected for background and physical radioactive decay. Organisms were placed in counting tubes filled with clean seawater during the counting period. The counting time was adjusted to obtain a propagated counting error less than 5% (e.g. Metian et al., 2008b), typically 25-60 min for whole organism radioanalysis. This counting duration did not affect organism welfare, as shown by their inter-counting activity and feeding behavior.

2.3. Experimental Procedure

2.3.1. Radiolabelling of feeds

Twelve grams of pellets were dipped for 1 h in 16.5 mL of seawater spiked with 1.8 kBq ^{110m}Ag mL^{-1}. Pellets were then dried for 48 hours at 50°C and kept in a dry environment in order to prevent mold growth. Preliminary tests were performed to determine the possible leakage into the water of radioisotopes from the pellets during the feeding. During the feeding, acclimated fish were consuming pellets in less than 1 min. Therefore, preliminary tests consisted in pouring radiolabelled dry pellets (100 mg per treatment) for 1 and 10 min in 50 mL seawater and to measure any radioactivity in the seawater. The leakage of pellet-radioactivity in water did not exceed 0.8% and 2% of the initial activity after 1 min and 10 min, respectively. Although these tests confirmed the single-pathway contamination (viz. food) of the fish, one turbot was used in each treatment, as a control to take into account the possibility of ^{110m}Ag recycling through water (see Section 2.3.2). Radiolabeling of the natural prey was carried out by exposing them for 7 to 21 days in seawater spiked with the radiotracer (nominal activity of 0.5 kBq^{110m}Ag L^{-1}, corresponding to 27 nmol L^{-1} equivalent stable Ag). No change in pH was detectable in the aquarium (close circuit) after tracer addition. The spiked seawater was renewed daily in order to keep the radiotracer activity as constant as possible.

Activity of the metal tracer in seawater was checked daily, before and after each seawater renewal, to determine time-integrated activities (Rodriguez y Baena et al., 2006). Prey were fed after each seawater renewal and just prior to radioisotope addition. Exposures of prey were made in aerated, 20-L aquaria. For shrimp exposure, each organism was kept individually in a cylindrical PVC container (drilled to allow for free water circulation) during the whole duration of the experiment in order to avoid cannibalism during moulting and to facilitate individual identification. For the worms, the walls of the aquarium were obscured and plastic tubes were added as artificial burrows.

2.3.2. Exposure of turbots via radiolabelled feeds

Four sets of experiments were realized for each type of feed considered. Each time, 8 to 12 juvenile turbots (11.9 ± 5.5 g, wet weight) were randomly picked and transferred in an aerated, open circuit, 70-L aquarium. One week before the exposure to radiolabelled feed, fish were daily fed the selected type of feed and individually identified by slits cut in fins. Each experiment consisted in a single exposure to radiolabelled feed (single feeding method also called pulse chase feeding; see e.g. Hédouin et al., 2010; Metian et al., 2010). For each experiment, turbot were fed ad libitum for 30 min; the uneaten food was then removed. Ragworms and shrimp were cut into pieces prior to the feeding in order to facilitate ingestion. Two hours after the initiation of the feeding, each fish was whole-body γ-counted alive and then replaced in clean, flowing seawater conditions (parameters as previously described). No regurgitation of the radiolabelled feeds was observed. Fish were then fed daily non-labeled pellets (2% of their biomass). In parallel, after the feeding period, an additional turbot was placed in each aquarium within in a net to control any possible radiotracer recycling from seawater due to radiotracer leaching from the contaminated food or from fish depuration. Fish were then whole-body γ-counted alive daily over 21 days (including control individuals). They were moved to another, clean 70-L aquarium after each counting to avoid contamination from ^{110m}Ag contained in faeces. After the depuration period, 4 individuals were dissected in 7 compartments: (1) the 4 fillets (skinned muscles), (2) the kidney, (3) the liver, (4) the gall bladder, (5) the digestive tract, (6) the head (including gills) and (7) the remaining parts (including skin, skeleton, fins, heart and muscle residues) and were separated, weighed (wet wt) and radioanalysed to determine the radiotracer body distribution. During experiments, no mortality was recorded.

2.3.3. ^{110m}Ag compartmentalization in feeds

For the natural prey, the distribution of ^{110m}Ag between the soluble and insoluble fractions was determined according to a method adapted from Bustamante and Miramand (2005). Briefly, an aliquot of contaminated feed stored at -80°C was crushed and tissues homogenized using a T25 Ultra-Turrax Basic (IKA®) in approximately 10 volumes (w:v) of TRIS-HCl buffer 0.02 M sucrose 0.25 M with 1 mM protease inhibitor (PMSF, phenylmethylsulfonylfluoride) and 5 mM reducing agent (DTT, dithiothreitol), at pH 8.06. The homogenates were centrifuged at 45 000 G for 2 h at 4°C (Sorvall Evolution RC Superspeed Centrifuge, Sorvall instruments®) to separate particle-free supernatant from the debris. Aliquots of the homogenates and trophically available metal (TAM) obtained were radioanalyzed in order to determine the radiotracers compartmentalization. The same procedure was repeated over time for each prey.

2.4. Data treatment and statistical analyses

Depuration of radiotracers was expressed as the percentage of remaining radioactivity (radioactivity at time t divided by the initial radioactivity measured in the organism at the beginning of the depuration period; Warnau et al., 1996). Depuration kinetics were fitted using non-linear model. They were best fitted using the following two-component model that includes an exponential component and a constant (Eq. 1) (decision based on F test and ANOVA tables):

$$A_t = A_{0s} x\ e^{-k_{es}\ x\ t} + AE \text{ (Eq.1)}$$

where At and A0s are the remaining activities (%) at time t (d) and 0, respectively; kes is the depuration rate constant (d^{-1}) and AE is the assimilation efficiency (%). The first component represents the depuration kinetics of the radiotracer fraction that is weakly associated to the organisms and rapidly eliminated, whereas the second component (AE) describes the radiotracer fraction that is retained in the body (Warnau et al., 1996). A short-term biological half-life can be calculated ($T_{b1/2}$) from the depuration rate constant according to the relation $T_{b1/2s} = \ln 2/k_{es}$. By definition, biological half-life of the "AE" component is infinite. Model constants were estimated by iterative adjustment of the model using the nonlinear curve-fitting routines in the Statistica® software 7.0. Distribution patterns of ^{110m}Ag among organs and tissues were compared by a G-test using R software 3.0.1 (R Development Core Team, 2014). The level of significance for statistical analyses was always set at $\alpha = 0.05$.

3. RESULTS

In order to evaluate the influence of food quality on ^{110m}Ag assimilation in the turbot *S. maximus*, 4 food items were first radiolabelled through seawater. The seabream *S. aurata*, the shrimp *P. serratus*, the ragworm *H. diversicolor* and the manufactured compounded feed concentrated ^{110m}Ag differently: the average activities measured in these feeds just before the feeding of the turbots were respectively 7 Bq g^{-1}, 37 Bq g^{-1}, 117 Bq g^{-1} and 1072 Bq g^{-1} (Table 1). When turbots were fed seabream or shrimp, activities could be measured accurately in turbots after feeding. However, after 48 h of depuration, uncertainties of counts became very high (> 20%). After two weeks, the activities were below detection limits. Therefore, depuration kinetics could not be modeled for these two types of food. When turbots were fed with pellets, whole-body depuration kinetics were best fitted by a two-component model (an exponential phase and a constant; Figure 1; R^2: 0.99). ^{110m}Ag was poorly assimilated by the fish (AE = 0.32 ± 0.75%, p>0.05). Virtually all ingested ^{110m}Ag was very rapidly lost ($T_{b½s}$= 0.77 ± 0.02 d; mean ± SD; Fig. 1).

Table 1. Activities (Bq g^{-1} wwt, mean ± SD) in the different feeds (n=4) and in the turbots (n = 8-12) after the single-feeding and at the end of depuration period. Assimilation efficiencies (%) related to the different feeds are also indicated.

Feed	Activity in feed (Bq g^{-1})	Activity in turbot (2h after the single-feeding, Bq g^{-1})	Activity in turbot (end of depuration, Bq g^{-1})	Remaining activity (%)
Seabream	7	0.67 ± 0.39	<0.03 ± 0.009[a]	<6.5 ± 5.2[b]
Shrimp	37	0.98 ± 0.45	<0.02 ± 0.007[a]	<2.1 ± 0.6[b]
Pellet	1072[c]	15.44 ± 7.17	0.19 ± 0.09	1.3 ± 0.65
Worm	117	11.65 ± 5.61	0.29 ± 0.10	2.3 ± 1.2

[a] Values indicated are the detection limits.
[b] Values calculated using detection limits.
[c] Concentration (Bq g^{-1}) dry weight.
[*] Different from 0 (p< 0.0001).

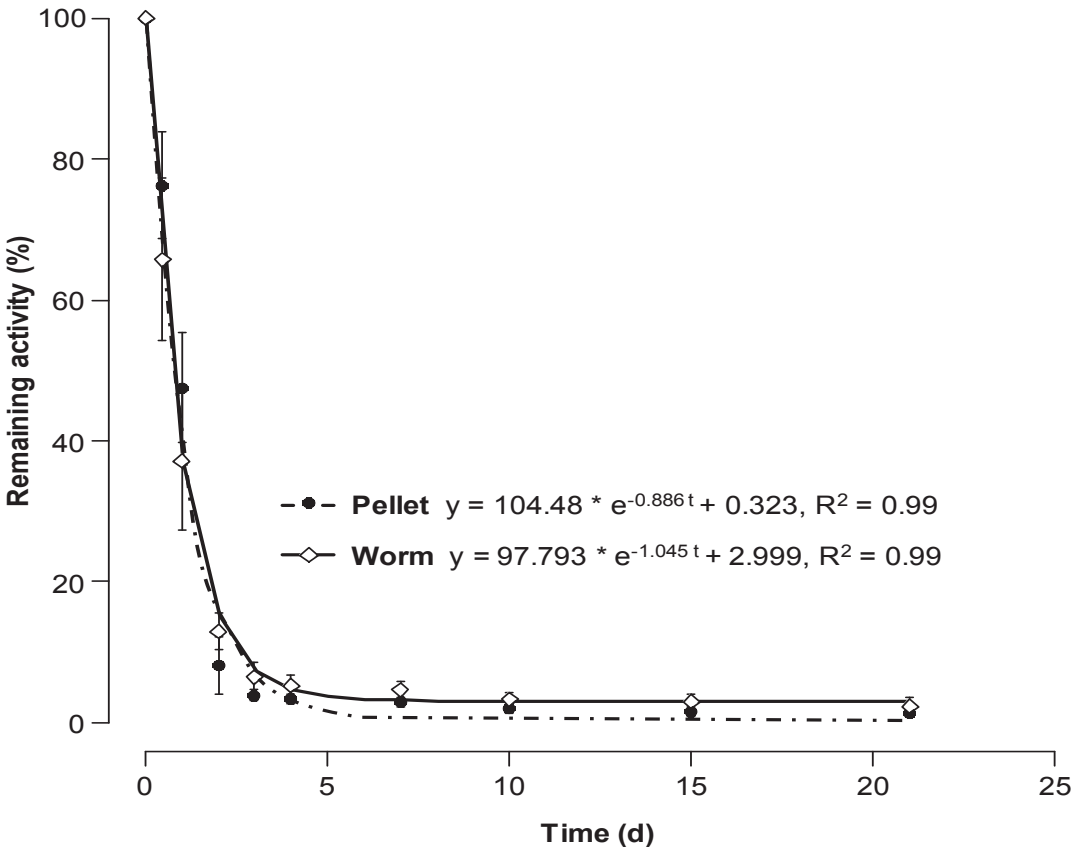

Figure 1. Influence of type of food on whole-body depuration of ^{110m}Ag in turbots (% remaining activities, mean ± SD).

When ragworms were used for feeding turbots, depuration kinetics were best fitted by the same two-component model as with pellets (Fig. 1; R^2: 0.99). The short-term depuration of ^{110m}Ag was hereto fast ($T_{b½s}$= 0.66 ± 0.03 d; mean ± SD; Fig. 1) but, conversely to the other feeds, ^{110m}Ag assimilation was high enough to be accurately estimated (AE: 3 ± 0.7%; mean ± SD; Table 1, $p<0.05$).

Post-feeding distribution of ^{110m}Ag in turbots at the end of the 21-d depuration period is shown in Table 2, for the different types of food. Similar patterns of ^{110m}Ag distribution among compartments were observed in turbots fed with the different feeds ($p>0.05$): liver and digestive tract accounting for until 65% of the total ^{110m}Ag load (Table 2). These body compartments represented, respectively, less than 10% and 1% of the body weight. Most of the remaining activity was distributed in the head and in remaining tissues.

Table 2. Body distribution (%, mean ± SD, n = 4) of ^{110m}Ag in the turbots fed with radiolabelled feeds and then maintained for 21d in uncontaminated seawater. Values with high uncertainty (>5%) are shown in italic. Weights were expressed as a percentage of total mass.

Compartments	Seabream[a]		Shrimp[a]		Pellet[b]		Worm	
	Weight (%)	Distribution (%)	Weight (%)	Distribution (%)	Weight (%)	Distribution (%)	Weight (%)	Distribution (%)
Digestive tract	9.7 ± 2.4	16.1 ± 14.5	5.9 ± 0.6	10.7± 2.9	6.0 ± 0.9	14.0 ± 2.8	5.4 ± 0.78	15.5 ± 4.5
Filets	18.6 ± 1.5	16.5 ± 3.0	20.8 ± 0.6	13.8 ± 11.2	22.1 ± 1.6	4.69 ± 1.4	22.9 ± 1.8	2.8 ± 1.2
Gall bladder	<1	5.1 ± 1.2	<1	7.0 ± 1.5	<1	2.4 ± 1.6	<1	2.2 ± 1.1
Head	23.3 ± 3.0	21.4 ± 8.1	20.1 ± 1.1	15.5 ± 4.3	20.2 ± 4.38	18.0 ± 6.68	26.2 ± 1.7	12.9 ± 4.8
Kidneys	<1	13.7 ± 9.9	<1	5.4 ± 1.6	<1	1.4 ± 0.7	<1	<1
Liver	1.2 ± 0.4	9.0 ± 3.3	<1	17.8 ± 15.1	<1	29.8 ± 13.9	<1	49.7 ± 17.3
Remaining parts	46.6 ± 2.0	18.3 ± 7.6	51.6 ± 0.7	29.7 ± 9.7	50.3 ± 4.8	29.7 ± 5.1	44.5 ± 1.18	16.1 ± 9.1

[a]For these experiments, activities are closed to the detection limits.
[b]For this experiment, counting errors exceed 5% due to the low activities measured.

Regarding subcellular partitioning of ^{110m}Ag in natural prey, the majority was found in the soluble fraction: the average proportion ranged from 50 to 72%. Significant difference was recorded for the two extreme values ($p < 0.01$): the highest proportion of ^{110m}Ag in the soluble fraction (72 ± 5%) was measured in shrimp whereas this proportion was 50 ± 4% in ragworms (Fig. 2).

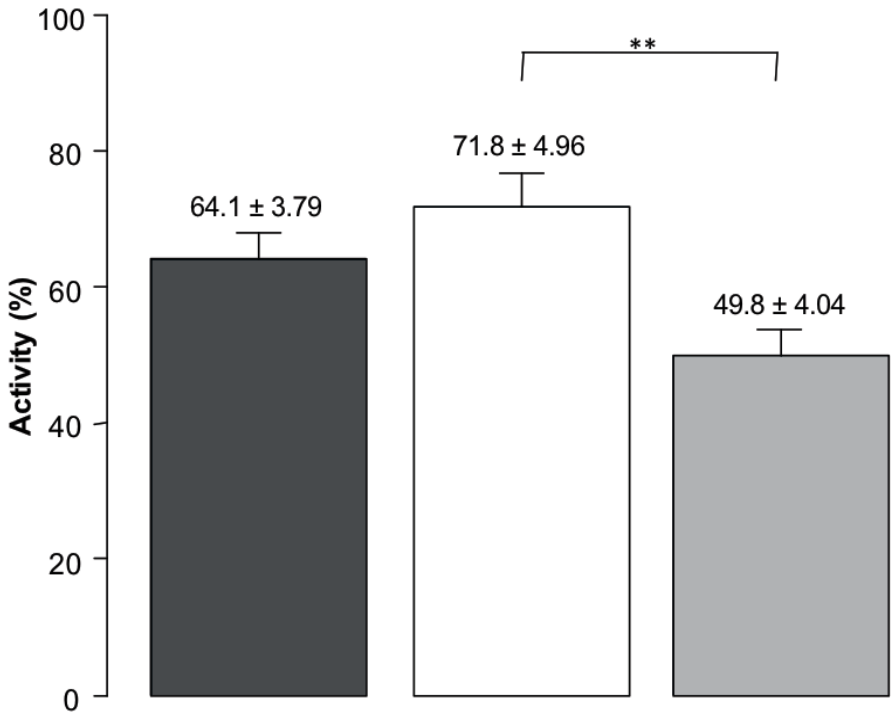

Figure 2. Percentage of ^{110m}Ag in soluble fraction of the three food items (seabream in black, shrimp in white and ragworm in grey, n = 4). * $p < 0.05$. ** $p < 0.01$.

4. DISCUSSION

^{110m}Ag can contribute up to 20% of the low level gamma radioactivity in liquid effluents from some pressurized reactors under normal operating conditions (Baudin and Garnier-Laplace, 1994). For several years, radioecological field studies have detected ^{110m}Ag in the main components of aquatic ecosystems downstream from nuclear power stations (e.g. Eyrolle et al., 2008; Aono et al., 2014). Moreover, the recent Fukushima Dai-ichi Nuclear Power Plant accident has led to the release of a massive amount of this radionuclide in the environment (Buesseler et al., 2012; Aono et al., 2014). In this context, it is essential to understand the transfer of this radionuclide in aquatic organisms to better assess the risk for the ecosystem (food chain) and for humans.

Despite the potential importance of trophic transfer, the contribution of the food pathway for ^{110m}Ag global bioaccumulation in marine fish has been poorly investigated (e.g. Pentreath, 1977; Rouleau et al., 2000; Wood, 2011). Experimental approaches appear to be the most appropriate to better understand, estimate and model radionuclide transfer from prey to predators (Adam et al., 2002).

To undertake this study, different natural prey and compound pellet were exposed to waterborne ^{110m}Ag. Concentration Factors (CFs) were calculated at the end of the exposure period; they were 76 ± 9 in shrimp, 119 ± 77 in seabream and 203 ±101 in ragworm. Although the exposure time varied among prey (7-21 days), results suggested that ragworms, exposed for 7 days in this study, have a higher ^{110m}Ag accumulation capacity than the other feeds.

To get a better understanding of ^{110m}Ag biological storage mechanisms at intracellular level in natural prey, ultracentrifugation was used for determining the subcellular fractioning of this element. The method used allows separating insoluble fraction (i.e., intracellular metal-rich granules and membrane fragments) from soluble fraction (i.e., cytosol and soluble metallothioneins and heat-sensitive proteins; Vijver et al., 2004). Most studies using such a technique were focusing on target organs and showed that ^{110m}Ag was mainly stored in an insoluble form, for example in the gills of the trout *Oncorhynchus mykiss* (80-95%; Galvez et al., 2002; Wood et al., 2002) or in the digestive gland of the scallop *Pecten maximus* (93 ± 2%; Metian et al., 2008a). Subcellular storage of ^{110m}Ag (and thus partitioning) may vary among organs for a given species, as shown in the bivalve species, *Gafrarium tumidum*; where 65% of ^{110m}Ag was found in the insoluble fraction of the visceral mass whereas 90% was found in this fraction of the gills (Metian et al 2005). Therefore, in the present study, we decided to centrifuge the whole organisms (prey) to get a better idea of the integrated subcellular partitioning. Results revealed that a large proportion of the ^{110m}Ag was included in the soluble fraction of prey (50 -72%).

Differences of ^{110m}Ag accumulation in food items (in terms of concentration or subcellular distribution) did not have any influence on assimilation of the radionuclide by turbots. Indeed, our results indicated clearly that ^{110m}Ag was poorly assimilated and retained by the turbots whatever the feed considered. The assimilation efficiency of the radioelement ingested with ragworms and pellets was <3%. AEs could not even be calculated in the case where turbots were fed with seabream and shrimp (activities measured in turbots were rapidly below the detection limits; Table 1). These results are consistent with the literature, although a wide range of food items was never looked at. Estimated AEs in individuals fed ragworms and pellets (3 ± 0.7% and 0.3 ± 0.8%, respectively; mean ± SD) are similar to the AE of 4.2 ± 2.8% estimated in plaice *Pleuronectes platessa*, fed with radiolabelled ragworms (46 days of depuration; Pentreath, 1977).

To the best of our knowledge, no study has been using pellets as food for studies on ^{110m}Ag AE. However, Rouleau et al. (2000) followed Ag depuration in individuals of the American plaice, *Hippoglossoides platessoides*, fed with a ^{110m}Ag-labelled wet paste (composition close to manufactured pellets; Provencher, 1995); authors found a very low retention of ^{110m}Ag with this food, with an estimated AE of 8.6 ± 4.8%. For this latter study, authors have used gravid females (adults) and thus direct comparison is not possible as we used juvenile fish and sexual maturity may affect the retention of trace elements due to the energy allocation for the oocyte production (Bang et al., 2008).

Some authors are linking AE of elements in predators with the subcellular partitioning of these elements in their prey. The concept of Trophically Available Metal (TAM), as defined by Wallace and Luoma (2003), tates that the fraction of elements found in the soluble fraction of prey should reflect the element fraction that is bioavailable to predators ingesting that prey. In our study, results revealed that although ^{110m}Ag was preferentially found in the soluble fraction of prey (50-72%), AEs were always low. Therefore, the positive relationship between TAM and AE stated by Wallace and Luoma (2003) could be true for simple organisms (as shown for bivalves fed phytoplankton; Wang and Fisher, 1996; Reinfelder et al., 1997) but was not verified in the case of our more complex prey (teleost, crustacean and polychaete). Even considering the lower value of soluble fraction of ^{110m}Ag (23.7-26.8%) previously found in the whole body *H. diversicolor* by Rainbow et al. (2006), the AE of ^{110m}Ag is still too low compared to what would be ex- pected by TAM concept. Possible explanations for this low AE in turbot despite a theoretically important source of bioavailable ^{110m}Ag are: (1) the occurrence of neutralization processes such as speciation or complexation of the element due to acidic conditions within the stomach of the fish, that would limit the element accessibility in the digestive tract or (2) the occurrence of detoxification mechanisms in the wall of the digestive tract, preventing ^{110m}Ag to penetrate into the internal compartments of the fish. The first hypothesis is backed up with our pellet experiment for which ^{110m}Ag was not biological bound but chemically adsorbed. Indeed, although the radionuclide was virtually 100% under “soluble” form in the latter experiment, ^{110m}Ag was not assimilated either by the fish. Regarding the second hypothesis, our body distribution results show that most of the assimilated ^{110m}Ag is located in the liver and digestive tract and not eliminated (AE component), suggesting storage of the element in these organs and tissues of the turbots.

This can be seen in relation to previous observations made, for example, in several bivalves (freshwater and seawater species; Berthet et al., 1992), the sea urchin *Paracentrotus lividus* (Warnau et al., 1996) and the fish *Oryzias latipes* (Chae et al., 2009), where Ag is stored within the intestinal wall as amorphous Ag (Ag_2S) generally found precipitated in the basement membranes and underlying connective tissues: Berthet et al., 1992).The occurrence of mechanism(s) impeding the transfer of the element across the fish digestive barrier was also mentioned in several studies, which suggested that intestinal tissues help buffer diet-borne metal uptake (Cu and Zn), by acting as a barrier (Maage and Julshamn, 1993; Clearwater et al., 2000; Handy et al., 2000).

Results from the transfer of ^{110m}Ag to the turbot via manufactured pellets highlighted a low risk of contamination of fish produced in aquaculture in the case of an environmental contamination by ^{110m}Ag. The potential for pellet to accumulate ^{110m}Ag by adsorption exists in the environment but the related AE in fish would be extremely low (Table 1). However, the bioaccumulation of ^{110m}Ag in fish through seawater was not investigated in our study and should be considered to establish a comprehensive risk to fish and consumers.

As radioactive isotopes have nearly identical physical and chemical properties to equivalent stable isotopes, the findings of the present study can also be extended to the stable element (viz. Ag) and its bioaccumulation mechanisms in fish (*e.g.* Metian et al., 2008a; Metian et al., 2010). Here, we demonstrated the low silver retention regardless the 4 type of feeds used (natural prey and manufactured pellet) in a marine fish. This information is also of interest in the context of the occurrence of silver pollution in the marine environment, *e.g.* in relation to the rapid increase in the use of silver nanoparticles (Savery et al., 2013) or to the urban poor sewage treatment (Sañudo-Wilhelmy and Flegal, 1992).

5. CONCLUSION

This present study confirmed the low assimilation efficiency of ^{110m}Ag from food in a marine fish, the turbot *S. maximus*, with the use of a range of food items that are the main components of the diet of the fish in natural conditions or in aquaculture production. Results showed a poor assimilation and retention of ^{110m}Ag by the predator fish regardless of the type of food.

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ANNEXE 5

Comparative study of trophic transfer of the essential metals Co and Zn in two tropical fish: a radiotracer approach

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(2017) Comparative study of trophic transfer of the essential metals Co and Zn in two tropical fish: A radiotracer approach. *Journal of Experimental Marine Biology and Ecology* 486: 42–51.

ABSTRACT: The trophic transfer of two essential metals (Co and Zn) was investigated in two tropical euryhaline fish species (*Monodactylus argenteus* and *Scatophagus argus*), at both the adult and juvenile stages, using the pulse-chase feeding method and radiotracers (^{57}Co and ^{65}Zn). The food selected was brine shrimp (*Artemia salina*) previously exposed to dissolved radiotracers. Depuration kinetics of both elements were followed for 45d. During this period, ^{57}Co and ^{65}Zn distribution was also determined at different time intervals among 15 different body compartments. Results showed that, in juveniles, the ingested Co was poorly assimilated by both species (AE < 6%), whereas *S. argus* assimilated ^{65}Zn more efficiently than *M. argenteus* (AE = 24% vs. 15%). In terms of body distribution of these essential elements, the trends were similar between adults and juveniles of both species: Co was concentrated and mainly distributed in the liver in S. argus, highlighting the role of this organ in the storage of Co. Conversely, in both species, ingested Zn was more diffusely distributed throughout the body. The differences observed between species could be related to a difference in the digestive enzymatic system in each studied species, which in turn is related to their slightly different feeding habits in the natural environment.

Keywords: Cobalt, Zinc, Assimilation, Kinetics, Tissue distribution, Interspecies differences

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1. INTRODUCTION

Essential metals are part of the functional groups of various enzymes, play a structural role in respiratory pigments and metalloenzymes, and can act as activating co-factors for various enzymes (see e.g. Simkiss 1979; Williams 1981). For example, Co is a key component of vitamin B12 (cyanocobalamin), which is a coenzyme in a number of cellular processes including the oxidation of fatty acids and the synthesis of DNA (e.g. Blust 2011). Zn has structural and catalytic roles in many proteins and almost 10% of all genes in sequenced fish genomes carry the annotation of Zn binding (e.g. Hogstrand 2011). Healthy conditions of fish are optimal if these elements are present in sufficient amounts in their tissues: depletion in these elements can provoke pathological damages and/or physiological alterations and their excess can also lead to toxic effects (e.g. Rainbow 2002; Förstner & Wittmann 2012).

In fish, food appears increasingly as an important pathway for acquiring essential metals (e.g. Xu & Wang 2002; Mathews & Fisher 2009). For example, the metal intake from the diet in the teleost Scophthalmus maximus was estimated to be responsible for more than 70% of the body burden of Co, Mn and Zn (Mathews & Fisher 2009). Despite a growing dataset showing the major role that food plays in essential and non-essential metal accumulation in fish (e.g. Spry et al. 1988; Zhang & Wang 2005), there is still limited understanding of the factors that influence metal assimilation at the interspecific level. Among these factors, feeding strategies have been reported to be crucial in the differential metal assimilation in coexisting species (e.g. Ni et al. 2000).

A key parameter for understanding and modeling metal trophic transfer in fish is the assimilation efficiency (AE). This variable can be easily determined under laboratory conditions, using radiotracer techniques (e.g. Mathews et al. 2008; Pouil et al. 2015). A complementary approach is the determination of the dynamics of inter-organ metal transfer, based on the measurements of metal in the different organs and tissues at different time intervals during the depuration phase. Although of interest, this approach is not very commonly reported in the literature. It has however been applied for toxicokinetic studies in fish (e.g. Hogstrand et al. 2003) or with other aquatic aquatic active predators like cephalopods (Bustamante et al. 2002; 2004).

In this context, the present work investigated the trophic transfer of two essential elements (Co and Zn) in adults and juveniles of two tropical euryhaline fish species, the silver moony *Monodactylus argenteus* and the spotted scat *Scatophagus argus*, using gamma-emitting radiotracers (^{57}Co and ^{65}Zn). This biokinetic study has focused on obtaining transfer rate data for tropical fish species given the fact that the majority of available information has to date come from studies carried out with temperate species. Recent reviews on the topic of trace element and radionuclide transfer in marine organisms have stressed the need for more data on tropical species in order to make valid comparisons on a global scale (see e.g. Fowler & Fisher 2005). Two levels of biological organization were considered in this study, i.e. the whole organism and the different organs and tissues, in order to evaluate the biokinetic parameters of the metal depuration and to identify metal transfer dynamics among the body compartments during the depuration phase.

2. MATERIALS AND METHODS

2.1. Acclimation of organisms

One hundred juveniles and 20 adults of each species (silver moony *M. argenteus* and spotted scat *S. argus*) were shipped to the International Atomic Energy Agency-Environment Laboratories' premises in the Principality of Monaco. Fish were acclimated for 3 months to laboratory conditions, adjusted to replicate as closely as possible their natural tropical environment (2000-L tank for adults and 700-L aquarium for juveniles; open circuit: 200 L h^{-1} in each tank; 0.45-µm filtered seawater; salinity: 35 p.s.u.; temperature: 25 ± 0.3°C; pH: 8.1 ± 0.1; light/dark: 12h/12h). During the acclimation period for both species, the juvenile fish were fed 3 to 4 times per day with adult brine shrimp (*Artemia salina*) at a daily ration of 130-290 mg wet weight (wwt) per individual. Adults were fed 4 times per day with compounded pellets (JBL Mariperls®) at a daily ration of 1.6-2 g dry weight (dwt) per individual. Virtually no mortality was observed during the acclimation period. Since body size (age) is known to affect metal bioaccumulation in marine organisms (Boyden 1974; Warnau et al. 1996a; Hédouin et al. 2006), only individuals with homogeneous sizes were used in the experiments.

2.2. Experimental Procedure

2.2.1. Radiolabelling of brine shrimp

In order to investigate the trophic transfer of the two essential elements (Co and Zn) by *M. argenteus* and *S. argus*, adult brine shrimp (*Artemia salina*) were used as prey. Preparation of the radiolabelled brine shrimp was carried out by exposing them for 18 h in aerated 20-L aquaria (approx. 300g wwt of adult brine shrimp per aquarium). At the end of the exposure period, brine shrimp were briefly rinsed and stored at -20°C. Exposure was repeated using 6 batches of brine shrimp in order to obtain enough radiolabelled food for trophic transfer experiments (with both juvenile and adult fish). Radiotracers of high specific activity were purchased from CERCA, France (^{57}Co, [$T_{1/2}$] = 271.8 days) and Amersham, UK (^{65}Zn, [$T_{1/2}$] = 243.9 days). Stock solutions of radiotracers were prepared in 0.1 M HCl. Seawater was spiked with the radiotracers (nominal activity of 3 kBq L^{-1} for Co and 2 kBq L^{-1} for Zn). Small volumes (1 mL) of the diluted radiotracer solution were added to the aquaria (close circuit), and no change in pH and salinity was detectable after the tracer addition. At the end of the exposure period, average activities in the brine shrimp were 239 Bq g^{-1} wwt for ^{57}Co and 40 Bq g^{-1} wwt for ^{65}Zn.

2.2.2. Exposure of juvenile fish

One batch of 86 juvenile silver moonies and one of 96 juvenile spotted scats (8.4 ± 1.4 g and 4.8 ± 1.4 g wwt, respectively) were each transferred into a 70-L aquarium (open circuit: 100 L h^{-1}; aerated, 0.45-µm filtered seawater; salinity: 35 p.s.u.; temperature: 25 ± 0.3°C; pH: 8.1 ± 0.1; light/dark: 12h/12h) prior to the exposure to radiolabelled brine shrimp.

The experiment consisted of a single exposure to radiolabelled brine shrimp via ingestion (thawed prey, single feeding method also called pulse-chase feeding; see e.g. Metian et al. 2010; Pouil et al. 2015). Juvenile fish were fed *ad libitum* for 1.25 h in a closed circuit system (247g wwt and 255g wwt of radiolabelled brine shrimp distributed to silver moony and spotted scat, respectively). Two hours after the beginning of ingestion, all fish were whole-body γ-counted alive and each batch of fish was removed and divided into two groups. Twenty-one individuals were transferred into a 20-L aquarium containing clean, flowing seawater (parameters as previously described). The remainder of the fish were returned into the initial 70-L aquarium.

No regurgitation of the ingested radiolabelled brine shrimp was observed. The 21 individuals from the 20-L aquarium were weighed separately (for individual recognition) and γ-counted alive (see section 2.3) at different time intervals over 45d to follow the whole-body depuration kinetics of ^{57}Co and ^{65}Zn. The aquarium was cleaned during each counting period to avoid contamination from radiotracers contained in the faeces. Fish from the 70-L aquarium were regularly sampled (n=3) using the same protocol, anesthetized and dissected (see section 2.2.4) after γ-counting.

2.2.3. Exposure of adult fish

Eighteen adult silver moonies and 13 adult spotted scats (81 ± 12g and 193 ± 35g wwt, respectively) were fed *ad libitum* in a 70-L aquarium during 6 h with thawed radiolabelled brine shrimp (i.e. 11 feedings for 30 min each time) in order to maximise the ingested activity in the fish (total distribution of 795g of radiolabelled brine shrimp for both species). Due to the size of adult individuals, γ-counting was not performed with living whole organisms; thus, 3 fish were randomly collected at different time intervals and dissected during the 45-d depuration period. For this purpose, fish were anesthetized, sacrificed and dissected into 15 body compartments (see details in section 2.2.4).

2.2.4. Body distribution of metals in fish

Juvenile fish maintained in the 70-L aquarium and all adults were regularly sampled, anesthetized using eugenol, and dissected in order to compare the metal organotropism in the tissues of both species. At each sampling time, 3 individuals (for both juveniles and adults) were dissected into the following body compartments: (1) muscles with the skin, (2) stomach and pyloric caeca, (3) intestine, (4) liver, (5) gall bladder, (6) pancreas and spleen, (7) kidneys, (8) heart, (9) gills, (10) eyes, (11) brain, (12) skeleton (13) head, and in addition for adult fish (14) gonads and (15) red gland. All compartments were separated, weighed (wwt) and radio-analysed (see section 2.3) to determine the radiotracer distribution among the various tissues. During all the experiments, no mortality occurred.

2.3. Radiotracers and Counting

The radioactivity of the tracers was measured using a high-resolution γ-spectrometer system composed of 3 Germanium N type detectors (EGNC 33-195-R, Intertechnique®) connected to a multi-channel analyser and a computer equipped with spectra analysis software (Interwinner 4, Intertechnique®). The radioactivity in live organisms and tissue samples was determined by comparison with known standards of appropriate geometry (calibration and counting). Measurements were corrected for background and physical radioactive decay. Live organisms were placed in polystyrene counting tubes (diameter: 80 mm, height: 50 mm), filled with clean seawater during the counting period. The volume of seawater was kept as low as possible to ensure at the same time the welfare of the fish and a constant geometry during the counting period (i.e. reduced fish movement).

The counting time was kept as short as possible and adjusted to obtain a propagated counting error less than 5% (e.g. Rodriguez y Baena et al. 2006; Metian et al. 2009). In the case of radioanalysis of live fish, counting times generally ranged between 15 and 55 min. Tests were performed prior to the experiments, where fish were placed in similar counting conditions in order to observe their behaviour, i.e. in a counting box for 1 hour in the dark. Dissolved O_2 concentration was monitored throughout these tests and was always > 3 mg L^{-1}. No alteration in organism health or behaviour was observed.

2.4. Data treatment and statistical analyses

2.4.1. Whole-body depuration kinetics in juvenile fish

Depuration of radiotracers was expressed as the percentage of remaining radioactivity (radioactivity at time t divided by the initial radioactivity measured in the organism at the beginning of the depuration period * 100; Warnau et al. 1996b). The depuration kinetics of the radiotracers were best fitted using a two-component exponential model (Eq. 1), with a decision based on F test and ANOVA tables (Hédouin et al. 2010):

$$A_t = A_{0s} x\, e^{-kes\, x\, t} + A_{0l} x\, e^{-kel\, x\, t} \quad (1)$$

where A_t and A_0 are the remaining activities (%) at time t (d) and 0, respectively; k_e is the depuration rate constant (d^{-1}); 's' and 'l' are the subscripts for the 'short-lived' and 'long-lived' components, respectively.

The short-lived component represents the depuration kinetics of the radiotracer fraction that is weakly associated with the organisms and rapidly eliminated (e.g. fraction in faeces), whereas the long-lived component describes the depuration kinetics of the radiotracer fraction that is assimilated by and tightly bound to the organism (Warnau et al. 1996b). The long-lived component allows assessing the assimilation efficiency (AE) of the radiotracer ingested with food (AE = A_{0l}). For each exponential component (s and l), a biological half-life can be calculated ($T_{b½s}$ and $T_{b½l}$) from the corresponding depuration rate constants (k_{es} and k_{el}, respectively) according to the relation $T_{b½} = \ln 2/k_e$. The model was fitted using non-linear routines and model constants were estimated by iterative adjustment of the model, using the Quasi-Newton method in the Statistica® software 7.0.

2.4.2. Distribution among body compartments (juveniles and adults)

For juveniles and adults of the two fish species, distribution of Co and Zn among the body compartments was determined over time and compared using the non-parametric Mann-Whitney U test (Zar 1996).

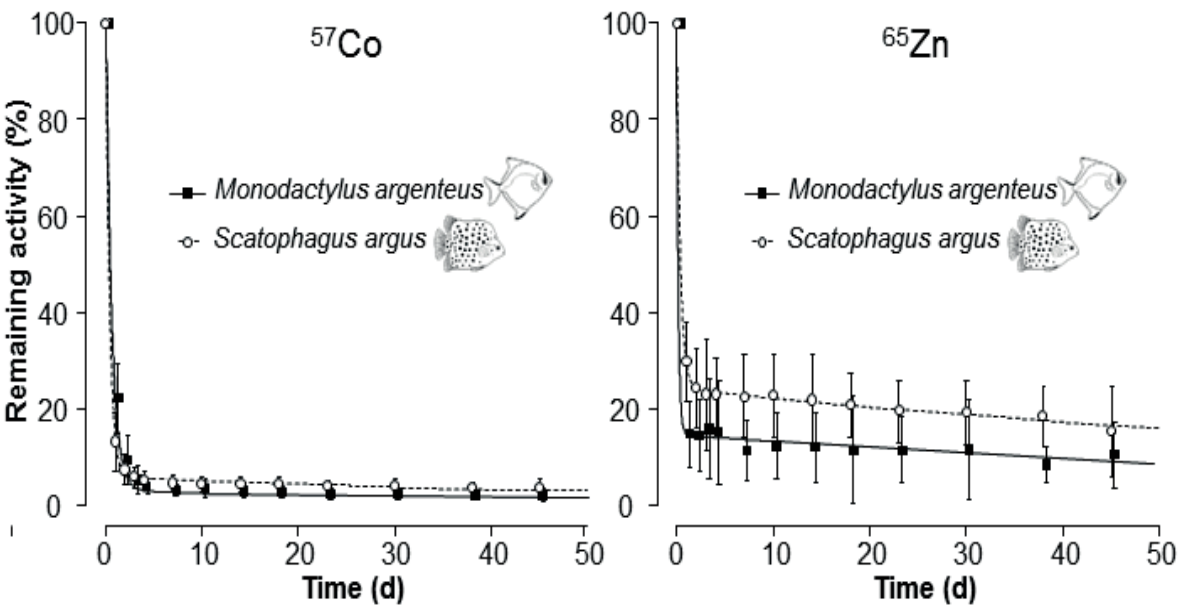


Figure 1. Whole-body depuration of ^{57}Co and ^{65}Zn after a single-feeding with radiolabelled brine shrimp in juvenile silver moony (*Monodactylus argenteus*, n=21) and spotted scat (*Scatophagus argus*, n=21) expressed as percentage of remaining activities (means ± SD). Parameters of depuration kinetics and their statistics are given in Table 1.

Furthermore, in order to quantify the transfer of each element over time among the internal body compartments, the ratio of the concentration of Co and Zn in each organ and/or tissue (Bq g^{-1} wwt) over the whole-body (Bq g^{-1} wwt) was calculated (sic concentration index, I_c, as defined by Rouleau et al. 2000). Values of $I_c > 1$ indicate that a tissue is enriched in Co or Zn compared to the average whole-body metal concentration. This index complements the information provided by metal body distribution and is useful for quantifying and comparing the storage capacities of the different body compartments in fish whose weight and ingested activity were variable. Comparisons of I_c values among body compartments were carried out using the Kruskall-Wallis non-parametric test, followed by a multiple-comparison test of Siegel and Castellan (Zar 1996). The level of significance for all statistical analyses was always set at $\alpha = 0.05$. All the statistical analyses were performed using either the Statistica® software 7.0 or R freeware 3.0.1 (R Development Core Team 2014).

3. RESULTS

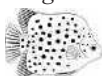
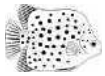
3.1. Depuration kinetics and assimilation efficiencies of ^{57}Co and ^{65}Zn in juveniles

The whole-body depuration kinetics of ^{57}Co and ^{65}Zn following the ingestion of radiolabeled A. *salina* by the juveniles of both fish species were best described by a two-component exponential model (Table 1 and Fig. 1). Assimilation efficiency (AE) and retention capacities are metal- and species- dependent. Both species assimilated poorly ^{57}Co (AE < 6%), whereas spotted scat assimilated ^{65}Zn more efficiently than silver moony (AE = 24.2 ± 1.0% vs. 14.7 ± 0.9%, respectively; $p < 0.05$). Assimilated ^{57}Co and ^{65}Zn were slowly eliminated, with biological half-life ($T_{b½l}$) values ranging from 33 to 55 d for ^{57}Co and from 67 to 83 d for ^{65}Zn (Table 1).

3.2. Body distribution and body dynamic of metals

During the 45-d depuration phase, dissections were carried out regularly in order to determine the metal distribution among selected body compartments (see section 2.2.4). Figures 2, 3 and 4 describe the distribution of Co and Zn, respectively, in the 4 main body compartments (i.e. stomach, intestine, liver, and muscles with skin representing 50-98% and 40-99% of total activity for Co and Zn, respectively) and in the remaining parts (i.e. the sum of the remaining body compartments) during the depuration phase in juveniles and adults of both species. The detailed distributions in each body compartment are provided in the supplementary material for this article.

Table 1. Estimated depuration kinetic parameters of ^{57}Co and ^{65}Zn in silver moony (*M. argenteus*) and spotted scat (*S. argus*) after a single feeding with radiolabelled brine shrimp and then maintained for 45 d in a clean flowing seawater. Depuration parameters: A_{0s} and A_{0l}: activity (%) lost according to the short- and the long-lived exponential component, respectively; the A_{0l} value is also the assimilation efficiency (AE; %), $T_{b½}$: biological half-life (d). ASE: asymptotic standard error; R^2: determination coefficient of kinetics.

Tracer	Species	Short-term		Long-term		R^2
		A_{0s} ± ASE (%)	$T_{b½s}$ ± ASE (d)	A_{0l} (=AE) ± ASE (%)	$T_{b½l}$ ± ASE (d)	
^{57}Co	*M.argenteus*	95.4 ± 0.7***	0.42 ± 0.01***	4.58 ± 0.44***	32.7 ± 9.0***	0.99
	S. argus 	94.2 ± 0.6***	0.28 ± 0.01***	5.77 ± 0.30***	54.5 ± 12.5***	0.99
^{65}Zn	*M.argenteus*	85.3 ± 1.9***	0.12^{NS}	14.7 ± 0.9**	66.8 ± 21.2***	0.95
	S. argus 	75.8 ± 1.9***	0.27 ± 0.04***	24.2 ± 1.0***	83.1 ± 19.3***	0.94

Significance of estimated parameters:
^{NS} p > 005 (not significant)
** p < 001
*** p < 0001

Considering element distribution, the depuration may be divided into three phases. The first phase (days 0-1), very rapid, corresponds to the passage of the food into the stomach which contains up to 80 % of the radioactivity body burden at the end of the feeding period (85 min). The second phase (days 1-4) is characterized by the occurrence of the digestive processes. During phases 1 and 2 (i.e. the first 4d of depuration), almost all the activity was distributed in the stomach and the intestine (up to 95% of the total body burden). Then, after 4 days a third phase can be depicted and corresponds to element translocations among the main compartments when whole-body activities are stable (i.e. when the absorbed fraction has stabilized; Fig. 1).

Gradually, the liver and the muscles with skin become then the predominant compartments in terms of trace element burden. Distributions are however relatively stable after day 10 except for Co in *S. argus*. Indeed, the percentage of ^{57}Co body burden in the liver tended to decrease between days 10 and 45 in both juvenile and adult fish, whereas a concomitant increase was observed in the muscles with skin (Fig. 2 and Fig. 4). Distribution patterns over time were similar between the species, juveniles or adults, for ^{65}Zn (Fig. 3 and Fig. 4), but not for ^{57}Co in which case the distribution in the liver was significantly higher in *S. argus* than in *M. argenteus* (Fig. 2 and Fig. 4; $p < 0.05$).

In terms of concentration index, I_c, Tables 2 and 3 provide a ranking of the I_c for Co and Zn, respectively, in each body compartment during the depuration phase in juveniles and adults of both species. The results indicate that after 2 days ^{57}Co was mainly concentrated in the liver for *S. argus* juveniles and adults (Table 2). In the case of *M. argenteus*, there was no significant difference between the calculated I_c values of juveniles and adults for all the compartments, although the liver and the pancreas seem to play an important role in the storage of ^{57}Co during the depuration phase. For ^{65}Zn, regardless of the species and the life stage, the highest values of I_c were always measured in the stomach and the intestine during the first 24h then, after this period, in the gall bladder, the pancreas and spleen (Table 3; $p < 0.05$).

Table 2. Concentration index (I_c) of ^{57}Co, throughout the 45-d depuration of juvenile silver moony (*M. argenteus*) and spotted scat (*S. argus*) after a single feeding with radiolabelled brine shrimp. Ic is the ratio between the activity concentration (Bq g^{-1} wwt) in the considered compartment and the activity in the whole fish (Bq g^{-1} wwt) multiplied by 100. A selection of body compartments having the highest and lowest Ic values (p<005) has been made. EYE: eyes, GAL: gall bladder, GIL: gills, GON: gonads, HRT: heart, INT: intestine, KID: kidney, LIV: liver, MUS: muscle, PAN: pancreas + spleen, RED: red gland, SKE: skeleton, STO: stomach. Values are means or means ± SD; n=3. Details of the data are available in supplementary materials.

Species		Time (d)																
		0	1	2	3		4	7	10		14	17	18	23		30	38	45
		Juvenile	Juvenile	Juvenile	Juvenile	Adult	Juvenile	Juvenile	Juvenile	Adult	Juvenile	Adult	Juvenile	Juvenile	Adult	Juvenile	Juvenile	Juvenile
M. argenteus	Highest I_c values	**Max:** STO (12±2) **Min:** INT (8±2)	**Max:** INT (39±3)	**Max:** INT (19±11) **Min:** PAN (7±2)	**Max:** KID (13±1) **Min:** STO (4±1)	**Max:** INT (63±4)	**Max:** HRT (14±6) **Min:** LIV (10±2)	**Max:** HRT (28±19) **Min:** LIV (15±1)	**Max:** GAL (21±13) **Min:** STO (4±1)	**Max:** LIV (21±13) **Min:** HRT (7±2)	**Max:** PAN (30±28) **Min:** LIV (16±5)	-	**Max:** PAN (47±36) **Min:** LIV (12±3)	**Max:** LIV (18±2) **Min:** HRT (10±2)	**Max:** LIV (16±11) **Min:** STO (6±3)	**Max:** PAN (60±9) **Min:** LIV (8±2)	**Max:** PAN (48±2) **Min:** LIV (8±3)	**Max:** PAN (51±34) **Min:** LIV (7±2)
	Lowest I_c values	**Max:** PAN (<1) **Min:** EYE (<1)	**Max:** KID (2) **Min:** EYE (<1)	**Max:** STO (3) **Min:** EYE (<1)	**Max:** GIL (2) **Min:** EYE (<1)	**Max:** GAL (6) **Min:** MUS (<1)	**Max:** I NT (10) **Min:** SKE (<1)	**Max:** KID (7) **Min:** SKE (<1)	**Max:** GIL (2) **Min:** EYE (<1)	**Max:** GON (5) **Min:** MUS (<1)	**Max:** GIL (2) **Min:** EYE (<1)	**Max:** GON (6) **Min:** MUS (<1)	**Max:** STO (4) **Min:** SKE (<1)	**Max:** GAL (7) **Min:** EYE (<1)	**Max:** GON (6) **Min:** MUS (<1)	**Max:** STO (4) **Min:** SKE (<1)	**Max:** STO (4) **Min:** SKE (<1)	**Max:** STO (4) **Min:** SKE (<1)
S. argus	Highest I_c values	**Max:** STO (9±2) **Min:** INT (3±2)	**Max:** INT (13±1) **Min:** LIV (8±3)	**Max:** LIV (13±6) **Min:** INT (10±7)	**Max:** LIV (14±2)	-	**Max:** LIV (18±1)	**Max:** LIV (26±3)	**Max:** LIV (18±4)	**Max:** LIV (30±2)	**Max:** LIV (33±6)	**Max:** LIV (21±1)	**Max:** LIV (21±1)	**Max:** LIV (25±4)	**Max:** LIV (29±6)	**Max:** LIV (17±2)	**Max:** LIV (17±3)	**Max:** LIV (15±2)
	Lowest I_c values	**Max:** LIV (<1) **Min:** EYE (0)	**Max:** KID (<1) **Min:** EYE (<1)	**Max:** KID (4) **Min:** EYE (<1)	**Max:** INT (7) **Min:** EYE (<1)	**Max:** LIV (10) **Min:** MUS (<1)	**Max:** HRT (4) **Min:** EYE (<1)	**Max:** INT (3) **Min:** EYE (<1)	**Max:** KID (2) **Min:** EYE (<1)	**Max:** INT (9) **Min:** MUS (<1)	**Max:** HRT (3) **Min:** EYE (<1)	**Max:** BRA (5) **Min:** MUS (<1)	**Max:** HRT (3) **Min:** EYE (<1)	**Max:** PAN (4) **Min:** EYE (<1)	**Max:** RED (4) **Min:** MUS (<1)	**Max:** HRT (4) **Min:** EYE (<1)	**Max:** HRT (5) **Min:** EYE (<1)	**Max:** HRT (6) **Min:** EYE (<1)

Table 3. Concentration index (I_c) of ^{65}Zn, throughout the 45-d depuration of juvenile silver moony (*M. argenteus*) and spotted scat (*S. argus*) after a single feeding with radiolabelled brine shrimp. Ic is the ratio between the activity concentration (Bq g^{-1} wwt) in the considered compartment and the activity in the whole fish (Bq g^{-1} wwt) multiplied by 100. A selection of body compartments having the highest and lowest Ic values (p<005) has been made. EYE: eyes, GAL: gall bladder, GIL: gills, GON: gonads, HRT: heart, INT: intestine, KID: kidney, LIV: liver, MUS: muscle, PAN: pancreas + spleen, RED: red gland, SKE: skeleton, STO: stomach. Values are means or means ± SD; n=3. Details of the data are available in supplementary materials.

Species		Time (d)																
		0	1	2	3		4	7	10		14	17	18	23		30	38	45
		Juvenile	Juvenile	Juvenile	Juvenile	Adult	Juvenile	Juvenile	Juvenile	Adult	Juvenile	Adult	Juvenile	Juvenile	Adult	Juvenile	Juvenile	Juvenile
M. argenteus	Highest I_c vaues	**Max:** STO (13±1) **Min:** INT (7±3)	**Max:** KID (40±27) **Min:** GAL (22±13)	**Max:** GAL (47±19) **Min:** PAN (26±16)	**Max:** PAN (25±13) **Min:** GAL (22±6)	-	**Max:** GAL (34±23) **Min:** HRT (13±8)	**Max:** GAL (61±41) **Min:** HRT (26±15)	**Max:** GAL (79±72) **Min:** KID (20±3)	-	**Max:** PAN (50±22) **Min:** GAL (29±7)	**Max:** PAN (198±93) **Min:** HRT (22±3)	**Max:** PAN (88±61) **Min:** KID (25±8)	**Max:** PAN (53±44) **Min:** GAL (31±28)	-	**Max:** PAN (102±78) **Min:** HRT (14±10)	**Max:** PAN (100±71) **Min:** HRT (31±14)	**Max:** PAN (88±20) **Min:** KID (17±7)
	Lowest I_c values	**Max:** PAN (3) **Min:** SKE (0)	**Max:** HRT (21) **Min:** EYE (<1)	**Max:** INT (4) **Min:** EYE (<1)	**Max:** HRT (10) **Min:** EYE (<1)	**Max:** PAN (50) **Min:** MUS (<1)	**Max:** BRA (4) **Min:** SKE (<1)	**Max:** KID (7) **Min:** SKE (<1)	**Max:** LIV (7) **Min:** SKE (<1)	**Max:** GAL (24) **Min:** MUS (<1)	**Max:** STO (4) **Min:** SKE (<1)	**Max:** BRA (4) **Min:** MUS (<1)	**Max:** BRA (7) **Min:** SKE (<1)	**Max:** LIV (3) **Min:** SKE (1)	**Max:** RED (66) **Min:** MUS (<1)	**Max:** BRA (6) **Min:** SKE (<1)	**Max:** BRA (4) **Min:** SKE (<1)	**Max:** BRA (6) **Min:** HED (<1)
S. argus	Highest I_c values	**Max:** STO (7±0) **Min:** INT (5±3)	**Max:** INT (6±2) **Min:** LIV (2±1)	-	-	-	-	-	-		-	-	-	**Max:** HRT (6±2)		**Max:** PAN (11±7) **Min:** MUS (3±0)	-	-
	Lowest I_c values	**Max:** HRT (<1) **Min:** SKE (<1)	**Max:** HED (1) **Min:** BRA (<1)	**Max:** KID (9) **Min:** BRA (<1)	**Max:** PAN (6) **Min:** BRA (<1)	**Max:** KID (7) **Min:** SKE (<1)	**Max:** PAN (5) **Min:** BRA (<1)	**Max:** PAN (5) **Min:** BRA (1)	**Max:** HRT (3) **Min:** GAL (<1)	**Max:** LIV (4) **Min:** MUS (<1)	**Max:** PAN (4) **Min:** BRA (<1)	**Max:** HRT (8) **Min:** MUS (<1)	**Max:** GAL (7) **Min:** INT (<1)	**Max:** MUS (4) **Min:** INT (<1)	**Max:** HRT (5) **Min:** MUS (<1)	**Max:** EYE (2) **Min:** INT (<1)	**Max:** HRT (5) **Min:** INT (<1)	**Max:** PAN (13) **Min:** INT (<1)

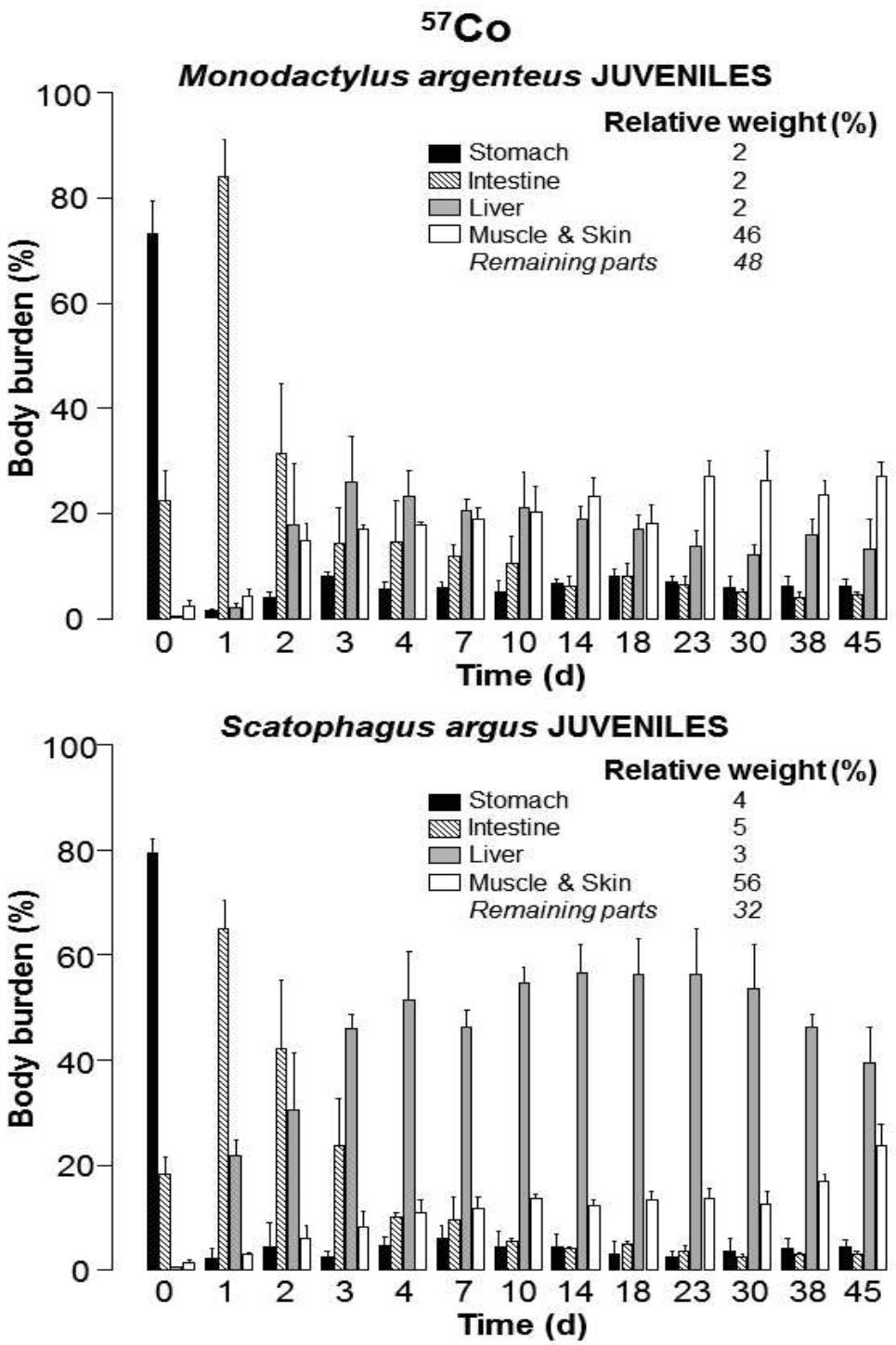


Figure 2. ^{57}Co distribution among 4 body compartments (intestine, stomach, liver, and muscles and skin) of juvenile silver moony (*M. argenteus*) and juvenile spotted scated (*S.argus*) over the depuration phase after feeding with radiolabelled brine shrimp. At each time three fish were dissected. All the values are expressed as percentage of the whole-body activity (means + SD).

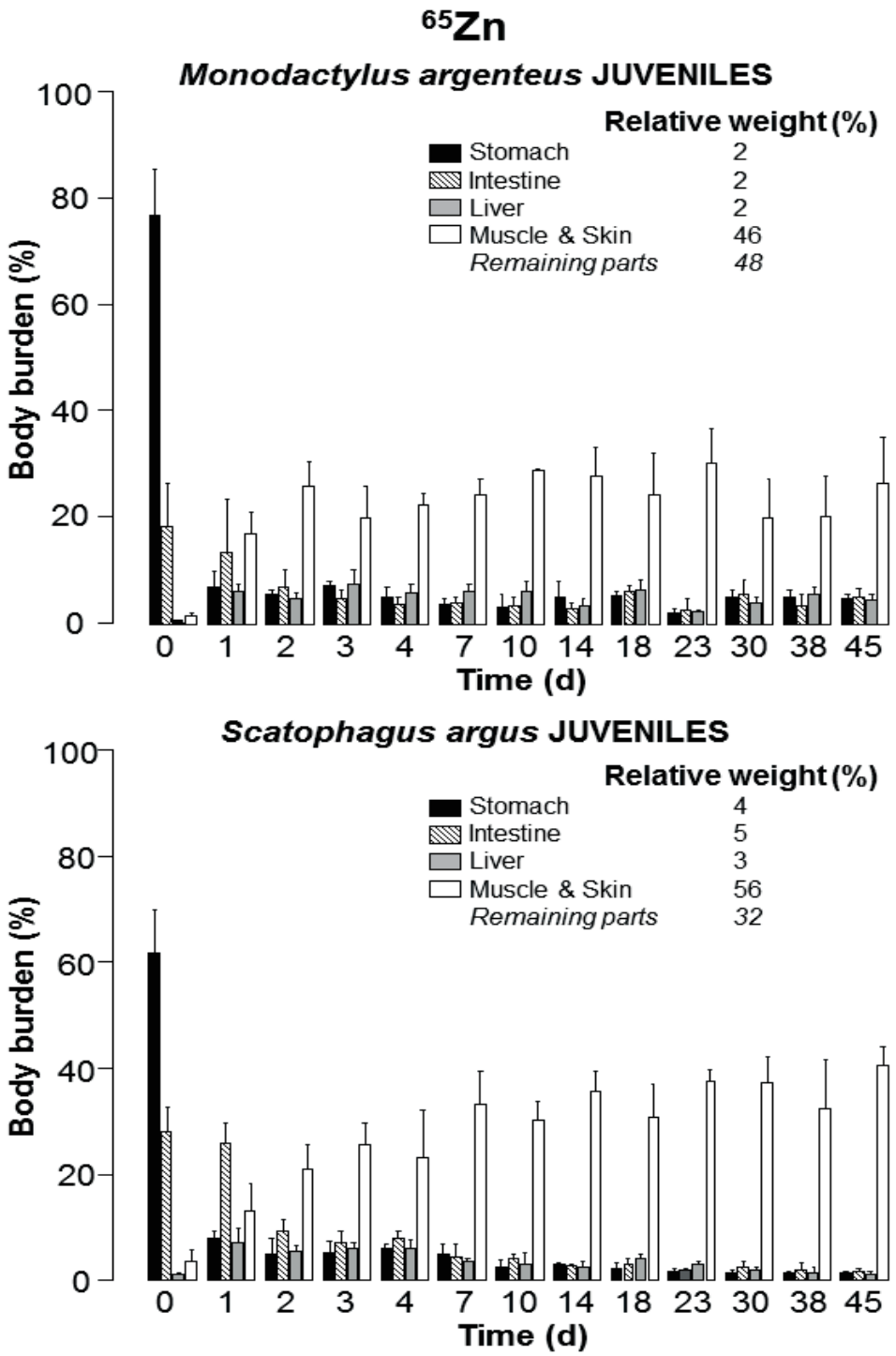


Figure 3. ^{65}Zn distribution among 4 body compartments (intestine, stomach, liver and muscles and skin) of juveniles silver moony (*M. argenteus*) and juvenile spotted scated (*S.argus*) over the depuration phase after feeding with radiolabelled brine shrimp. At each time three fish were dissected. All the values are expressed as percentage of the whole-body activity (means + SD).

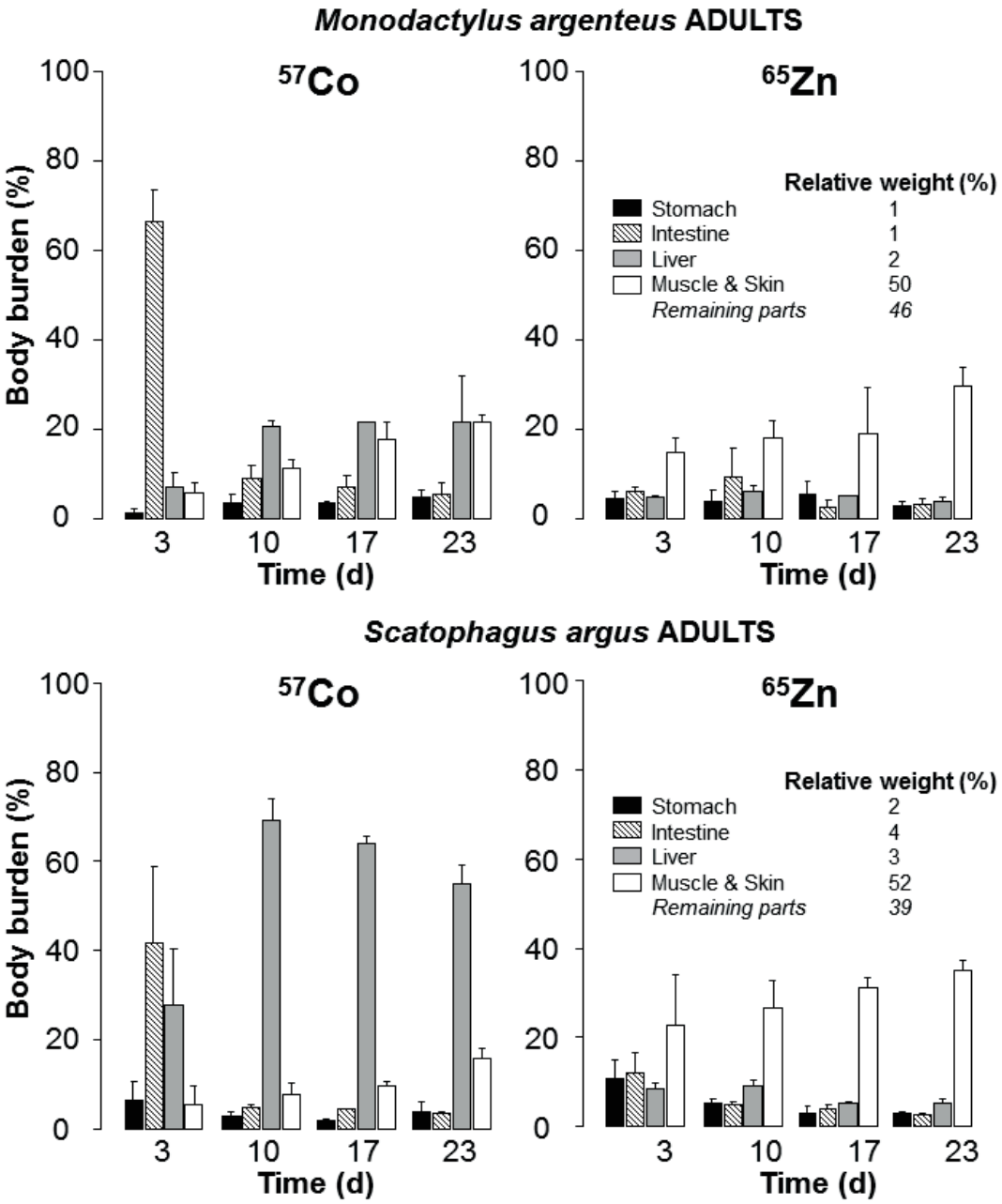


Figure 4. ^{57}Co and ^{65}Zn distribution among 4 body compartments (intestine, stomach, liver and muscles with skin) of adult silver moony (*M. argenteus*) and adult spotted scat (*S. argus*) over the depuration phase after feeding with radiolabelled brine shrimp. At each time three fish were dissected. All the values are expressed as percentage of the whole-body activity (means + SD).

4. DISCUSSION

The tropical silver moony, *Monodactylus argenteus*, and the spotted scat, *Scatophagus argus*, were selected to investigate the assimilation, retention and transfer among organs and tissues of Co and Zn ingested from radiolabeled food under controlled laboratory conditions. Experimental approaches commonly used, based on whole-body kinetics (e.g. Zhao et al. 2001; Mathews & Fisher 2009; Pouil et al. 2015), do not allow easily discerning the dynamics of metal transfer among organs and tissues. This fact underscores the originality of the present study since it combines different approaches to provide new information regarding the mechanisms of assimilation and the subsequent dynamics of translocation of these trace metals among the tissues and organs of these euryhaline fish. At the whole-body level, results show that AEs of Co are quite similar for the two fish species whereas they differ for Zn. Indeed, ranges of AE for ^{57}Co and ^{65}Zn in silver moony and spotted scat were 5-6% and 15-24%, respectively. These AEs are in accordance with those reported in the literature for other species of marine fish, for which AEs ranged from 2 to 44% for Co and 1 to 52% for Zn (Table 4).

Many factors could explain the variations of AEs. Wang (2001) proposed in his review a ranking of these factors classified in 3 different categories: (1) environmental quality (such as food quality and quantity), (2) metal geochemistry and (3) feeding physiology and biology (such as ingestion rate and gut passage time). Although only bivalves were considered in that review, these factors are generally relevant for fish as well. In the present study, interspecific difference in Zn AE was observed. Indeed, results indicated a higher AE for *S. argus*. Exposure conditions (i.e. the use of radiolabeled brine shrimp as unique food for trophic transfer in both species maintained under the same experimental conditions) did not allow addressing categories (1) and (2) mentioned by Wang (2001). Although both species are omnivorous, as indicated by their identical Fishbase trophic levels (3.0 for the both species; Froese and Pauly 2015a, b), differences exist in their trophic ecology. Indeed, analyses of the stomach contents from field surveys indicated that detritus is the main diet of *M. argenteus* (Blaber 1980; Rainboth 1996), whereas *S. argus* feeds mainly on zoobenthos such as worms and nekton such as finfish (Mills & Vevers 1989; Monkolprasit 1994; Jeyasselan 1998). These observations suggest that spotted scat has a stronger predatory behavior than silver moony.

Dietary habits are related to specific mixes of digestive enzymes or at least to variability of some enzymes' activity (e.g. Furne et al. 2005). For example, among 6 fish species, Hidalgo et al. (1999) have demonstrated that amylase displayed higher enzymatic activity in omnivorous species than in carnivorous predators.

Thus, in the present study, the higher Zn AE measured in *S. argus* could be due to a more efficient enzymatic system for the digestion of brine shrimp than in silver moony. In short, spotted scat might be better "equipped" to deal with digesting complex multicellular prey. This suggested interspecific variation in the fish digestive enzyme array cannot explain the trends observed for Co. For this element, AEs were very low and very similar between the two fish species. Differences, however, occurred in terms of body distribution and I_c. The results highlight the major role played by the liver in Co storage in spotted scat. This organ is involved in the mechanisms of detoxification and storage of Co, as previously shown in the European plaice *Pleuronectes platessa* fed radiolabeled annelid worms *Arenicola marina*, where Amiard-Triquet & Amiard (1974) reported a low Co AE with almost all the activity found in liver and kidney. Thus, trophic ecology could lead to the occurrence of specific mechanisms of storage and detoxification. Further investigation is however needed to obtain a better understanding of the mechanisms of detoxification and storage of Co in the two species examined in this study.

Regarding the potential effect of the life-stage, juveniles and adults of both species had comparable patterns of distribution and concentrations (i.e. "concentration index") for Co and Zn, i.e. the major fraction of both elements was found in the same organs and the metal concentrations in the organs were similar between the two life-stages. These comparable patterns can result from a similar metabolism in juveniles and adults (viz. similar digestive activity and AE) although there were differences in size (adults of silver moony and spotted scat were respectively 10 and 40 times larger than juveniles). In addition, it is important to note that adults of both species were not sexually mature at the moment of the experiment. This would potentially affect the results due to the non-negligible allocation of some essential elements to reproductive organs, especially in mature females with oocyte production (e.g. Protasowicki 1986; Rajkowska & Protasowicki 2013).

5. CONCLUSION

The biokinetic approach at two levels of biological organization, (1) the whole organism and (2) the different organs and tissues, has provided an improved understanding of the mechanisms responsible for the difference in Co and Zn assimilation efficiency observed between *M. argenteus* and *S. argus*. Dissections carried out at different times during the depuration provided new information about the dynamics of metal translocation in the different organs and tissues. Thus, changes observed in the Co distribution between the liver and the muscles in *S. argus* are a good example of the contribution of this radiotracer-based methodology. These results also demonstrated interspecific variations in the assimilation and tissue distribution of Co and Zn in tropical marine fish.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at
http://dx. doi.org/10.1016/j.jembe.2016.09.005.

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ANNEXE 5

SUPPLEMENTARY MATERIAL

Comparative study of trophic transfer of the essential metals Co and Zn in two tropical fish: a radiotracer approach

Table S1. Co and Zn concentration index (I_c) values during depuration phase in body compartments of *Monodactylus argenteus* juveniles fed brine shrimp.

Elements	Compartments	Time (d)																									
		0		1		2		3		4		7		10		14		18		23		30		38		45	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Co	brain	0.0	0.0	0.4	0.5	0.5	0.3	0.7	0.5	1.1	0.2	2.7	1.7	1.0	0.5	3.1	1.3	3.0	1.6	1.9	0.4	3.4	0.8	3.0	0.9	4.1	1.6
	eyes	0.0	0.0	0.1	0.0	0.5	0.1	0.4	0.1	0.5	0.1	0.7	0.2	0.6	0.1	0.7	0.3	0.7	0.0	0.7	0.1	0.6	0.1	0.7	0.1	0.7	0.3
	gall bladder	0.2	0.1	1.4	1.0	16.0	2.4	7.4	4.1	12.0	10.5	20.7	4.7	21.2	12.5	28.5	18.4	15.6	7.5	6.8	7.3	18.1	15.3	23.6	10.5	19.5	14.2
	gills	0.1	0.0	0.5	0.2	2.5	0.7	2.1	0.1	1.9	0.2	2.5	0.5	2.3	0.2	2.5	0.5	2.8	0.8	3.6	0.7	2.1	0.4	2.7	0.5	2.5	0.1
	head	0.0	0.0	0.1	0.1	0.8	0.1	0.7	0.1	0.8	0.1	0.7	0.1	0.9	0.2	0.8	0.1	0.7	0.2	1.1	0.2	0.8	0.1	0.9	0.0	0.9	0.2
	heart	0.1	0.0	0.5	0.5	9.1	4.0	7.1	3.6	13.6	6.2	27.6	19.2	7.9	2.6	25.5	10.5	28.1	8.5	9.3	2.2	25.0	17.6	25.8	17.2	30.9	14.2
	intestine	8.4	2.2	39.2	2.7	19.0	10.9	8.3	4.3	10.0	8.6	6.0	1.1	4.5	0.7	3.0	0.5	4.2	0.9	3.6	0.9	2.6	0.4	2.4	1.0	2.4	1.0
	kidneys	0.1	0.0	1.9	1.6	8.6	2.5	12.7	1.3	10.5	3.3	7.8	6.6	5.7	3.2	27.8	15.3	21.9	3.6	10.0	9.1	25.8	11.1	18.5	14.5	13.8	10.2
	liver	0.1	0.0	1.4	0.4	10.3	5.7	9.7	2.5	10.3	1.7	15.1	1.3	12.8	4.9	15.5	5.4	12.4	2.6	17.8	1.5	7.9	1.6	8.4	2.9	7.2	2.4
	muscles+skin	0.1	0.0	0.2	0.1	1.1	0.1	1.0	0.0	1.1	0.1	1.1	0.2	1.3	0.2	1.2	0.2	1.2	0.3	3.5	3.3	1.3	0.1	1.2	0.1	1.2	0.1
	pancreas+spleen	0.4	0.4	0.5	0.3	6.8	1.5	7.6	1.3	11.7	4.8	17.6	4.9	9.1	3.7	29.6	27.7	46.5	36.0	16.5	15.4	59.7	9.0	47.6	2.2	51.0	33.5
	skeleton	0.0	0.0	0.2	0.1	0.7	0.1	0.5	0.1	0.5	0.1	0.5	0.0	0.6	0.1	0.5	0.0	0.4	0.1	0.7	0.1	0.5	0.1	0.6	0.1	0.6	0.1
	stomach	12.3	1.5	0.2	0.2	2.8	0.8	4.4	0.5	3.4	0.7	3.7	0.8	4.0	0.9	4.3	0.1	5.7	1.8	5.1	0.1	3.9	0.8	3.6	1.1	4.4	0.8
Zn	brain	0.3	0.1	1.9	0.9	1.6	1.1	2.0	0.8	3.8	1.9	4.9	2.7	1.4	0.6	2.2	0.9	7.2	3.0	2.3	1.4	5.8	2.1	4.2	1.2	5.7	1.2
	eyes	0.0	0.0	0.5	0.4	0.9	0.0	0.6	0.2	0.8	0.2	1.0	0.5	1.2	0.6	1.0	0.4	1.0	0.3	1.4	0.6	1.6	0.4	1.3	0.7	1.5	0.4
	gall bladder	0.6	0.0	22.2	13.4	47.1	18.8	21.5	6.4	33.8	22.9	60.8	41.1	78.7	71.8	29.0	7.3	25.9	17.0	31.4	27.9	32.3	11.9	47.9	27.7	28.9	18.0
	gills	0.1	0.0	4.4	1.5	3.8	0.4	2.7	0.3	2.5	0.2	2.9	0.5	1.8	1.3	2.2	0.3	2.3	0.8	2.8	0.6	1.1	0.5	1.4	0.9	1.6	0.5
	head	0.0	0.0	1.5	0.4	1.8	0.3	1.4	0.1	1.3	0.2	1.2	0.3	1.1	0.1	1.0	0.2	0.6	0.2	1.1	0.3	1.1	0.1	1.0	0.2	0.7	0.2
	heart	0.6	0.2	21.2	25.6	27.2	11.5	10.3	5.4	13.1	7.8	25.7	14.6	26.8	19.0	36.8	14.8	52.7	17.8	36.0	5.1	14.3	9.9	30.8	13.7	34.3	21.3
	intestinee	6.7	3.1	6.6	4.7	4.3	2.7	2.7	0.7	2.1	0.3	2.0	0.3	1.8	1.5	1.5	0.2	3.2	0.1	1.9	1.8	2.7	1.0	2.1	0.9	2.8	1.8
	kidneys	1.1	0.6	40.3	27.1	42.5	8.7	10.2	0.5	14.1	4.3	6.9	5.8	20.4	3.3	44.4	34.8	25.4	7.8	36.6	30.9	23.4	6.0	40.4	22.9	16.6	6.9
	liver	0.1	0.1	4.0	0.9	3.0	0.5	2.8	0.6	2.7	1.3	4.6	0.2	3.8	0.5	2.8	0.9	4.7	1.7	3.2	0.4	2.6	1.5	3.1	1.1	2.5	0.9
	muscles+skin	0.1	0.0	1.1	0.5	2.3	0.3	1.3	0.7	1.7	0.1	1.8	0.2	3.4	1.8	2.0	0.4	1.9	0.9	3.1	0.5	1.4	0.4	1.3	0.4	1.6	0.3
	pancreas+spleen	2.8	3.1	8.1	5.0	26.3	16.4	24.9	12.6	29.6	11.8	51.6	22.0	42.5	26.9	50.4	21.8	87.9	60.9	52.9	43.9	102.3	78.4	100.4	71.0	87.9	20.2
	skeleton	0.0	0.0	0.7	0.4	1.1	0.1	1.0	0.1	0.8	0.2	0.7	0.1	0.8	0.1	0.8	0.2	0.5	0.4	1.1	0.2	0.8	0.2	0.9	0.0	1.0	0.2
	stomach	12.7	1.2	1.5	0.8	4.3	0.4	3.9	1.0	3.0	1.0	2.2	0.9	2.6	2.1	3.5	2.2	3.7	0.4	1.8	0.9	3.5	1.7	3.2	1.2	3.7	1.6

Table S2. Co and Zn concentration index (I_c) values during depuration phase in body compartments of *Monodactylus argenteus* adults fed brine shrimp.

Elements	Compartments	Time (d)							
		3		10		17		23	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Co	brain	0.7	0.5	0.8	0.2	1.2	0.2	1.3	0.5
	eyes	0.2	0.1	0.9	0.2	0.7	0.2	0.7	0.1
	gall bladder	5.7	2.8	9.0	1.4	6.9	5.0	12.4	10.2
	gills	1.3	0.7	4.3	1.9	3.4	0.6	4.8	1.0
	gonads	0.4	0.1	4.8	4.1	6.2	3.8	5.6	6.6
	head	0.2	0.1	1.0	0.1	0.9	0.0	0.9	0.1
	heart	1.7	0.6	6.6	2.2	9.5	1.6	10.1	6.5
	intestine	62.8	3.6	7.3	1.9	7.1	2.6	3.8	2.5
	kidneys	1.9	1.0	7.7	1.6	7.0	1.4	11.7	4.0
	liver	4.6	3.6	13.4	2.8	12.5	0.2	16.3	10.8
	muscles+skin	0.1	0.0	0.2	0.0	0.3	0.1	0.4	0.0
	pancreas+spleen	4.3	2.6	7.0	1.4	17.3	9.0	8.5	4.0
	red gland	1.1	0.8	8.0	2.9	10.9	7.4	8.2	2.9
	skeleton	0.3	0.0	0.9	0.2	0.5	0.1	0.5	0.1
	stomach	1.2	1.0	3.4	0.8	3.8	0.3	6.3	2.9
Zn	brain	14.6	1.4	1.4	1.1	13.2	2.4	7.2	2.1
	eyes	0.9	0.2	1.5	0.0	1.2	0.4	2.0	0.1
	gall bladder	31.9	13.0	24.3	23.7	45.2	15.5	11.0	8.1
	gills	4.2	1.3	4.5	1.8	2.6	1.0	3.7	0.8
	gonads	2.3	2.7	4.6	0.5	4.5	1.1	5.1	2.6
	head	0.9	0.0	1.4	0.1	0.8	0.2	1.3	0.3
	heart	30.0	13.0	5.8	2.4	22.0	3.0	4.0	3.4
	intestineee	5.7	1.0	4.5	0.6	2.7	2.0	2.2	1.4
	kidneys	11.6	7.5	4.3	1.3	22.3	3.6	14.2	5.5
	liver	2.8	0.7	4.0	0.3	2.9	0.1	2.7	1.0
	muscles+skin	0.3	0.0	0.4	0.1	0.4	0.2	0.6	0.1
	pancreas + spleen	49.8	31.2	14.5	7.2	198.0	93.4	53.0	36.0
	red gland	6.9	4.4	4.6	1.3	27.1	14.5	65.8	30.1
	skeleton	0.6	0.1	1.1	0.1	0.7	0.1	0.9	0.1
	stomach	4.3	1.7	3.8	0.6	6.5	3.7	4.0	1.9

Table S3. Co and Zn concentration index (I_c) values during depuration phase in body compartments of *Scatophagus argus* juveniles fed brine shrimp.

		Time (d)																									
		0		1		2		3		4		7		10		14		18		23		30		38		45	
Elements	Compartments	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Co	brain	0.0	0.0	0.1	0.1	0.2	0.1	0.3	0.1	0.4	0.1	0.5	0.2	0.4	0.1	0.6	0.1	0.7	0.3	0.7	0.1	1.0	0.2	0.9	0.3	1.1	0.7
	eyes	0.0	0.0	0.1	0.0	0.2	0.0	0.2	0.1	0.3	0.1	0.3	0.1	0.3	0.0	0.3	0.1	0.3	0.2	0.3	0.1	0.3	0.1	0.2	0.2	0.4	0.0
	gall bladder	0.1	0.1	0.5	0.2	1.3	0.7	1.5	0.1	1.2	0.8	1.5	1.0	0.3	0.2	2.2	1.5	2.7	1.8	0.8	0.4	1.2	0.7	1.3	0.5	1.7	0.3
	gills	0.0	0.0	0.7	0.4	1.9	0.5	1.4	0.3	1.7	0.3	2.7	1.6	1.6	0.6	2.0	0.4	1.7	0.5	1.4	0.8	1.4	0.6	1.8	0.7	2.2	0.5
	head	0.0	0.0	0.2	0.0	0.3	0.1	0.4	0.1	0.5	0.2	0.6	0.1	0.4	0.0	0.5	0.0	0.5	0.0	0.6	0.1	0.4	0.1	0.5	0.0	0.6	0.1
	heart	0.1	0.1	0.8	0.2	2.0	0.3	3.6	1.0	3.5	0.4	2.7	1.7	1.8	0.6	2.7	0.2	2.9	2.3	3.6	0.1	4.4	1.6	4.5	1.7	5.9	2.0
	intestine	3.3	1.6	13.3	0.8	10.2	6.7	7.0	4.5	2.9	1.0	3.4	2.0	1.2	0.1	1.8	0.4	1.4	0.2	1.0	0.3	0.5	0.1	0.6	0.1	0.7	0.2
	kidneys	0.0	0.0	0.8	0.1	3.7	1.9	2.5	0.5	1.7	0.8	2.6	1.1	2.2	0.6	3.0	0.5	1.9	1.6	3.6	1.9	2.5	1.0	2.6	0.7	2.7	1.7
	liver	0.1	0.0	7.6	2.7	13.2	5.8	14.3	2.1	17.5	1.3	26.1	3.4	18.4	3.6	32.7	6.3	20.6	1.4	25.1	4.2	16.7	1.9	16.7	3.0	15.3	2.2
	muscles+skin	0.1	0.0	0.2	0.0	0.5	0.2	0.6	0.2	0.8	0.1	0.9	0.1	0.7	0.1	0.9	0.1	1.0	0.2	1.0	0.4	0.7	0.1	0.9	0.1	1.0	0.1
	pancreas+spleen	0.1	0.1	0.6	0.3	0.9	0.2	2.0	0.9	1.8	1.7	1.6	0.8	1.5	0.3	1.3	0.3	2.3	0.5	3.7	3.5	4.1	0.7	2.7	1.0	4.5	4.0
	skeleton	0.0	0.0	0.1	0.0	0.3	0.1	0.3	0.1	0.4	0.1	0.4	0.1	0.3	0.0	0.4	0.0	0.4	0.1	0.4	0.1	0.4	0.1	0.4	0.0	0.5	0.0
	stomach	8.8	1.5	0.2	0.1	1.5	2.0	1.1	0.5	2.1	1.0	2.3	0.8	1.3	0.7	1.9	0.6	1.4	0.6	1.4	0.5	1.2	0.5	1.7	0.5	1.5	0.9
Zn	brain	0.1	0.0	0.5	0.2	0.7	0.2	0.4	0.1	0.9	0.2	1.0	0.4	0.6	0.2	0.7	0.3	1.5	0.5	0.7	0.2	1.3	0.3	1.3	0.3	1.0	0.7
	eyes	0.1	0.0	0.8	0.6	1.3	0.4	1.5	0.4	1.2	0.6	1.6	0.2	0.9	0.5	1.8	0.2	2.0	0.9	1.7	0.0	1.7	0.1	1.0	0.9	1.7	0.7
	gall bladder	0.2	0.1	1.1	0.7	2.3	2.8	4.1	0.6	3.0	2.2	2.2	0.5	0.4	0.0	1.8	0.6	7.4	3.8	1.7	0.4	4.2	1.8	3.5	1.9	4.7	1.5
	gills	0.3	0.2	3.2	1.6	4.4	2.1	2.6	0.4	2.3	0.1	2.3	0.5	1.8	0.2	2.1	0.3	1.3	0.4	0.9	0.4	0.5	0.3	0.7	0.2	0.6	0.2
	head	0.1	0.1	1.2	0.2	1.6	0.2	1.6	0.1	1.9	0.6	1.9	0.5	1.7	0.5	1.8	0.1	1.8	0.3	1.7	0.1	1.4	0.1	1.5	0.1	1.4	0.3
	heart	0.8	0.5	3.5	2.1	4.5	2.6	4.7	1.6	4.8	1.4	3.9	1.5	2.9	0.7	3.2	0.8	3.7	3.2	6.1	1.8	6.9	2.3	5.1	1.8	8.0	2.3
	intestine	5.3	3.0	5.5	1.6	2.2	1.2	2.0	0.9	2.3	0.8	1.7	1.1	0.9	0.2	1.3	0.3	0.8	0.4	0.5	0.1	0.5	0.2	0.4	0.3	0.4	0.1
	kidneys	0.3	0.1	4.2	2.8	8.8	5.1	2.9	0.8	2.3	1.4	2.8	1.5	1.5	1.2	3.1	1.5	2.0	1.4	2.0	1.2	3.9	1.3	2.1	1.1	2.1	0.6
	liver	0.3	0.1	2.4	0.8	2.4	0.5	2.1	0.7	2.2	0.9	2.1	0.5	1.1	0.6	1.3	0.6	1.6	0.4	1.4	0.2	0.6	0.2	0.5	0.2	0.4	0.2
	muscles+skin	0.2	0.1	0.9	0.4	2.3	0.3	2.5	0.4	2.3	1.0	3.5	0.4	3.5	3.0	3.9	0.7	3.6	0.7	3.9	0.3	3.2	0.3	2.3	0.8	3.0	0.2
	pancreas+spleen	0.5	0.2	5.0	4.3	2.5	1.8	5.5	1.9	5.4	4.0	5.3	3.1	1.3	0.3	3.6	0.9	6.8	3.6	1.2	0.7	10.6	6.8	4.7	1.3	13.3	10.8
	skeleton	0.1	0.0	0.5	0.4	1.1	0.1	1.1	0.0	1.2	0.1	1.0	0.5	1.2	0.1	1.1	0.0	1.4	0.2	1.2	0.1	1.1	0.1	1.1	0.1	1.3	0.2
	stomach	6.8	0.2	1.0	0.3	1.3	0.8	2.3	0.7	2.8	0.8	2.1	0.6	0.8	0.4	1.5	0.3	1.1	0.5	0.9	0.3	0.6	0.3	0.6	0.3	0.4	0.2

Table S4. Co and Zn concentration index (I_c) values during depuration phase in body compartments of *Scatophagus argus* adults fed brine shrimp.

Elements	Compartments	Time (d)							
		3		10		17		23	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Co	brain	0.8	0.3	0.4	0.2	0.9	0.2	0.6	0.0
	eyes	0.3	0.1	0.3	0.1	0.4	0.1	0.5	0.1
	gall bladder	1.0	0.4	1.1	0.5	1.5	0.8	1.3	0.7
	gills	0.9	0.5	1.5	0.3	2.2	0.9	2.5	0.1
	gonade	6.4	3.9	1.7	1.2	3.5	0.7	3.7	3.2
	head	0.3	0.1	0.4	0.0	0.6	0.1	0.5	0.1
	heart	0.6	0.2	0.9	0.1	1.7	0.4	1.6	0.0
	intestine	8.6	4.3	1.6	0.6	0.9	0.3	1.1	0.4
	kidneys	2.1	1.2	2.5	0.8	4.2	1.2	3.6	1.0
	liver	9.9	4.0	29.5	2.0	20.7	0.7	28.9	5.7
	muscles+skin	0.1	0.1	0.2	0.1	0.2	0.0	0.3	0.0
	pancreas+spleen	1.0	0.4	0.9	0.2	1.8	1.1	2.4	0.5
	red gland	2.0	0.9	3.1	1.2	5.0	2.2	4.3	0.7
	skeleton	0.4	0.1	0.2	0.0	0.3	0.0	0.4	0.0
	stomach	2.3	1.8	1.4	0.4	1.1	0.2	2.1	0.7
Zn	brain	5.2	3.2	2.1	1.8	6.5	2.7	1.1	0.1
	eyes	1.5	0.9	1.7	0.5	1.4	0.4	1.5	0.7
	gall bladder	2.4	0.3	0.9	0.1	2.6	1.1	1.4	0.4
	gills	3.9	1.6	3.5	0.8	2.7	1.8	2.1	0.2
	gonade	1.9	1.1	2.2	1.4	2.1	0.7	3.7	3.2
	head	1.8	0.3	1.2	0.9	1.4	0.1	1.4	0.2
	heart	5.6	2.6	3.5	2.8	7.7	0.9	4.5	1.9
	intestine	2.4	1.0	1.5	0.3	0.8	0.1	0.9	0.2
	kidneys	6.9	1.4	3.7	1.1	2.9	0.8	2.1	0.7
	liver	3.1	0.3	3.9	0.3	1.7	0.2	2.7	0.4
	muscles+skin	0.4	0.2	0.6	0.1	0.6	0.1	0.8	0.0
	pancreas+spleen	5.6	3.1	2.4	0.5	4.8	2.5	2.4	1.1
	red gland	5.7	1.3	3.3	0.5	4.2	0.8	2.2	1.9
	skeleton	0.3	0.1	1.1	0.1	1.3	0.1	1.5	0.1
	stomach	3.6	1.9	2.5	0.4	1.4	0.5	1.7	0.2

ANNEXE 6

How ingestion of algal toxins is influencing transfer of essential nutrients (Mn and Zn) in fish

Pouil S[1,2], Clausing R[1], Metian M[1], Bustamante P[2], Dechraoui Bottein M-Y[1] (en préparation). How ingestion of algal toxins is influencing transfer of essential nutrients (Mn and Zn) in fish.

ABSTRACT: Studying the dietary transfer of essential metals is important to better understand how these metals are accumulated by fish. Because of their role in fish physiology, there is an increasing body of literature examining the effects of environmental variables such as water temperature, pH and salinity on fish nutrition. Other stressors such as algal biotoxins also occur in the natural environment and are known to affect the physiology of aquatic organisms; however, to the best of our knowledge, the potential effects of biotoxins on fish nutrition have never been investigated. We assessed the influence of ingested brevetoxins (PbTxs), biotoxins produced by the dinoflagellate *Karenia brevis*, on trophic transfer of essential metals (Mn and Zn) in fish using highly sensitive radiotracer techniques (i.e. β- and γ-spectrometry). Using PbTxs-containing and radiolabelled mussels *Mytilus edulis* under controlled laboratory conditions, juvenile turbots *Scophthalmus maximus* were exposed to both dietary PbTxs and radiotracers (^{54}Mn and ^{65}Zn) simultaneously in a single feeding or were exposed daily for 3 weeks to dietary PbTxs and then single-fed with dietary radiotracers. After a 21-day depuration period, the assimilation efficiencies (AEs) of Mn and Zn in juvenile turbots were determined and found not to be affected by the ingestion of PbTxs ($p>0.05$). These results can be explained by the rapid turnover and elimination of ingested PbTxs in the body, likely before distribution to all body compartments suggesting that dietary PbTxs have limited effects on metal assimilation in fish.

Keywords: *Karenia brevis*, Brevetoxins; Turbots; Multiple stressors; Metals; HABs; Nuclear applications, Radiotracers

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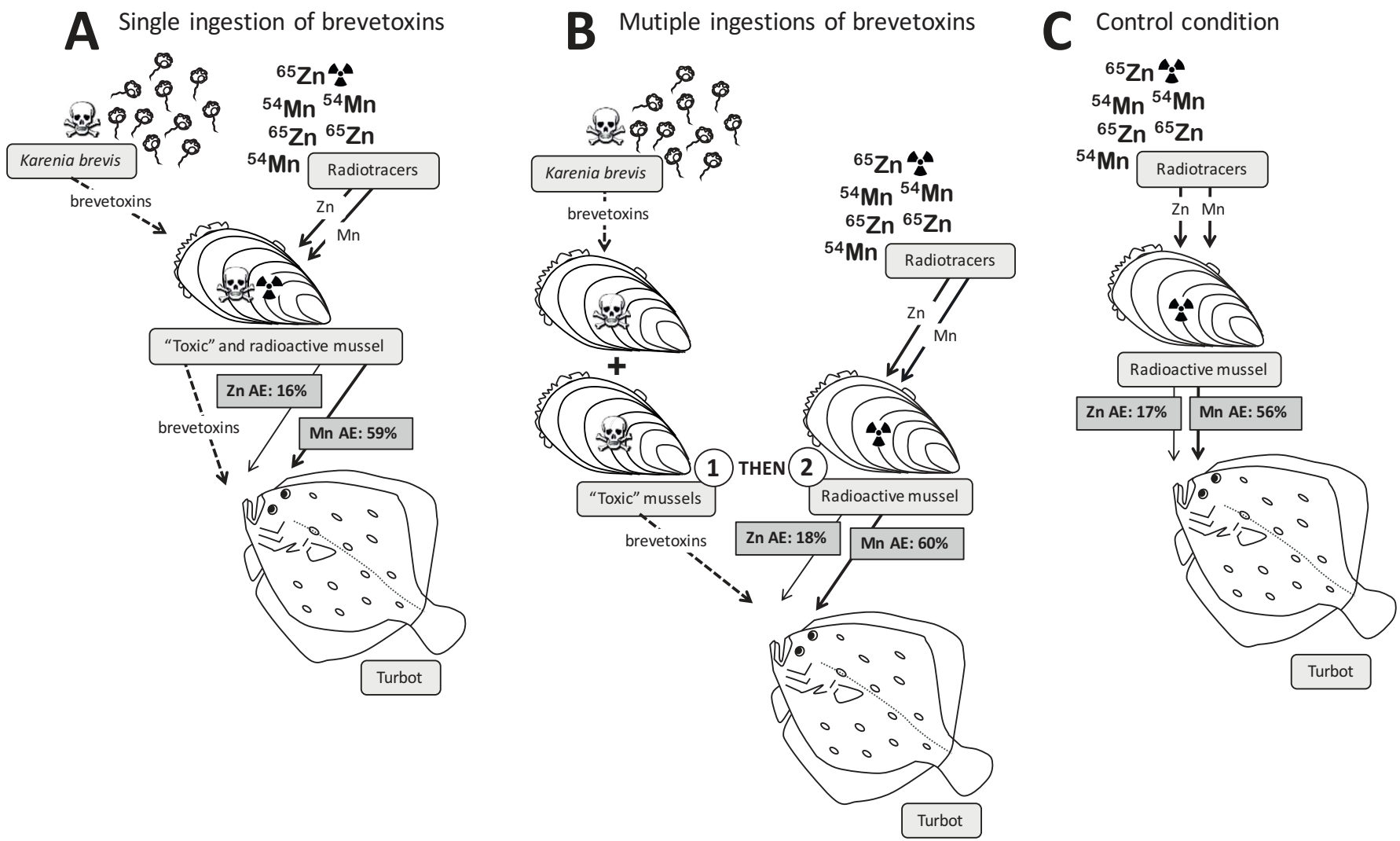
GRAPHICAL ABSTRACT:



1. Introduction

Some metals found in the marine environment, such as Mn and Zn, are called essential metals because marine organisms such as fish require them for essential physiological processes. For example, these metals are part of the functional groups of various metabolic enzymes, they play a structural role in respiratory pigments and metalloenzymes, and they constitute co-factors for various proteins (see e.g. Simkiss 1979, Williams 1981, Watanabe et al. 1997). Fish are dependent of food for metal intakes. As insufficient or excess quantities of these metals, which are obtained primarily through the diet (e.g. Xu & Wang 2002, Mathews & Fisher 2009), can provoke physiological alterations (e.g. Förstner & Wittmann 2012),. investigating the environmental factors influencing the trophic transfer of essential metals in fish is a key research area.

In the field, fluctuations in environmental variables such as the temperature, pH or salinity of the surrounding water are known to influence the digestive physiology of fish. For example, laboratory experiments on multiple marine fish species have shown that seawater temperature and pH can strongly affect gut transit time or the activity of enzymes involved in the digestion process (Edwards 1971, Miegel et al. 2010, Pimentel et al. 2015, Rosa et al. 2016). Salinity also plays a major role in fish physiology, not only in osmoregulation processes, but also in feeding and food conversion (reviewed by Bœuf & Payan 2001). Although no significant effect of pH and salinity was demonstrated on the trophic transfer of essential metals in several species of fish (Pouil et al. 2017, Ni et al. 2005, Jacob et al. 2017), previous studies have already shown a positive relation between temperature and Zn trophic transfer in the carp *Cyprinus carpio* and the turbot *Scophthalmus maximus* (Pouil et al. 2017, Van Campenhout et al. 2007). However, naturally produced toxic compounds in the marine environment such as algal toxins can also act as stressors but their effects on the physiology of fish are relatively unknown.

Although toxic compounds are produced by less than 2% of the described species of marine microalgae (Landsberg 2002), the occurrence of blooms of these harmful algae (harmful algal blooms: HABs) appear to be increasing over the last decades (Smayda 1990, Hallegraeff 1993, Sournia 1995, Van Dolah 2000). These algal biotoxins can be transferred up aquatic food chains, impacting species interactions, organismal health and population dynamics (e.g. van den Bergh et al. 2002, Smith & Schindler 2009, Anderson 2009), particularly in fishes (reviewed by Landsberg 2002).

Much research effort has focused on the bioaccumulation of biotoxins in fish and their toxicological effects during acute or chronic exposures (e.g. Lewis et al. 2003, Woofter et al. 2005, Naar et al. 2007, Ledreux et al. 2014), but little attention has been paid to the possible effects of biotoxin ingestion on the digestive physiology of fish.

The aim of the present study was to investigate the influence of a single and multiple ingestions of brevetoxins (PbTxs), the biotoxins produced by the dinoflagellate *Karenia brevis*, on the trophic transfer of essential metals in fish. One critical physiological parameter for understanding metal trophic transfer in fish is the assimilation efficiency (AE), which is the fraction of the ingested metals that is incorporated into biological tissues (Wang & Fisher 1999). Thus, we experimentally assessed the AEs of two essential elements (Mn and Zn) in juvenile turbots *Scophthalmus maximus* after a single or multiple feedings of mussels *Mytilus edulis* containing PbTxs. Highly sensitive radiotracer techniques were used (1) to determine the AEs of the studied essential metals and (2) to quantify the PbTxs ingested in juvenile turbots.

2. Materials and Methods

2.1. Origin, acclimation and maintenance of organisms

Juvenile turbots *Scophthalmus maximus* were purchased from a fish farm (France Turbot, France). Fish were acclimated to laboratory conditions for at least 6 months (constantly aerated, open-circuit 700-L plastic tank; flux: 350 L h^{-1}; salinity: 38; temperature: 20 ± 1 °C; pH: 8.0 ± 0.1; light/dark cycle: 12 h/12 h). During the acclimation period, the fish were fed a daily ration of 2% of their biomass with 1.1-mm pellets (proteins: 55% and lipids: 12%; Le Gouessant, France). All experimental fish were individually identified by slits cut into the fins.

Common mussels *Mytilus edulis* were purchased from a seafood seller (Les Halles du Midi, Monaco). They were transported to IAEA-EL premises in Monaco and were acclimated in the same conditions as the turbot for 1 month prior to the experiments. During this period, mussels were fed daily with phytoplankton (*Isochrysis galbana*).

Cultures of *Karenia brevis* (NOAA-1 strain isolated from Charlotte Harbor, FL, USA) were grown in an aerated 20-L plastic container (aerated; 0.45µm filtered aged seawater; salinity: 38; temperature: 20 ± 1°C; pH: 8.0 ± 0.1; bilateral luminosity 66–80 µE m^{-2} s^{-1}; light/dark: 12h/12h) spiked with an atypical f10k medium (Kellmann et al. 2010). The culture was started using 2 L of f10k medium to have an initial cell concentration of approx. 1000 cells mL^{-1}. At regular intervals when the concentration reached 5000 cells mL^{-1}, 2 L of f10k medium was added in order to get 20 L of culture in stationary phase (i.e. approx. 10000 cells mL^{-1}) for experiments. Cell counting was performed in Lugol 10% using a Sedgewick Rafter counting chamber (20 × 50 µL) under light microscope.

2.2. Experimental procedures

Independent experiments were conducted to investigate the influence of the ingestion of PbTx on the AE of Mn and Zn in juvenile turbots. To do this, in the first condition (Treatment 1), turbot were fed with radiolabelled (^{54}Mn and ^{65}Zn) and "toxic" (PbTxs mussel a single time (see section 2.2.2). In the second condition (Treatment 2) turbots were fed daily for three weeks with toxic mussels and then single-fed with radiolabelled mussels (see section 2.2.3). The last condition (Treatment 3) consisted of the identical design without the PbTxs (see section 2.2.4) and served as the experimental control. Details of the all the experimental food used are provided in Table 1. One week before the experiment, all the juvenile turbots were fed daily with non-radiolabelled non-toxic gel food (NRNT, Table 1) to acclimate them to the food matrix.

Table 1. Brevetoxin and radiotracers contents in the different gel foods prepared from homogenized mussels' soft tissues mixed with gelatin (see details in section 2.2.1). Data are Means ± SD.

Gels	Characteristics	Brevetoxin concentration (ng g^{-1})	^{54}Mn concentration (Bq g^{-1})	^{65}Zn concentration (Bq g^{-1})	Uses
NRNT	Non-radioactive No toxin content	-	-	-	To acclimate all the turbot used in experiment to gel food
RNT	Radioactive No toxin content	-	48 ± 1	1059 ± 30	For the single-feeding of turbots from Treatments 2 & 3
NRHT	Non-radioactive High toxin content	525 ± 88	-	-	For multiple brevetoxin ingestions by turbots (Treatment 2)
RLT	Radioactive Low toxin content	188 ± 38*	37 ± 2	1012 ± 6	For single ingestion by turbots (Treatment 1)
NRLT	Non-radioactive Low toxin content	188 ± 38	-	-	As proxy to estimate the brevetoxin content in the gel RLT

To quantify the accumulated toxin in fish tissue after both single and multiple exposures, experiments 1 and 2 were repeated in the absence of radiotracers, as quantification of PbTx concentrations was only possible on samples without radiotracers due to the interference γ-emitters can cause in liquid scintillation used for tritiated PbTx ([^{3}H]PbTx-3; a β-emitter) detection (see section 2.3.1). Each time, 8 turbots were single-fed or multi-fed (3 weeks) with non-radiolabelled-low toxin content mussels (NRLT) or non-radiolabelled high toxin content mussels (NRHT), respectively, sacrificed 2 h after feeding, and dissected to collect four body compartments: (1) digestive tract, (2) gall bladder, (3) liver and (4) muscle. Methods of extraction and quantification of toxin concentrations in these tissues are described in section 2.3.

2.2.1. Preparation of the food items

To prepare a series of toxic and/or radiolabelled foods for experimental exposure of turbots, common mussels *M. edulis* (3.1 ± 5.1 g wet wt) were randomly placed in three 20-L aquaria (n=40, constantly aerated, open-circuit; salinity: 38; temperature: 20 ± 1 °C; pH: 8.0 ± 0.1; light/dark cycle: 12 h/12 h).

Mussels in two 20-L aquaria were exposed daily (four days total) to brevetoxin producing *K. brevis* at an environmentally relevant cell concentration of 980 ± 20 cells mL^{-1}. Each day, a quantity of *K. brevis* culture was carefully added to the aquaria maintained in a closed-circuit during the feeding time to preserve *K. brevis* cell integrity. Feeding lasted for 1 h after which mussels were fed I. galbana for 30 min. At the end of the exposure, a fraction of exposed mussels from each aquarium was used to prepare the NRHT gel food (Table 1). Briefly, the soft tissues of the mussels were collected, homogenized and mixed with gelatin that had been dissolved in hot seawater (0.1 g mL^{-1}) and cooled to 30°C (ratio of 0.25 mL g^{-1}).

After exposure to *K. brevis*, the remaining mussels from one of the 20-L aquaria (aquarium 1; n=20) were then exposed daily for 23 days to dissolved radiotracers of high specific activity purchased from Isotope Product Lab, USA (^{54}Mn as MnCl2 in 0.5 M HCl, [$T_{1/2}$] = 312.2 d and ^{65}Zn as $ZnCl_2$ in 0.1M HCl, [$T_{1/2}$] = 243.9 d). Seawater was spiked in closed-circuit and repeated regularly in order to keep radioactivity constant. The radioactivity in seawater was checked before and after each spike renewal (e.g. Metian et al. 2009). After each water renewal, mussels were fed 30 min with *I. galbana*.

Mussels were collected at different time intervals and were γ-counted alive and put back in the aquarium. After the 23 d of exposure to the radiotracers, the radiolabelled, Pb-Txs-containing mussels from aquarium 1 were processed to prepare radiotracers and low toxin content gel food (RLT; Table 1) using all mussels' soft tissues and gelatin as described above. During the same period, remaining mussels from the second 20-L aquarium (aquarium 2; n=20) were kept in seawater and fed daily with *I. galbana*. Twenty three days after the last *K. brevis* culture exposure, mussels were dissected and soft tissues processed for toxin analysis as proxy for mussels exposed to radiotracers (aquaria 1) or to prepare a non-radiolabelled-low toxin content gel food (NRLT; Table 1) as previously described.
Mussels from the third 20-L aquarium (aquarium 3, n=20) were exposed to dissolved radiotracers for 23 d as described above without previous exposure to toxic *K. brevis* and their soft tissue collected in order to prepare the radiolabelled gel food without toxin (RNT).
All the five experimental gel foods (no-radiotracers with high toxin content – NRHT –; radiotracers and low toxin content – RLT –; radiotracers no toxin – RNT –; no-radiotracers and low toxin content – NRLT –; no radiotracers no toxins – NRNT –; Table 1) were stored at -4°C, and radioactivity and PbTxs concentrations were measured as described in the section 2.3 before use. Gels were thawed and cut into small, homogenous pieces (size < 2 mm) a few minutes before being offered to the turbot.

2.2.2. Treatment 1: Influence of a single PbTx ingestion on Mn and Zn AE in fish

Seven juvenile turbots (18.2 ± 2.0 g wet wt) were given a single-feeding of radiotracers-low toxin gel food RLT (see section 2.2.1 and Table 1), and AEs of Mn and Zn were followed over 21 d. Prior to the experiment, juvenile turbots were chosen at random and transferred into an aerated, open circuit 20-L aquarium (water renewal: 60 L h^{-1}; 0.45-µm filtered seawater; salinity: 38; temperature: 20 ± 1°C; pH: 8.0 ± 0.1; light/dark: 12h/12h). Turbots were single-fed with the gel food RLT for a maximum of 15 min in order to avoid radiotracers leaching from the food. One additional turbot was placed within a net in the same aquarium to restrict access to food, and served as control for possible radiotracer leaching from the contaminated food or from fish depuration. Two (2) hours after the feeding period, all turbots were whole-body γ-counted alive (section 2.3.2) at different time intervals over a 21-d period to follow the depuration kinetics of the radiotracers.

2.2.3. Treatment 2: Influence of multiple PbTx ingestions on Mn and Zn AE in fish

Seven juvenile turbot (22.1 ± 3.7 g wet wt), kept in the same conditions as described in section 2.2.2, were pre-exposed to gel food NRHT for 3 weeks (5 feedings/week) and then single-fed with radiotracers gel food RNT (Table 1). One additional, non-fed turbot was placed in the aquarium as in experiment 1. After the radiotracer-food single-feeding, each fish was γ-counted and then replaced in clean, flow-through seawater (parameters previously described). Depuration was followed in each individual by regular, live whole-body γ-counting (as detailed in section 2.3.2) over 21 d as before.

Because abiotic factors such as seawater pH are known to affect the digestive physiology of fish (e.g. Pimentel et al. 2015, Rosa et al. 2016), the same experiment was repeated under two seawater pH conditions although this was not the scope of the present paper. A description of the collection methodology as well as a brief analysis of the results is available in the Supplementary Material.

2.2.4. Treatment 3: Mn and Zn AE in fish without dietary PbTx (control condition)

In order to described the depuration kinetics of Mn and Zn, a single-feeding was performed using gel food with radiotracers (RNT) in a batch of juvenile turbots (n=7, 21.1 ± 4.3 g wet wt) maintained in the same conditions as previously described.

2.3. PbTxs and radiotracers quantification

2.3.1. PbTxs quantification using β-spectrometry

PbTxs in the four mussel based-gel foods (RLT, NRLT and NRHT; Table 1) and turbot tissue samples (pooled for homogeneity and to obtain sufficient quantity for measurements) were extracted as previously described in Poli et al. (2000) with minor modifications. Briefly, samples were homogenized in 3 volumes of acetone, sonicated (min. 1 min in ultrasonic water bath) and centrifuged at 3000 g for 10 min. Each supernatant was collected and transferred into a 50-mL Falcon tube and the process was repeated two more times. Combined supernatants were evaporated under a stream of nitrogen in a water bath at 40 °C. Dried samples were resuspended in 6 mL of 80% methanol and 6 mL of n-hexane. Samples were then manually mixed and centrifuged at 3000 g for 1 min. The methanol phase was collected in glass tubes and evaporated under a stream of nitrogen.

The extract was then resuspended in 100% methanol and stored at -18°C. The activity based radioligand-receptor binding assay (RBA) was used to detect and quantify composite PbTxs concentrations in the different tissue extracts (following Twiner et al 2007 with modification) using the PbTx-3 analogue as standard. This competitive-inhibition assay measures the binding of a constant low concentration of [^{3}H]PbTx-3 to its specific receptor on voltage gated sodium channels in the presence of extract. The reduction in [^{3}H]PbTx-3 binding is directly proportional to the amount of PbTx-3 standard or of unlabelled toxins present in the sample (Poli et al. 1986, Dechraoui 2014). Measurements were carried out in triplicates in a 96-well filterplates (MSFB N6B 50 MultiscreenHTS). The microplates were then incubated for 1 h at 4 °C before filtration and washed twice of cold binding buffer (Phosphate buffered saline pH 7.4, Tween 20). After filtration, scintillation cocktail was added in each well and the microplate stored in the dark for 2 h before radioactivity measurement using a scintillation liquid counter (MicroBeta Plate Counter, PerkinElmer).

2.3.2. Radiotracer quantification by γ-spectrometry

The radioactivity of the tracers was measured using a high-resolution γ-spectrometer system composed of 4 Germanium - N or P type - detectors (EGNC 33-195-R, Canberra® and Eurysis®) connected to a multi-channel analyser and a computer equipped with a spectra analysis software (Interwinner 6, Intertechnique®). The radioactivity of living organisms and samples was determined by comparison with standards of known activity and of appropriate geometry (calibration and counting). Measurements were corrected for background and physical radioactive decay. Triplicates of each radiolabelled experimental gel foods (gel RNT and gel RLT) were separated, weighed (wet wt) and placed in plastic tubes (diameter: 42mm, height: 65mm) for further radioactivity counts. Then, 25 mL of 2M HCl were added in each tube in order to get an appropriate geometry and samples were stored overnight before radioanalyses. Living organisms were counted in tubes (diameter: 160 mm, height: 80 mm) filled with clean seawater. The counting period was adjusted to obtain a propagated counting error less than 5% (e.g. Rodriguez y Baena et al. 2006) and varied between 15 and 60 min in order to maintain fish health and ensure normal behaviour.

2.4. Data treatment and statistical analyses

Depuration of radiotracers was expressed as the percentage of remaining radioactivity [(radioactivity at time t divided by the initial radioactivity measured in the organism at the beginning of the depuration period; following methods developed in Warnau et al. (1996)]. The depuration kinetics of Ag were best fitted using a two-component exponential model Eq. (1):

$$(1)\ A_t = A_{0s} x\ e^{-k_{es}\ x\ t} + A_{0l} x\ e^{-k_{el}\ x\ t}$$

where A_t and A_0 are the remaining activities (%) at time t (d) and 0, respectively; k_e is the depuration rate constant (d^{-1}). "s" and "l" subscripts are related to the short- and long-lived component, respectively. The "s" component represents the depuration of the radiotracer fraction that is weakly associated with the organisms and rapidly eliminated (i.e. proportion associated with the faeces). The "l" component describes the depuration of the radiotracer fraction that is actually absorbed by the organism and eliminated slowly. The long-lived component allows estimating the assimilation efficiency (AE) of the radiotracer ingested with food (AE = A_{0l}).

A biological half-life can be calculated for both short- and long-lived component ($T_{b1/2s}$ and $T_{b1/2l}$) from the corresponding depuration rate constants (k_{es} and k_{el}) according to the relation $T_{b1/2} = \ln 2/k_e$. Model constants and their statistics were estimated by iterative adjustment of the model and Hessian matrix computation, respectively, using the non-linear curve-fitting routines in the Statistica® software 7.0.

Comparison of assimilation of metals among the different experimental conditions was performed using Kruskal-Wallis and Siegel and Castellan non-parametric tests AE calculated for each individual turbot (the best fitting model obtained for the entire set of turbots was applied to individuals; Zar 1996). For Mn, two individuals per condition with an insufficient initial activity (i.e. activity measured 2 hours after the radiolabelled feeding) have been excluded from statistical analysis. The level of significance for statistical analyses was always set at $\alpha = 0.05$. All the statistical analyses were performed using R software 3.0.1 (R Core Team 2014).

3. Results

3.1. PbTx concentrations in food and fish

Aliquots taken from the no-radiotracers and toxin containing gel foods (i.e. NRLT and NRHT) confirmed accumulation of brevetoxins in mussels after 4 exposures to *K. brevis* culture. The values measured in 3 aliquots confirmed the homogeneity of the mussel gel food preparation. PbTx concentrations measured were 188 ± 38 ng PbTx-3 equivalents g^{-1} food and 525 ± 88 ng PbTx-3 equivalents g^{-1} food respectively for the NRLT and the NRHT gel (Table 1). Thus, the average quantities of PbTx given to each turbot during the single (Experiment set 1) and the multiple (Experiment set 2) dietary exposure were 4 ng PbTx-3 equiv g^{-1} fish and 222 ng PbTx-3 equiv g^{-1} fish, respectively (Table 2).

Table 2. Brevetoxins eaten by turbot exposed for a unique (Experiment set 1) and chronic (Experiment set 2) ingestions and subsequently measured in the selected body compartments. Data are Means.

Experimental conditions	PbTx ingested per feeding and per turbot	Total PbTX ingested per turbot	Brevetoxin measured in the selected body compartments						
			Digestive tract		Gall bladder		Liver		Muscles
	ng g^{-1} and [ng]	ng g^{-1} and [ng]	ng g^{-1} and [ng]	% of ingested	ng g^{-1} and [ng]	% of ingested	ng g^{-1} and [ng]	% of ingested	ng g^{-1} and [ng]
Single ingestion (Treatment 1)	4.3 [94.0]	4.3 [94.0]	12.5 [11.1]	12	60.7 [17.6]	19	7.6 [5.6]	6	< LOQ
Multiple ingestions (Treatment 2)	14.8 [262.5]	222 [3937.5]	8.4 [4.9]	0.1	80.1 [20.1]	0.5	9.6 [3.6]	<0.1	< LOQ

Measurements of PbTxs in the four body compartments of juvenile turbots (the digestive tract, the gall bladder, the liver and the muscles) found the highest concentrations in the gall bladder (ranging from 60 to 80 ng PbTx-3 equiv g^{-1}, Fig 1, Table 2). Lower concentrations were found in the digestive tract and the liver (8-12 ng PbTx-3 equiv g^{-1} and 8-10 PbTx-3 equiv ng g^{-1}, respectively; Fig 1, Table 2). Toxin concentrations in muscle tissue were below the limit of quantification (i.e. 0.3 ng g^{-1}; Fig. 1). For all the body compartments measured, similar PbTxs concentrations were found between the turbots from the different experimental conditions.

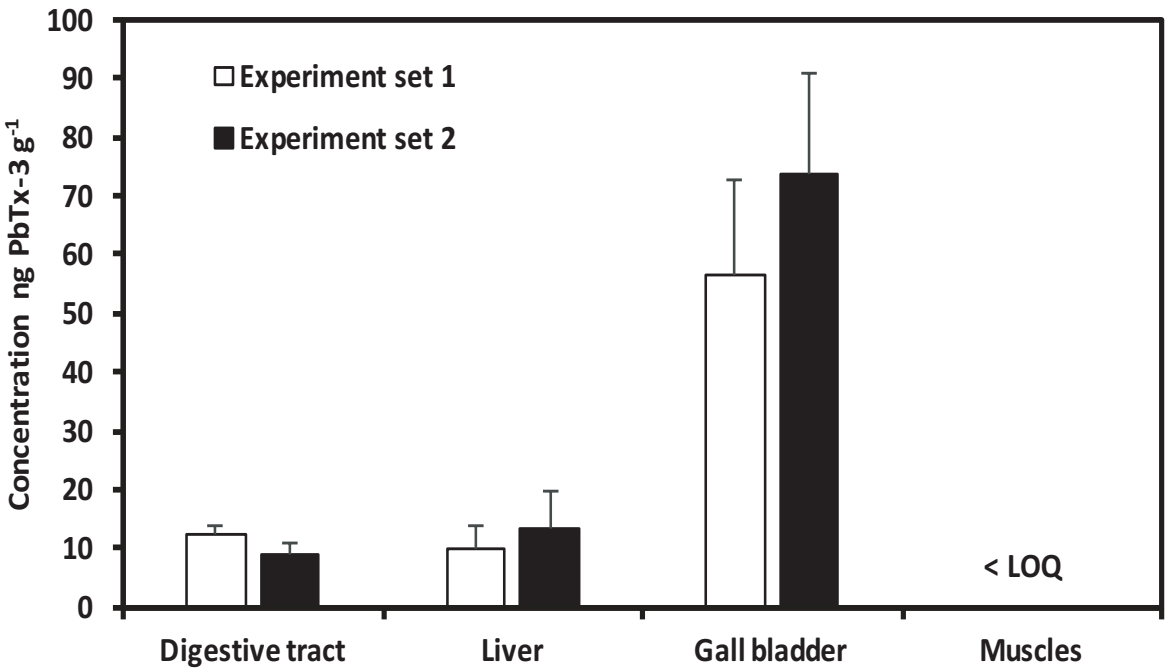


Figure 1. Concentration of brevetoxins (expressed as PbTx-3 equiv.) in each body compartments of turbots (digestive tract, liver, gall bladder and muscles), pooled before measurements as explained in section 2.3.1, after a unique (Experiment set 1) or chronic ingestions (Experiment set 2) to "toxic" mussels.

3.2. Effects of PbTx on essential metal trophic transfer in fish

After a single (Treatment 1) and multiple (Treatment 2) PbTxs ingestions, depuration kinetics of ^{54}Mn and ^{65}Zn were followed in juvenile turbots maintained for 21 d in uncontaminated seawater. No growth and mortality were recorded during the experiment. Before the single-feeding, the activity levels of each tracer in both radiolabelled gel foods (RLT and RNT) were measured, giving 48 ± 1 Bq and 37 ± 2 Bq g^{-1} wet wt for ^{54}Mn and 1059 ± 30 Bq and 1012 ± 63 Bq g^{-1} wet wt for ^{65}Zn, respectively (Table 1).

No activity was measured in the control turbot at any time. Depuration kinetics of ^{54}Mn and ^{65}Zn were most accurately described by 2-component exponential models (Fig. 2, Table 3; R^2: 0.51 - 0.96). For both Mn and Zn, a large proportion (40 - 84%) of the ingested radiotracer was associated with the short-lived component. The short-lived component was characterized by a very rapid loss with $T_{b1/2s}$ ranged from 0.27 to 0.35 d for Mn and 0.23 to 0.28 d for Zn. The long-lived component indicated that Zn (AEs: 16-18%) was less assimilated than Mn (AEs: 56-60%). Comparison of AE determined for each individual turbot showed no significant difference among experimental conditions for either metal ($p > 0.05$, Fig. 2). For Mn, $T_{b1/2l}$ average values ranged from 23 to 73 d, suggesting long-term retention of this metal in juvenile turbots. In all experimental conditions, the long-term depuration rate constant (k_{el}) of Zn was not significantly different from 0 ($p > 0.05$; Table 3), resulting in $T_{b1/2l}$ values tending toward $+\infty$.

Table 3. Depuration kinetic parameters of ^{54}Mn and ^{65}Zn in juvenile turbot (A) single-fed with toxins, (B) and multi-fed with toxins (C) non-exposed to toxins (n = 5-7 per treatment) and then maintained for 21d in unspiked seawater. k_e: depuration rate constant (d^{-1}); $T_{b1/2}$: biological half-life (d), A_0: remaining activities (%); ASE: asymptotic standard error; R^2: determination coefficient. Probability of the model adjustment: ^{NS} $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Tracer	Experimental conditions	Short-lived			Long-lived			R^2
		A_{0s} ± ASE	k_{es} ± ASE	$T_{b1/2s}$ ± ASE	A_{0l} (=AE) ± ASE	k_{el} ± ASE	$T_{b1/2l}$ ± ASE	
^{54}Mn	A	40.89 ± 5.88***	2.61 ± 1.81*	0.27 ± 0.19	59.12 ± 3.63***	0.025 ± 0.007***	27 ± 7	0.72
	B	39.68 ± 4.80***	1.98 ± 0.81*	0.35 ± 0.14	60.38 ± 2.87***	0.010 ± 0.004*	73 ± 35	0.75
	C	44.27 ± 9.36***	2.21 ± 1.18*	0.31 ± 0.17	55.73 ± 5.66***	0.014 ± 0.010^{NS}	50 ± 36	0.51
^{65}Zn	A	83.60 ± 3.63***	3.06 ± 0.92**	0.23 ± 0.07	16.40 ± 2.26***	0.015 ± 0.014^{NS}	$+\infty$	0.94
	B	82.22 ± 3.31***	2.48 ± 0.47***	0.28 ± 0.05	17.79 ± 1.95***	0.011 ± 0.011^{NS}	$+\infty$	0.94
	C	82.52 ± 3.27***	2.74 ± 0.60***	0.25 ± 0.06	17.48 ± 2.05***	0.015 ± 0.012^{NS}	$+\infty$	0.96

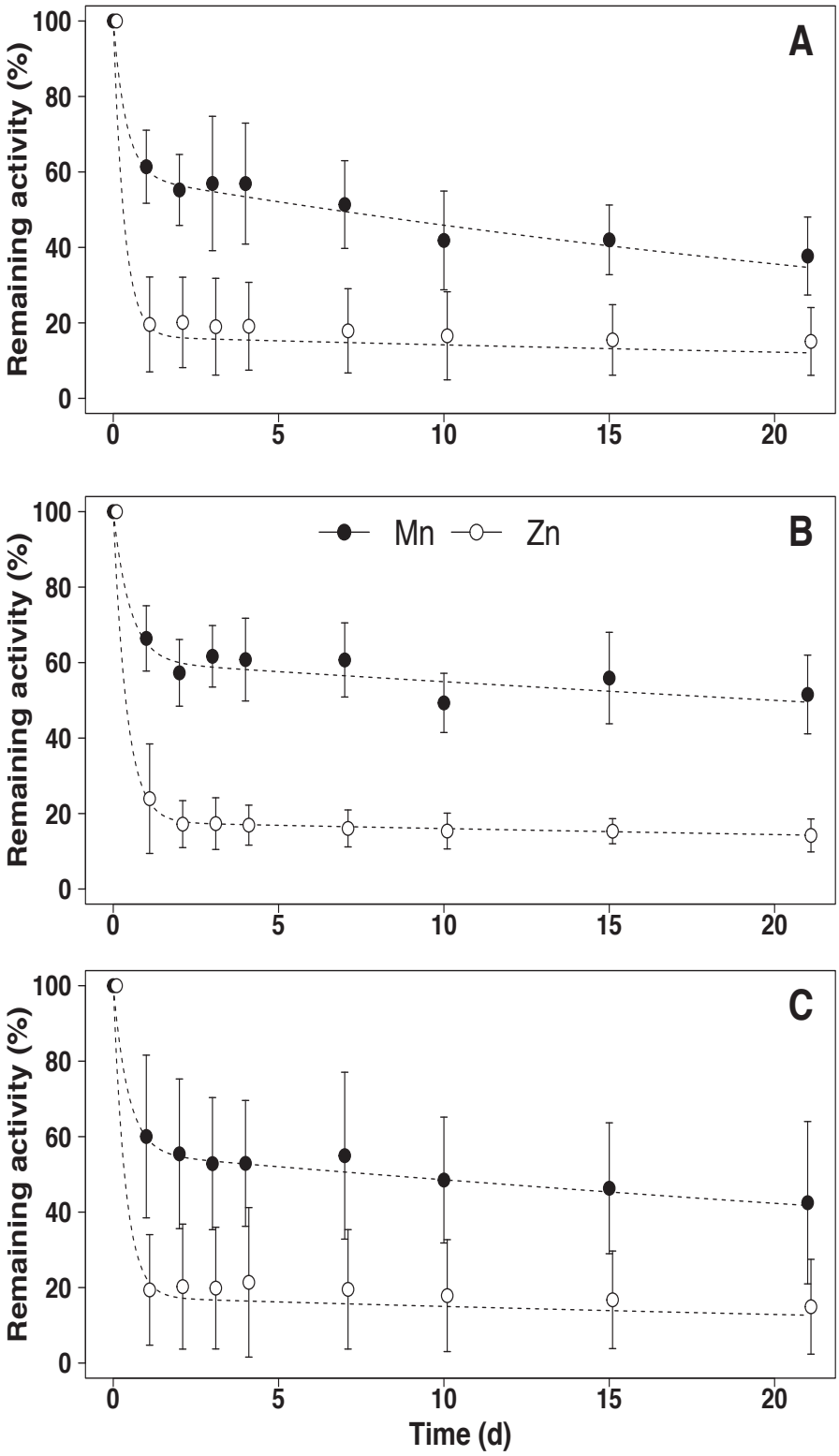


Figure 2. Whole-body depuration, ^{54}Mn and ^{65}Zn in juvenile turbots (A) single-fed with toxins, (B) multi-fed with toxins and (C) non-exposed to toxins. Parameters and statistics of depuration kinetics are given in Table 3.

4. Discussion

The most common routes of PbTxs exposure in aquatic species are by absorption of the toxin from lysed cells across gill epithelium or by direct ingestion of *K. brevis* cells and absorption of its toxins across the gastrointestinal epithelia (Kimm-Brinson & Ramsdell 2001). Although blooms of *K. brevis* are usually associated with massive fish mortalities, PbTx accumulation and toxicological effects in this taxon has not been well characterized. In mammals, the ingestion of shellfish containing PbTxs causes neurotoxic shellfish poisoning (NSP). NSP is characterized by both neurological and gastrointestinal symptoms (Kimm-Brinson & Ramsdell 2001). Brevotoxins specifically activate voltage gated sodium channel Na_y which are present in most excitable cells, inducing an influx of sodium and consequently cell regulation through activation of ion pump and/or channels such as the NA^+/K^+-ATPase. Mechanisms for metal transport have been proposed. Dietary uptake of Zn occurs primarily in the intestine where it is transported by ZIP4 protein across the epithelia (McAllister and Dyck, 2017). Zinc acts essentially as a neurotransmitter and was recently shown to play a role in cell signalling by binding to ion channels including Na_y (Noh et al, 2015). Manganese is a calcium analogue that can enter neurons and other excitable cells through voltage gated calcium channel as well as Na^+/Ca^{2+} exchanger (Inoue et al, 2011). Therefore, we tested the impact of dietary PbTxs exposure on the assimilation of metals in fish. The present study provides first answers to this assumption by characterizing Mn and Zn assimilation efficiencies (AEs) and tissue incorporation efficiency in juvenile turbots after one or after multiple ingestions of mussels *Mytilus edulis* containing PbTx at two no observable adverse effect level (4.3 and 14.8 ng PbTx-3 equiv/g) and by comparing the results obtained with the AEs of the same metals in turbots not exposed to PbTx.

In this study, we found a relatively limited trophic transfer of dietary PbTx in juvenile turbots that consumed mussels contaminated with PbTx (188 and or 525 ng g^{-1}) through four 1 hour exposure to environmentally relevant *K. brevis* concentration (approximately 1000 cells mL-1 in natural blooms, Stumpf et al. 2003, Gannon et al. 2009).Maximum PbTx concentrations are detected in the gall bladder of the fish whereas lower concentrations were found in the liver and the digestive tract. All the PbTx measurements done in the muscles were below the limit of quantifications (LOQ). The concentration were not found significantly different 2 hours after the last feeding of the 3 weeks exposure period.

These results suggest (1) a rapid turn-over of the ingested PbTxs that are absorbed in the digestive tract and then quickly eliminated by the hepatobiliary system already recognized as the key route for metabolizing and excreting brevetoxin in fish (Landsberg 2002) (2) the absence of re-distribution of these compounds in organs and tissues, such as muscles, that are not involved in digestion and excretion processes. Although this assumption cannot be fully confirmed by our results, Naar et al. (2007) have already shown similar findings in field measurements done on several species of fish. Indeed, these authors showed that, in 42 species of fish exposed to *K. brevis* blooms in the field, although PbTx concentration may reach level in the ug/g range in liver, PbTx were generally below the LOQ or were in low concentrations in the muscles (11-414 ng g^{-1}; i.e. in average 10 and 30 times lower than in digestive tract and liver, respectively). Therefore, the observed rapid turn-over of the PbTx in turbots, likely due to the relatively low exposure dose used in our experiment, can explain the absence of differences in the AEs of Mn and Zn between the experimental treatments. Our results show that assimilation efficiencies (AEs) of Mn and Zn were not affected by the single or multiple ingestions of dietary PbTxs with AEs ranging from 55 to 60% and 16 to 18% for Mn and Zn, respectively. For Zn, these values are in accordance with the values reported in the literature for this species but fed with other prey (Pouil et al. 2017, 2016, Mathews et al. 2008). Mn AEs measured in the present study were generally lower than those mentioned in the literature. For example, Pouil et al. (2016) found that the Mn AEs in turbot fed with shrimp, fish and ragworms were ranged from 23 to 44% showing that Mn AE is highly dependent on the prey type. To the best of our knowledge, the potential effects of biotoxin ingestions on metal AE have never been assessed in fish or other aquatic organisms. Based on our results, the occurrence of biotoxins in the food (in low concentrations, i.e. < 600 ng g^{-1}) of fish has no influence of the subsequent metal AE.
The current study presents the first findings regarding to the effects of the ingestion of biotoxins on the trophic transfer of selected essential elements in fish. Care needs to be taken in how to interpret and expand on these results. For example, bivalves were used as prey for the juvenile turbots. Therefore, the assimilation efficiencies observed in this study might not reflect actual assimilation of these elements in natural conditions, as bivalves are not natural prey of this species (Sparrevohn & Støttrup 2008, Florin & Lavados 2010).

Furthermore, in connection with ecological relevance of this work, in natural environment, higher concentrations of PbTxs can be observed in bivalves during *K. brevis* blooms (Landsberg et al. 2009) which could lead to greater physiological effects on their consumers such as fish.

5. Conclusion

This study revealed no statistically significant differences in the essential metal assimilation efficiency of juvenile turbots *S. maximus* after (1) a single ingestion or (2) multiple brevetoxin ingestions when compared to the fish in the control treatment. It was also shown that no difference was observed in the efflux rate constant (k_e) during the rapid depuration phase, between fish pre-exposed or not to the dietary PbTxs. Thus, these results suggest that the occurrence of PbTxs in the food do not appear to affect the trophic transfer of Mn and Zn essential elements in juvenile fish (integrative process). Nevertheless, since harmful algae can produce a variety of toxins with diverse mode of actions, further investigations are needed to study the influence of other biotoxins on essential metal assimilation in fish.

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ANNEXE 6

SUPPLEMENTARY MATERIAL

How ingestion of toxins related to algal bloom events is influencing transfer of essential nutrients (Mn and Zn) in fish

Objectives

Because seawater pH are known to affect the digestive physiology of fish (e.g. Pimentel et al. 2015, Rosa et al. 2016), effects of ocean acidification in combination with the occurrence of brevetoxins in the food were investigated in the trophic transfer of essential metals (Mn and Zn) of juvenile turbots.

Acclimation to the targeted pH and exposure of turbots

For each pH condition, 10 juvenile turbots (pH 7.5: 31.9 ± 3.0 g w wet and pH 8.0: 30.1 ± 2.9 g w wet) were exposed under controlled pH conditions in two 20-L aquaria. The two pH values were 8.00 (pCO_2 of approx. 450 µatm) and 7.50 (pCO_2 of approx. 1800 µatm). These values were chosen based on the current projections provided by the literature for the next two centuries (ΔpH −0.5; (Orr et al. 2005, IPCC 2013).

Concerning the method used to regulate the seawater pH, we followed the recommendations of the Guide to best Practices for Ocean Acidification Research and Data Reporting (Riebesell et al. 2010). The pH_{NBS} was monitored every 15 min in each aquarium to within ± 0.05 pH_{NBS} units using a pH probe connected to a multi-probe aquaristic computer (IKS ComputerSysteme, www.iks-aqua.com) that bubbled pure CO_2 into the aquaria. Temperature in each aquarium was also monitored, using a dedicated probe connected to the same computer. The pH probes were regurlarly calibrated using Tris-HCl and NBS buffer solutions.

Turbots, acclimated to the targeted pH, were pre-exposed to gel food NRHT for 3 weeks (5 feedings/week) and then single-fed with radiolabelled gel food RNT. Turbot were individually identified as described previously. After the radiolabelled single-feeding and depuration was followed in each individual by regular whole-body γ-counting over 21 d.

Results

Similar brevetoxin concentrations were found in the measured organs (the digestive tract, the gall bladder, the liver and the muscles) between the turbots from the different pH conditions (Table S1).

We observed no significant different (p>0.05) between the Mn and Zn remaining activities (Fig. S1) of turbots acclimated to the two pH values for 2 months and pre-exposed for 3 weeks to the dietary brevetoxins (Fig. S1).

Table S1. Brevetoxins eaten by turbots acclimated at 2 different weawater pH (7.5 and 8.0) and subsequently measured in the selected body compartiments. Data are Means.

Experimental conditions	Brevetoxin ingested per feeding and per turbot (ng g^{-1} and [ng])	Total brevetoxin ingested per turbot (g g^{-1} and [ng])	Brevetoxin measured in the selected body compartments (ng g^{-1} and [ng])			
			Digestive tract	Gall bladder	Liver	Muscles
pH 7.5	8.2 [262.5]	123.4 [3937.5]	10.7 [10.8]	105.9 [65.6]	10.7 [13.0]	< LOQ
pH 8.0	8.7 [262.5]	130.8 [3937.5]	8.5 [8.0]	120.1 [64.7]	10.5 [10.2]	< LOQ

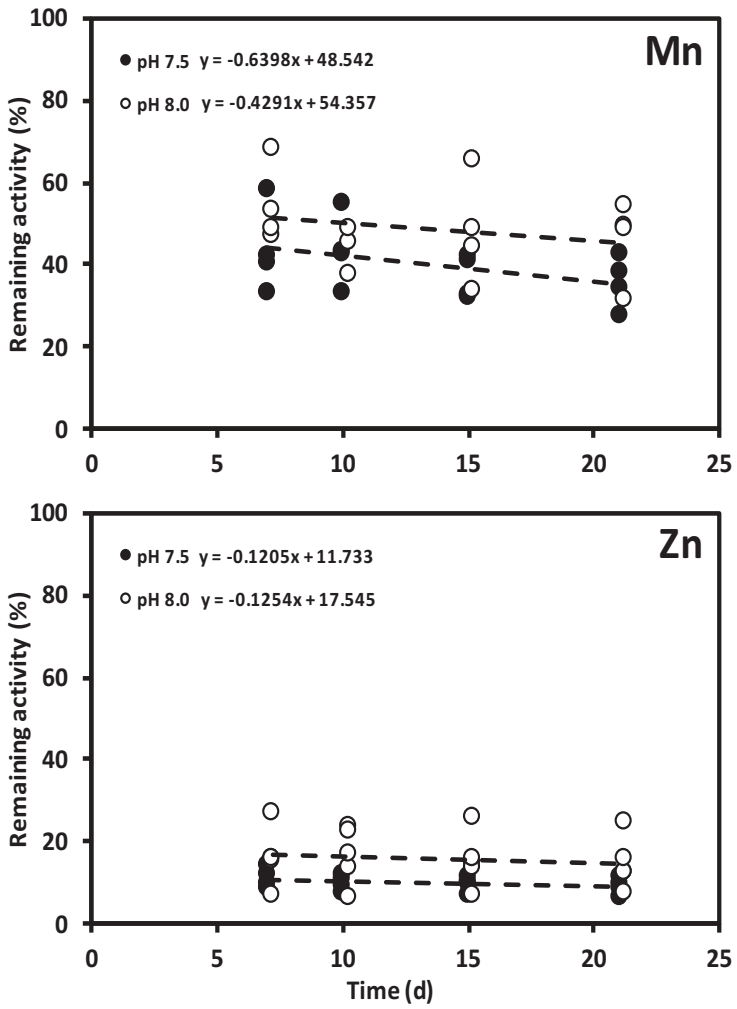

Figure S1. Remaining activities of Mn and Zn after a radiolebeled single-feeding using mussels in turbots acclimated at two different pH (7.5 and 8.0) and pre-exposed for three weeks to dietary brevetoxins.

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ANNEXE 7

Trophic transfer of essential elements in the clownfish *Amphiprion ocellaris* in the context of ocean acidification

Jacob H[1,2], **Pouil S**[1,3], Lecchini D[2,4], Oberhänsli F[1], Swarzenski P[1], Metian M[1] (2017). Trophic transfer of essential elements in the clownfish *Amphiprion ocellaris* in the context of ocean acidification. *PLoS One* 12(4): e0174344.

ABSTRACT: Little information exists on the effects of ocean acidification (OA) on the digestive and post-digestive processes in marine fish. Here, we investigated OA impacts (Δ pH = 0.5) on the trophic transfer of select trace elements in the clownfish *Amphiprion ocellaris* using radiotracer techniques. Assimilation efficiencies of three essential elements (Co, Mn and Zn) as well as their other short-term and long-term kinetic parameters in juvenile clownfish were not affected by this experimental pH change. In complement, their stomach pH during digestion were not affected by the variation in seawater pH. Such observations suggest that OA impacts do not affect element assimilation in these fish. This apparent pCO_2 tolerance may imply that clownfish have the ability to self-regulate pH shifts in their digestive tract, or that they can metabolically accommodate such shifts. Such results are important to accurately assess future OA impacts on diverse marine biota, as such impacts are highly species specific, complex, and may be modulated by species-specific metabolic processes.

Keywords: pH decrease, Coral reef fish, Food, Assimilation, Essential elements

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1. INTRODUCTION

The absorption of increased atmospheric CO_2 concentrations by the oceans is changing seawater chemistry, with forecasts estimating a drop of 0.3–0.4 units in ocean pH by the year 2100 [1]. This process is known as ocean acidification (OA) and has been shown to affect vital functions of many marine biota [2]. While the majority of research regarding the effects of CO_2-driven ocean acidification have focused on calcifying marine organisms, fewer studies exist on the impacts of OA on fish health [3]. Effects of increased pCO_2 on fish vary from species to species, life stage, and biological processes [4]. For example, Moran and Stottrup [5] found weight and growth rates substantially reduced in juvenile Atlantic cod (Gadus morhua) exposed to increasing levels of pCO_2. Conversely, other studies have showed that elevated levels of pCO2 had no clear effect on growth rates in juvenile spiny damselfish, *Acanthochromis polyacanthus* [6] and juvenile scups, *Stenotomus chrysops* [7]. Furthermore, Welch and Munday [8] found that high level of pCO_2 increased the reproductive output in the clownfish *A. percula*, but decreased the reproductive output in A. polyacanthus in similar conditions.

Among the biological processes studied in the context of OA research on marine fishes, digestion has received little attention to date. Frommel et al. [9,10] found morphological and physiological impairments in the digestive system of fish during early life stages under ocean acidification scenarios, but no direct correlations were made to physiological digestion mechanisms. However, a few recent studies that examined the digestive process found strong evidence that ocean acidification may affect the digestive capabilities of some fishes. For example, Rosa et al. [11] showed that hypercapnic conditions led to a substantial decrease in digestive enzyme activity; 42% in the activity of trypsin and 50% in the activity of alkaline phosphatase in the juvenile of the Bamboo shark *Chiloscyllium punctatum*. Moreover, Pimentel et al. [12] showed that similar conditions led to a decrease in both pancreatic (up to 26.1% for trypsin and 74.5% for amylase) and intestinal enzymes (up to 36.1% for alkaline phosphatase) of the post-metamorphic larvae of the flatfish *Solea senegalensis*. However, if new observations indicate that ocean acidification may interfere with fish digestion, the association between a decrease in enzyme activity and an essential element's assimilation deficiency has not yet been investigated.

One critical parameter for understanding metal trophic transfer in fish is the assimilation efficiency (AE) of an element from ingested food. If derived under controlled experimental conditions, AE is a first-order physiological parameter that can be compared quantitatively among different trace elements, organisms, food types, or environmental conditions [13]. The present study aimed to investigate the possible effects of ocean acidification on the assimilation efficiency of three essential elements using radiotracers (^{57}Co, ^{54}Mn, ^{65}Zn) in juvenile clownfish *Amphiprion ocellaris*. Juveniles were exposed to projected future pCO_2 levels over the next two centuries (pH 7.5) [1] as well as present-day conditions (pH 8.0) and their trophic transfer and assimilation efficiency (AE) for each element was compared between the two conditions using radiotracer techniques. It is worth noting that the selected low pH condition may also represent a low pH that can occasionally occur at the current time in coral reef lagoon [14].

2. MATERIAL AND METHODS

2.1. Origin and acclimation of the organisms

In February 2016, one hundred juvenile clownfish *A. ocellaris* just under two months old were obtained from a fish farm (Ecorecif, France) and transported to the IAEA Radioecology Laboratory in Monaco. Juveniles were randomly separated into two groups and acclimatized for seven weeks using two 20-L aquaria, configured as follows: (open circuit, water filtration = 0.45 µm; salinity = 38; temperature = 25.8 ± 0.3 °C; renewal of seawater = 70% h^{-1}; photoperiod = 12h / 12h), at either pH 8.0 ± 0.07 (pCO_2: 456 µatm) and 7.5 ± 0.06 (pCO_2: 1775 µatm). The pH_{NBS} was monitored every 15 minutes in each aquarium to within ± 0.05 pH_{NBS} units using a continuous pH-stat system (IKS, Karlsbad) that purged a pre-set rate of pure CO_2 into the aquaria. Temperature in each aquarium was also monitored with the same system. The pH probes were regularly calibrated using Tris-HCl and NBS buffer solutions [15]. Total alkalinity was measured by titration using Methrom 809 Titrando® calibrated with NBS buffers, Tris-HCl (Dickson, Batch #137) and standards (Dickson, Batch #150). The pCO_2 was determined from pH, temperature and total alkalinity measurements using the R package seacarb [16].

2.2. Kinetics of stomach pH in juvenile clownfish after a single feeding

The influence of seawater pH on juvenile clownfish was investigated since stomach pH can directly affect the digestive process [17] and it may thus affect assimilation efficiencies of essential elements by fish. For this purpose, a total of 24 acclimatized, juvenile clownfish were selected for each pH treatment (control condition: 0.51 ± 0.18 g; acidified conditions: 0.57 ± 0.17 g) and were acclimated to a single 9:00am feeding (pellets) for two weeks prior to the experiment. The experiment consisted of feeding fish *ad libitum* to characterize the acidification capacity in the fish stomach in two different conditions. Three fish (from both conditions) were subsequently sampled at various times over 8 hours. Three other individuals were sampled right before the feeding in order to determine the preprandial level of stomach pH. For pH determinations, measurements were conducted in living animals using a pH microelectrode (ThermoScientific, 9810BN). In order to minimize adverse impacts on the welfare of fish during this experiment, fish were anaesthetized using Eugenol and, immediately after anesthesia, the tip of the microelectrode was inserted into a small slit made in the stomach [17]. The CRIOBE committee approved the sampling protocol and gave the ethical permission CRIOBE-Fish-2016-012. At the end of the experiment, the fish were sacrified by decapitation and disposed of following IAEA radioactive waste procedures. A statistical comparison between pH values as a function of time for the two conditions was made using the two-tailed Mann-Whitney U test.

2.3. Long-term depuration of ^{57}Co, ^{54}Mn, and ^{65}Zn in juvenile clownfish

A total of 15 acclimatized juvenile clownfish were selected for each pH treatment (control condition: 0.32 ± 0.05 g; acidified conditions: 0.34 ± 0.06 g). Juveniles were transferred into two preconditioned 20-L aquaria that were set up in conditions similar to those of acclimatization tanks (with pH set to 7.5 ± 0.05 and 8.0 ± 0.08, temperature = 25.5 ± 0.3 °C; and the same seawater renewal rate). During the two weeks prior to exposure to radiolabeled food, the fish were fed daily with pellets of the same size as those used in the experiment.

The experiment consisted in one single exposure of fish to radiolabeled feed (single-feeding method; e.g. [18]). Clownfish were fed *ad libitum* and all uneaten pellets were removed after 5 min. From that moment, all fish (including control fish; fed with non-radiolabelled food in order to assess potential seawater contamination) were radioanalysed regularly to monitor the depuration kinetics of radiotracers over a period of 20 days. During the entire experiment, there were no negative effects observed on the well-being of the specimens.

2.4. Short-term depuration of ^{57}Co, ^{54}Mn and ^{65}Zn in juvenile clownfish

In order to get a best description of the early process of trophic transfer of the ^{57}Co, ^{54}Mn and ^{65}Zn in juvenile clownfish, a short-term experiment (<1d) was conducted. A total of six acclimatized clownfish were selected for each treatment (control condition = 0.38 ± 0.1 g; acidified conditions: 0.40 ± 0.1 g), and transferred to two 20-L aquaria in conditions identical to those of the acclimatization tanks. For this experiment (method being similar to long-term exposure one), individuals were radioanalysed at various times during one day (3, 6, 9, 12 and 24h).

2.5. Radiotracers and counting

Uptake and depuration kinetics of the three essential elements (Co, Mn, and Zn) were determined using matched radiotracers (^{57}Co as $CoCl_2$ in 0.1 M HCl, [$t_{1/2}$ = 271.8 days]; ^{54}Mn as $MnCl_2$ in 0.5 M HCl, [$t_{1/2}$ =312.2 days]; ^{65}Zn as $ZnCl_2$ in 0.1M HCl, [$t_{1/2}$ = 243.9 days]) purchased from Isotope Product Lab., USA. The radioactivity of the tracers was quantified using high-resolution γ-spectrometer systems that consisted of a suite of four high purity germanium (HPGe) N or P type - detectors (EGNC 33-195-R, Canberra® and Eurysis®) controlled by a multi-channel analyser and a computer equipped with spectral analysis software (Interwinner® 6, Intertechnique).

The radioactivity of each isotope was determined using calibrated standards of the same geometry. Measurements were corrected for background activity and radioactive decay. Live clownfish specimens were placed into custom designed holding tubes filled with non-contaminated seawater for gamma spectrometric analyses. This method maintained sufficient dissolved oxygen levels while minimizing counting errors due to geometry effects from movement of the live specimen during counting [19].

Counting time was adjusted to obtain propagated counting errors typically less than 5%; run times were typically 10-20 min for whole specimen radioanalysis (longer count times (up to 40 min) were required towards the end of long-term depuration experiment), which was also shown to not affect the specimen's wellbeing. Tests were performed prior to the experiments by placing fish into identical counting conditions to assess their behavior during counting (e.g. water temperature was stable and dissolved O_2 concentrations were always > 3 mg L^{-1}).

2.6. Radiolabelled processed feed pellet

Radiolabeling of the compound food pellets was performed as described by Pouil et al. [20] using the selected radiotracers described above. Briefly, 1.2 gr of pellets were dipped for 1 h in 1 mL of seawater spiked with ^{57}Co, ^{54}Mn and ^{65}Zn. The food pellets were then dried for 48 hr (50 °C) to prevent nutritional loss and mold growth. Potential discharge of the radioisotopes into seawater, which may then lead to a double exposure of the fish (food and water) was tested and confirmed not to be an issue as long as the fish consumed the food pellets within 1 min. Although these tests confirm the single-pathway contamination (viz. food) of the fish, additional clownfish were used as controls against possible contamination by seawater.

2.7. Kinetic data from the single-feeding experiment

Observation of depuration kinetics of these radiotracers were fitted using nonlinear regression routines and iterative adjustment using Statistica software 7.0. These kinetics were best fitted using a one- or two-component exponential model [21] or a two-component model that includes an exponential component and a constant as described by Pouil et al. [20,22]. A comparison between the remaining activities at different times for the short- and long-term experiments was made using the two-tailed Wilcoxon-Mann-Whitney test. The level of significance for statistical analyses was always set at $\alpha = 0.05$. All the statistical analyses were performed using R software (R-3.2.1).

3. RESULTS AND DISCUSSION

In order to evaluate the influence of pH on the absorption kinetics of essential elements in *A. ocellaris*, depuration of ^{57}Co, ^{54}Mn and ^{65}Zn were followed after a single feeding using radiolabeled pellets for both 24 hours and 20 days. The average activities in pellets were 10.5 ± 0.5 kBq g^{-1} of ^{57}Co, 5.4 ± 0.3 kBq g^{-1} of ^{54}Mn, and 5.2 ± 0.3 kBq g^{-1} of ^{65}Zn.

During the 20-day experiment, depuration kinetics of ^{7}Co, ^{54}Mn and ^{65}Zn in control and acidified conditions were described by a single exponential component model for ^{57}Co, a double exponential component model for ^{65}Zn, and a dual component model with a constant for ^{54}Mn (Table 1 and Fig 1A). A large proportion (70-99%) of ingested radiotracer has been associated with the short-term depuration. This component was characterized by a rapid loss ($t_{b1/2s} < 1$) independently of pH conditions.

At the end of the depuration period (20 days), the total retained activity (i.e. assimilation efficiency: AE) was respectively 5 ± 1% and 5 ± 5% for ^{54}Mn, and 28 ± 4% and 29 ± 5% for ^{65}Zn in control and acidified treatments, however ^{57}Co was not assimilated in both conditions (Table 1). There was no significant difference in the percentage of activity remaining between the two pH conditions for each element (Mann-Whitney U test, U = 91-146, N1 = 15-16, N2 = 16, P > 0.1). For the short-term depuration experiment (Fig 1B), there were also no significant differences in the remaining activities of individuals followed at each radioanalysed times (3, 6, 9, 12 and 24h) between pH conditions (Mann-Whitney U test, U = 12-26, N1 = 6, N2 = 6, P > 0.1).

Being one of the first studies to investigate the effects of increased pCO_2 on the trophic transfer of essential elements in fish, it is premature to broadly generalize the absence of direct effects found here to other fishes. Indeed, sensitivities may vary among species, especially among species with between different life cycles; coral reef species appears to be particularly tolerant to the effects of ocean acidification.

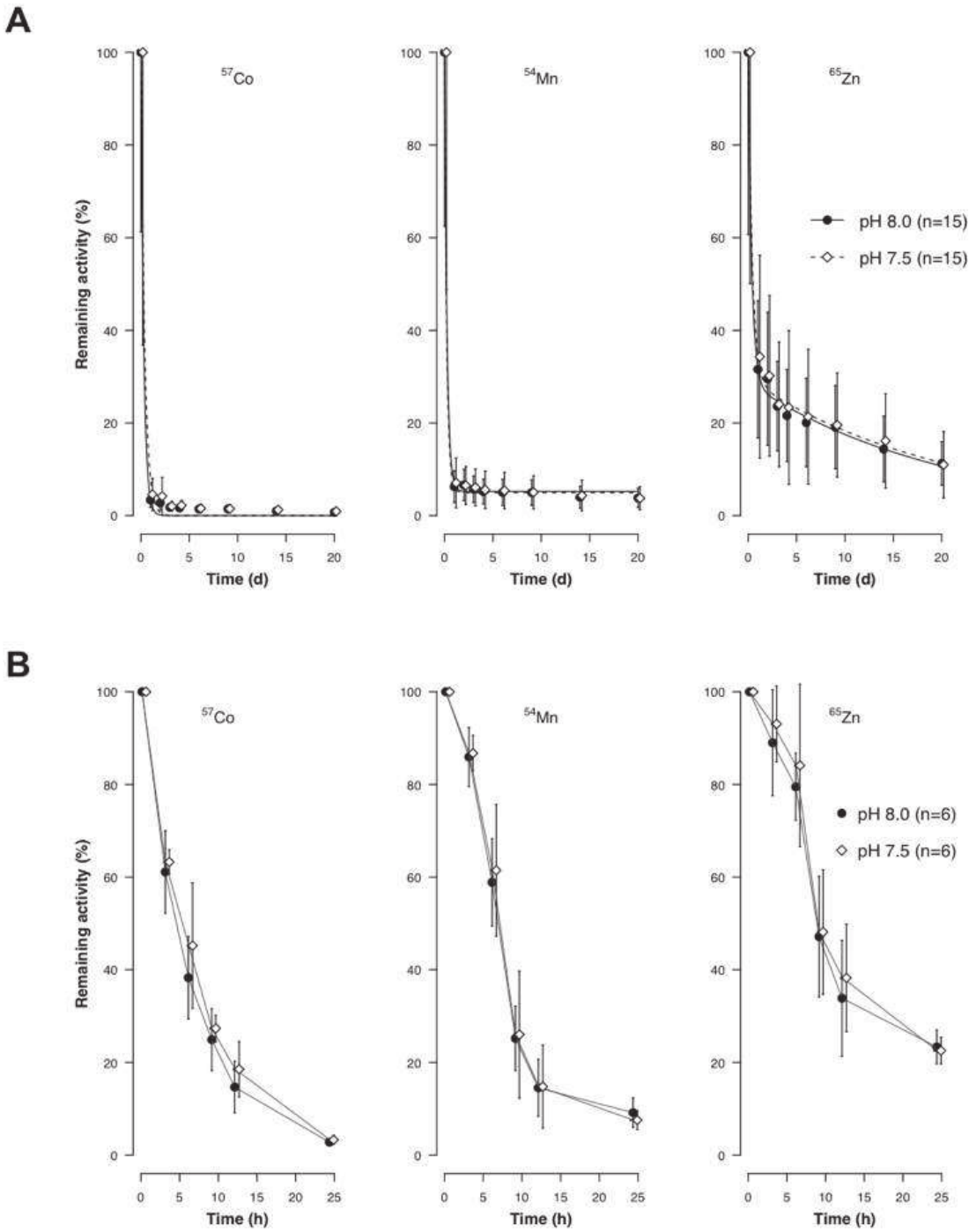

Figure 1. Short-term depuration experiment: Influence of pH on the depuration of Co, Mn and Zn during 20 days (A) and 24 hours (B) in juvenile clownfish *A. ocellaris* fed with radiolabelled pellets (single feeding approach). Values are means ± SD.

Many studies have found no effect of increased pCO_2 on growth rates in juvenile coral fish species [6,23], which further corroborate the absence of effect on assimilation in our study (stomach's pH of juvenile *A. ocellaris* was not affected, Mann-Whitney U test, U = 3-12, N1 = 3, N2 = 3, P > 0.1; Fig 2).This resistance may result from physiological adaptations from residing in a variable pH environment, such as coral reefs [24]. In our study, enzyme activity was not measured in the digestive tract while stomach pH was: this parameter regulates the activity of various enzymes such as pepsin [25] and it was not modified by an increase of pCO_2 in seawater (Fig 2).

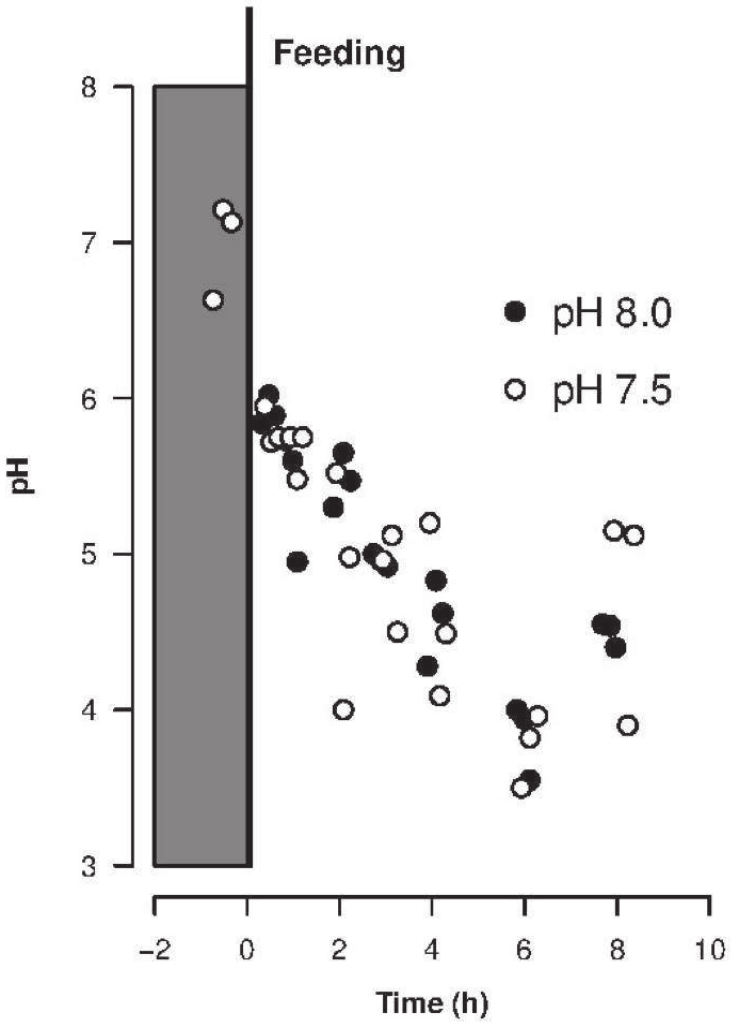

Figure 2. Influence of environmental pH condition on stomach pH of juvenile clownfish *A. ocellaris* before a single feeding and during 8 hours after feeding. Values on the left-hand side of the figure are individual pH measurements and values on the right-hand side are means of pH ± SD.

Table 1. Long-term depuration experiment: estimated depuration kinetic parameters of ^{57}Co ^{54}Mn and ^{65}Zn in juvenile *A. ocellaris* exposed to radiotracers by radiolabelled pellets (single feeding approach) in two pH treatments (7.5 and 8.0) and held for 20 days in unspiked water.

Isotope	pH	Model type	First component (Short lived)			Second component (Long lived)			
			k_{es}	A_{0s} ± ASE	$T_{b1/2s}$ ± ASE	k_{el} ± ASE	AE± ASE	$T_{b1/2l}$	R^2
^{57}Co	8.0	S	3.32 ± 0.92***	99.99 ± 3.09***	0.21 ± 0.06	-	-	-	0.87
	7.5	S	2.50±0.59***	99.95±4.55***	0.28±0.07	-	-	-	0.74
^{54}Mn	8.0	DC	4.33±2.75*	95.02±3.23***	0.16±0.10	-	4.98 ± 1.15***	∞	0.87
	7.5	DC	3.91±2.53*	94.77±4.71***	0.18±0.11	-	5.22 ± 4.72***	∞	0.76
^{65}Zn	8.0	D	2.71±1.19*	71.34±5.46***	0.26±0.11	0.05 ± 0.02**	28.65 ± 3.78***	14.15	0.74
	7.5	D	2.34±1.10*	70.61±7.53***	0.30±0.14	0.05± 0.02*	29.36 ± 5.22***	14.75	0.59

S: depuration model with one exponential component ($A_t = A_{0s} x\ e^{-kes\ x\ t}$); D: depuration model with two exponential components ($A_t = A_{0s} x\ e^{-kes\ x\ t} + A_{0l} x\ e^{-kel\ x\ t}$); DC: Two-component depuration model with constant ($A_t = A_{0s} x\ e^{-kes\ x\ t} + A_{0l}$ where $A_{0l}=AE$); kes and kel: depuration rate constant (d^{-1}) according to the short- and the long-lived exponential component (d^{-1}); A_{0s} and A_{0l} (=AE): remaining activity (%) according to the short- and the long-lived exponential component; $T_{b1/2}$: biological half-life (days); ASE: asymptotic standard error; R^2: determination coefficient.

*** $p < 0.001$

** $p < 0.01$

* $p < 0.05$

In past studies where the effects of ocean acidification on digestive enzyme activity was studied in the flatfish *Solea senegalensis* [12] and the Bamboo shark *Chiloscyllium punctatum* [11], the fitness and the survival of the species were also negatively impacted by ocean acidification [26, 27]. One possible explanation for the differences in response to ocean acidification observed between these two groups of species (coral fish vs. flatfish and benthic sharks) might come from their distinct metabolic rates. Indeed, Melzner et al. [28] suggested that marine animals with higher metabolic rates might be less affected by ocean acidification due higher extracellular pCO_2 values and advanced metabolism for the elimination of CO_2 and associated acid-base disturbances. Clownfish have higher metabolic rates many other fish species, including flatfish and bamboo shark [29], which should make them more tolerant to hypercapnia. Future studies should therefor focus on the effects of increased pCO_2 on food assimilation for lower metabolic rate fish species.

While species with higher metabolic rate may better cope with changes of pCO_2 in their environment, it is well reported that a series of fish physiological processes can be disturbed by higher pCO_2 [4]. Among the affected processes, it is interesting to focus on the acid-base balance and the ionoregulation in the context of the trophic transfer of essential elements. Indeed, dietary trace element absorption occurs mainly in the intestine [30,31], and in marine fish (drinking water species), the intestine is also involved in acid-base balance and the ionoregulation (with gills and kidney, 32,33). Hereto, even if previous studies provided evidence of disturbances caused by pCO_2 on these processes in the fish intestine [34,35], our results indicated that it does not affect dietary essential element transfer in clownfish.

The current study presents intriguing findings in the response of one fish species to the effects of OA through the trophic transfer of select essential elements. Care needs to be taken in how to interpret and expand on these results. For example, compound feed was used for the juvenile clownfish. Therefore, assimilation efficiencies observed in this study might not reflect actual assimilation of these elements in natural conditions, as artificial food has been found to influence the assimilation of different metals in comparison to natural preys although we used the same type of food for both treatments. Pouil et al. [22] showed that the assimilation efficiencies of essential elements were influenced by the nature of food provided to the flatfish *Scophthalmus maximus*, as the specific physicochemical forms and metabolites will likely play a role in the bioavailability of these elements for predators [36].

Therefore, depending on the experimental design, it is foreseeable that the AE of the essential elements in clownfish might vary depending on the initial treatments.
Furthermore, and in connection with ecological relevance of this work, changes in seawater chemistry due to ocean acidification will affect the solubility, speciation, and bioavailability of trace metals in water, biota, and sediment [37]. Therefore, also affecting the bioavailability, accumulation and storage of essential elements; the trophic transfer of elements might thus also change along the food chain. The integration of natural prey, also exposed to increased pCO_2, will provide a more realistic view of trophic transfer mechanisms under such environmental stressors.

4. CONCLUSION

This study revealed no statistically significant differences in the assimilation efficiency of juvenile clownfish exposed to high-level treatments of pCO_2 when compared to the fish in the control treatment. It was also shown that during the rapid depuration phase, no difference was observed between fish placed in the two treatments. Thus, these results suggest that the effects of ocean acidification effects do not appear to affect the trophic transfer of essential elements in juvenile clownfish A. ocellaris, although recent studies found that hypercapnia may decrease the activity of certain digestive enzymes in juvenile fish, sometimes by as much as 50% [10,11]. If ocean acidification can reduce the enzyme activity of juvenile fish, it may thus not ultimately result in a decrease in assimilation (integrative process). Indeed, some metabolic functions can be affected by a variation of seawater pH (or pCO_2) but, according to the present study, the ultimate process (viz. trophic transfer of essential element) may not.

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ANNEXE 8

Investigations of temperature and pH variations on metal trophic transfer in turbot (*Scophthalmus maximus*) fish

Pouil S[1,2], Oberhänsli F[1], Bustamante P[2], Metian M[1] (sous presse) Investigation of temperature and pH variations on the metal trophic transfer in turbot (*Scophthalmus maximus*). *Environmental Science and Pollution Research*. DOI: 10.1007/s11356-017-8691-4

ABSTRACT: Studying dietary metal transfer kinetics is essential to gain a better understanding in global metal accumulation rates and its impacts in marine fish. While there exists a solid understanding on the influence of various biotic factors on this transfer, metal assimilation in fish might be also affected by abiotic factors, as has been observed in marine invertebrates. The present study therefore aim to understand the potential effects of two climate-related master variables, temperature and pH, on the assimilation efficiency (AE) of essential (Co and Zn) and non-essential (Ag) metals in the turbot *Scophthalmus maximus* using radiotracer tools. Juvenile turbots were acclimated for 8 weeks at two and two temperatures (17° C and 20° C) and pH (7.5 and 8.0) regimes, under controlled laboratory conditions and then fed with radio-labelled shrimp (^{57}Co, ^{65}Zn and ^{110m}Ag). Assimilation efficiencies of Co and Ag in juvenile turbot, determined after a 21-d depuration period, were not affected by pre-exposition to the different environmental conditions. In contrast, temperature did significantly influence Zn AE ($p<0.05$), while pH variations did not affect the assimilation of any of the metals studied. In fact, temperature is known to affect gut physiology, specifically the membrane properties of anterior intestine cells where Zn is adsorbed and assimilated from the ingested food. These results are relevant to accurately assess the influence of abiotic factors in AEs of metals in fish as they are highly element-dependant and also modulated by metabolic processes.

Keywords: Metal trophic transfer, Trace elements, Teleost, Ocean acidification, Global warming

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1. INTRODUCTION

Metals are typically found in the marine environment at low concentrations. Some metals are metabolically required at the correct amount for organisms, such as Co and Zn (i.e. essential metals), and others can be toxic even at very low concentrations, such as Ag (i.e. non-essential metals). Anthropogenic activities tend to increase metal concentrations in coastal environments, which can cause detrimental effects to the organisms living in these areas. This is particularly problematic due to the emergence of new emission sources, especially for Ag, including cloud seeding, nano-particles, or electronic component manufacturing (Lanceleur et al. 2011). Fish are exposed to these metals from both the dissolved and the particulate phases (Warnau and Bustamante 2007). Since food has been recognized as a pathway of major importance for metal intake in fish (Xu and Wang 2002; Mathews and Fisher 2009), investigating the factors influencing the trophic transfer of metals in fish is of paramount importance.

A key parameter for understanding metal trophic transfer in fish is the assimilation efficiency (AE; Wang and Fisher 1999). Numerous studies have focused on the determination of factors that influence metal AE in several aquatic species, including fish (e.g. Xu et al. 2002, Zhang and Wang, 2005, Pouil et al. 2016). For example, the importance of the composition and nature of the food source, both qualitatively and quantitatively, on metal AE has been determined in different species of marine fish (e.g. Wang and Wong 2003; Wang et al. 2012; Pouil et al. 2016). Similarly, the influence of the physiological state and life-stage of the organism on metal AE, have also been studied in several fish species (e.g. Zhang and Wang 2005; Zhang and Wang 2007; Pouil et al. 2017). These different studies have shown that biological, physiological, and ecological factors can importantly influence AE of trace metals. Nevertheless, the trophic transfer of metals can also be impacted by environmental variables (abiotic factors) as it has been shown in marine invertebrates (Lee and Lee, 2005) or in freshwater fish (Van Campenhout et al. 2007). Surprisingly, such an influence, to the best of our knowledge, has been not documented in the literature on marine fish.

Temperature and pH are two key environmental variables influencing marine fish physiology. For example, temperature, one of the main abiotic driver of fish physiology (Beitinger and Fitzpatrick 1979), was shown to affect gut transit time or the activity of the enzymes involved in the digestion process when fish are chronically exposed to temperatures away from their thermic preferences (Edwards 1971; Miegel et al. 2010).

Effects of environmental pH on fish physiology seems to be, in the other hand, more limited (Kroeker et al. 2010). However, few studies indicated that pH can alter the structure and functioning of the digestive tract (e.g. Frommel et al. 2014), and even the digestive enzyme activities (Pimentel et al. 2015; Rosa et al. 2016) of early stages of marine fish. The variation of temperature and pH may occur simultaneously, and organisms can be affected differently by them. Indeed, interactions of temperature with pH could theoretically generate a simple sum of the effect of each individual factor (additive effect) or more complex situations (antagonistic or synergistic effects) as explained by Flynn et al. (2015). In their natural environment, marine fish are most probably facing these possible complex interactions. In this context, the present study aims to assess the possible effects of two environmental variables (temperature and pH) on the assimilation of two essential (Co and Zn) and one non-essential (Ag) metals in the juvenile turbot *Scophthalmus maximus*. Radiotracer techniques were used to determine depuration parameters in controlled conditions of juvenile turbot previously acclimated at two temperatures (17° C and 20° C) and pH (7.5 and 8.0) after a single-feeding with radiolabelled shrimp.

2. MATERIALS AND METHODS

2.1. Origin and acclimation of fish

Juvenile turbot *Scophthalmus maximus* were purchased from a fish farm (France Turbot, www.france-turbot.com) and shipped to the International Atomic Energy Agency premises in the Principality of Monaco. Fish were randomly placed in four 20-L aquaria (n=8) and acclimated for minimum of 1 month to laboratory conditions (open circuit, water renewal: 60 L h^{-1}; 0.45 µm filtered seawater; salinity: 38; light/dark: 12h/12h; temperature: 17°C; pH: 8.00). During this period, the fish were fed one time per day (as described by Pouil et al. 2015 and Pouil et al. 2016) with a ration of 1.5% of their biomass with 1.1-mm pellets (proteins: 55% and lipids: 12%; Le Gouessant, www.legouessant.com). After this period, fish were acclimated to the target temperature and pH values (see Table 1) for 8 weeks prior to a unique radiotracer exposure (i.e. one single-feeding using radiolallebed shrimp following by 21 days of depuration as described in the section 2.2.2.).

Table 1. Summary of seawater parameters during the different phases (acclimation and depuration) of the experiment on the assimilation of metals in juvenile turbot exposed to different conditions of temperature and pH.

Experimental phase	Temperature (°C)	pH_{NBS}	Total alkalinity (µmol kg^{-1})	*p*CO2 (µatm)
Acclimation	16.94±0.22	7.98±0.07	2539±4	513±67
	19.76±0.08	7.98±0.04	2540±3	525±33
	16.96±0.18	7.48±0.06	2536±6	1843±134
	19.77±0.12	7.48±0.04	2534±6	1896±45
Depuration	16.79±0.05	7.97±0.05	2541±8	563±75
	19.62±0.43	7.95±0.04	2537±4	550±25
	16.79±0.06	7.47±0.05	2540±4	1867±111
	19.57±0.42	7.50±0.08	2537±2	1879±82

Juveniles were exposed under controlled temperature and pH conditions in a crossed experimental design (2 temperatures x 2 pH levels). The two temperatures were 17° C and 20° C and the two pH values were 8.00 (pCO_2 of approx. 450 µatm) and 7.50 (pCO_2 of approx. 1800 µatm). These values were chosen based on the optimal food conversion efficiency ratio (FCE) of juvenile turbot at 17.4±0.5°C at normal pH (Imslad et al. 2001) and the current projections provided by the literature for the next two centuries (ΔT°C: +3° C and ΔpH: -0.5; Orr et al. 2005; IPCC 2013).

Concerning the method used to regulate the seawater pH, we followed the recommendations of the Guide to best practices for ocean acidification research and data reporting (Riebesell et al. 2010). The pH_{NBS} was monitored every 15 minutes in each aquarium to within ±0.05 pH_{NBS} units using a pH probe connected a multi-probe aquaristic computer (IKS ComputerSysteme, www.iks-aqua.com) that bubbled pure CO_2 into the aquaria.

Temperature in each aquarium was also monitored, using a dedicated probe connected to the same computer. The pH probes were calibrated weekly using Tris-HCl and NBS buffer solutions (Dickson et al. 2007). Total alkalinity was measured by titration using Methrom 809 Titrando calibrated with NBS buffers, Tris-HCl (Batch 150, Dickson 2016) and reference materials (Batch 137, Dickson 2016). The pCO_2 was determined from pH, temperature and total alkalinity measurements using the R package seacarb (Lavigne et al. 2011).

2.2. Experimental procedures

2.2.1. Shrimp radiolabelling

Since crustaceans dominated the natural diet of turbot (Sparrevohn and Støttrup 2008; Florin and Lavados 2010) we used shrimp as radiolabelled prey. Preparation of the 80-radiolabelled shrimp *Palaemon* sp. (approx. 1 to 2 cm in total length) was carried out by exposing them for 7 days to dissolved radiotracers in an aerated 20-L aquarium (closed circuit; shrimp density: 4 shrimps L^{-1}, 0.45 µm filtered seawater; salinity: 38; light/dark: 12h/12h; temperature: 17°C; pH: 8.00). Radiotracers of high specific activity were purchased from Polatom, Poland (^{57}Co as $CoCl_2$ in 0.1 M HCl, $t_{1/2}$ = 272 days; ^{65}Zn as $ZnCl_2$ in 0.1M HCl, $t_{1/2}$ = 244 days and ^{110m}Ag as $AgNO_3$ in 0.1 M HNO_3, $t_{1/2}$ = 252 days). Seawater was spiked with small volumes (> 0.2 mL) of radiotracers (nominal activity of 2 kBq L^{-1} for ^{57}Co and 8 kBq L^{-1} for ^{65}Zn and ^{110m}Ag). No change in pH was detectable in the aquarium (close-circuit) after the tracer additions. During the 7-day exposure, seawater was renewed and spiked 4 times to eliminate ammonia generated by shrimp excretion and keep the radiotracer activity constant. The activity of the radiolabelled metal tracers in seawater was checked before and after each seawater renewal, to determine time-integrated activities (Warnau et al. 1996; Rodriguez y Baena et al. 2006).

Each organism was kept isolated during the duration of the experiment in a buoyant cylindrical polystyrene container (drilled to allow for free water circulation) in order to avoid cannibalism. The shrimps were fed with non-contaminated minced mussels one time between each water renewal.

2.2.2. Exposure of turbot via radiolabelled shrimp

A total of 8 acclimatized turbot were randomly selected for each experimental treatment (viz. 4*20-L tanks with each time 8 organisms, wet weights were: 22.4±3.4g, 22.1±3.8g, 22.3±5.4g and 23.7±4.2g respectively for the turbot exposed to pH 8.0 at 17° C, pH 8.0 at 20° C, pH 7.5 at 17° C and pH 7.5 at 20° C). Slits cut into the fins were performed on anesthetized fish to facilitate individual recognition, ensuring at the same time the welfare of the fish (see e.g. Pouil et al. 2016). For the last three feedings before the exposure to radiolabelled shrimp (5% of their biomass per day), fish, previously fed with pellets, were fed with non-labelled shrimp. The experiment consisted of a single feeding of fish in the different experimental conditions with radiolabelled shrimp.

To facilitate ingestion, radiolabelled shrimp were cut into pieces (Pouil et al. 2016). During and after the 5-min radiolabelled feeding, an additional turbot was placed in each aquarium to assess any possible radiotracer recycling from seawater due to leaching from the radiolabelled food or, later on, from fish depuration. Two hours after the radiolabelling feeding, all the fish (including control individual of each condition) were whole-body γ-counted alive (Pouil et al. 2016). They were then replaced in the same open-circuit aquarium and were regularly radio-analysed to follow the radiotracer depuration kinetics over 21 days. During the first week of depuration turbot were fed using non-labelled shrimp and then fed daily with non-labelled pellets (1.5% of their biomass) to cover their nutritional needs.

2.3. Radioanalysis

The radioactivity of the tracers was measured using a high-resolution γ-spectrometer system composed of 4 Germanium - N or P type - detectors (EGNC 33-195-R, Canberra® and Eurysis®) connected to a multi-channel analyser and a computer equipped with a spectra analysis software (Interwinner 6, Intertechnique®). The radioactivity in living organisms and samples was determined by comparison with standards of known activity and of appropriate geometry (calibration and counting). Measurements were corrected for background and physical radioactive decay. Living organisms were placed in counting tubes (diameter: 160 mm, height: 80 mm) filled with clean seawater (at the appropriated conditions of pH and temperature) during the counting period.

The counting period was adjusted to obtain a propagated counting error less than 5% (e.g. Rodriguez y Baena et al. 2006) and varied between 15 and 60 min in order to maintain fish health and ensure normal behaviour. Variations of temperature and pH during the counting have not exceeded +2° C and -0.2 respectively. These recorded values were the extreme variation measured at the end of long counting times which occurred the last days of depuration; at the beginning, average increase temperature and decrease of pH were negligible (+2º C and -0.2 respectively).

2.4. Data treatment and statistical analysis

Depuration of radiotracers was expressed as the percentage of remaining radioactivity [(radioactivity at time t divided by the initial radioactivity measured in the organism at the beginning of the depuration period; following methods developed in Warnau et al. (1996)]. The depuration kinetics of Ag were best fitted using a two-component exponential model:

$$A_t = A_{0s} x\ e^{-k_{es}\ x\ t} + A_{0l} x\ e^{-k_{el}\ x\ t}$$

where A_t and A_0 are the remaining activities (%) at time t (d) and 0, respectively; k_e is the depuration rate constant (d^{-1}). “s” and “l” subscripts are related to the short- and long-lived component, respectively. The “s” component represents the depuration of the radiotracer fraction that is weakly associated with the organisms and rapidly eliminated (i.e. proportion associated with the faeces). The “l” component describes the depuration of the radiotracer fraction that is actually absorbed by the organism and eliminated slowly. The long-lived component allows estimating the assimilation efficiency (AE) of the radiotracer ingested with food (AE = A_{0l}). When depuration of the assimilated fraction of Co and Zn elements was extremely slow, the long-term depuration rate constant (k_{el}) might not be significant different from 0, then $T_{b1/2l}$ tends $+\infty$ and thus the “l” component of the model could therefore be simplified and replaced by a constant (as shown by Pouil et al. 2016). The equation becomes:

$$A_t = A_{0s} x\ e^{-k_{es}\ x\ t} + AE$$
$$\text{with } A_{0l} = AE$$

For the short-lived component, a biological half-life can be calculated ($T_{b1/2}$) from the corresponding depuration rate constant according to the relation $T_{b1/2s} = \ln 2/k_{es}$. Model constants and their statistics were estimated by iterative adjustment of the model and Hessian matrix computation, respectively, using the non-linear curve-fitting routines in the Statistica® software 7.0.

Comparison of assimilation of metals among the different experimental conditions was performed using two-way ANOVA on k_{es} and AE calculated for each individual turbot (the best fitting model obtained for the entire set of turbots was applied to individuals; Zar 1996). For Co, two individuals per condition with an insufficient initial activity (i.e. activity measured 2 hours after the radiolabelled feeding) have been excluded from statistical analysis. The level of significance for statistical analyses was always set at α=0.05. All the statistical analyses were performed using R software 3.0.1 (R Core Team 2014).

3. RESULTS

In order to evaluate whether different abiotic factors (i.e. temperature and pH) affect metal assimilation in the juvenile turbot *Scophthalmus maximus*, depuration kinetics of two essential (Co and Zn) and one non-essential metals (Ag) were followed after a pulse-chase feeding, using radiolabelled shrimp. During the whole experimental period (i.e. 8 weeks of acclimation to the targeted temperature and pH values and 3 weeks of depuration) where the fish were exposed to four different conditions (combinations of two temperatures and two pH; see the Material and methods section), only a limited growth of the individuals was measured and no mortality was recorded. Before the pulse-chase feeding of the fish, the activity level of each metal in the shrimps was measured: the average activities (per wet wt) were 20±5 Bq ^{57}Co g^{-1}, 213±65 Bq ^{65}Zn g^{-1} and 134±62 Bq ^{110m}Ag g^{-1}. During the entire experiment, no activity was measured in the control turbot.

Whole-body depuration kinetics of ^{57}Co, ^{65}Zn, and ^{110m}Ag in turbot were best fitted by a two-phase model (simple-exponential model and a constant; Fig. 1; R^2: 0.89 - 0.99). A large proportion (71 - 96 %) of the ingested radiotracers was associated with the short-term component for all the studied elements. This component was characterized by a very rapid loss ($T_{b1/2s}$ ranged from 0.3 to 0.7 d).

Comparison of k_{es} determined for each individual turbot indicated that, for all the elements (Co, Zn and Ag), there is no significant difference (pANOVA > 0.05, Fig. 2) independently of the pH and temperature conditions.

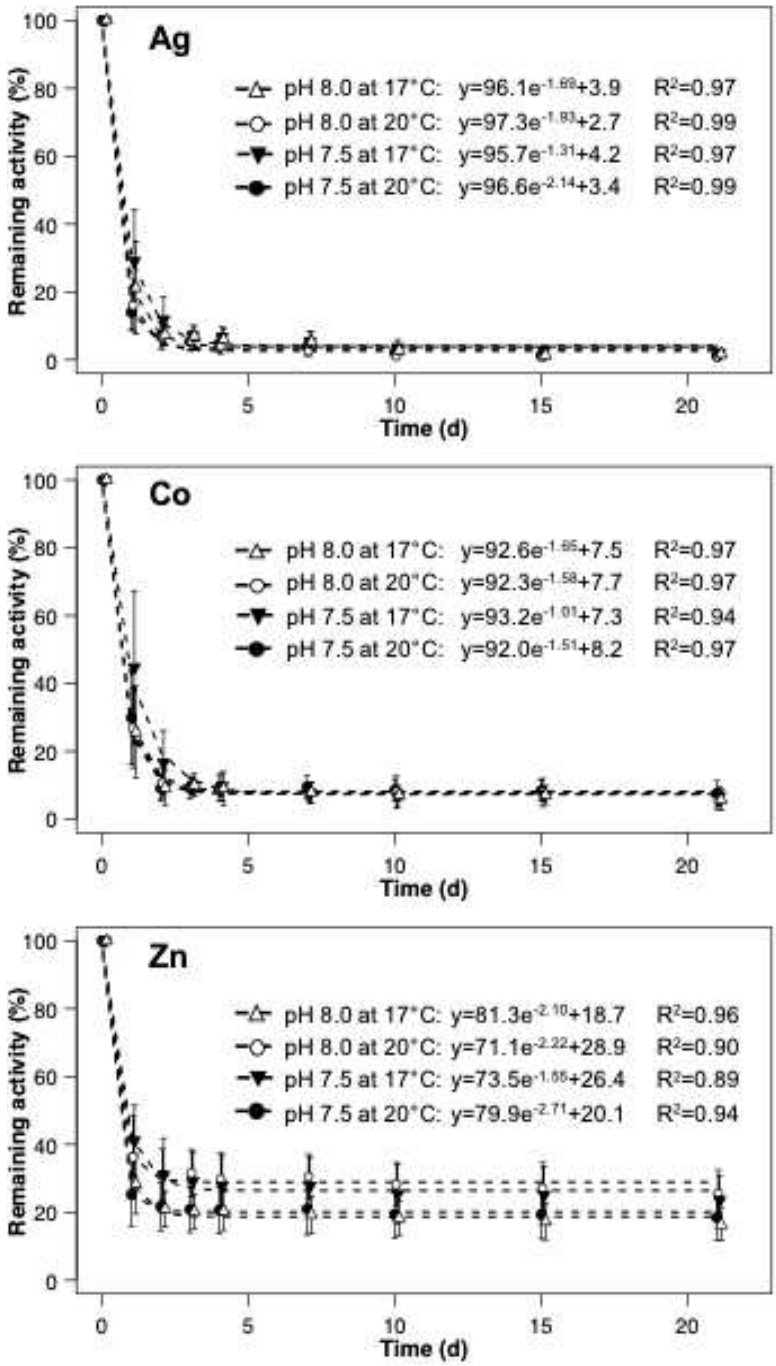

Figure 1. Influence of temperature and pH (see details of experimental conditions in Table 1) on whole-body depuration of ^{110m}Ag, ^{57}Co and ^{65}Zn in juvenile turbot (n = 6-8; percent remaining activities, means ± SD).

Estimated AEs in turbot were ranged from 19 % to 29 % for Zn whereas Co and Ag were very poorly assimilated by turbot (AE < 9 % for Co and AE < 5 % for Ag; Fig. 2). Statistical analyses carried out on individual estimated AEs revealed that neither temperature nor pH significantly affected the trophic transfer of Ag and Co in turbots (p>0.05; Fig. 2). In contrast, a significant effect of the temperature was observed between the two treatments at pH 8.0 for Zn (pANOVA = 0.03; Fig. 2) but not at the lower pH.

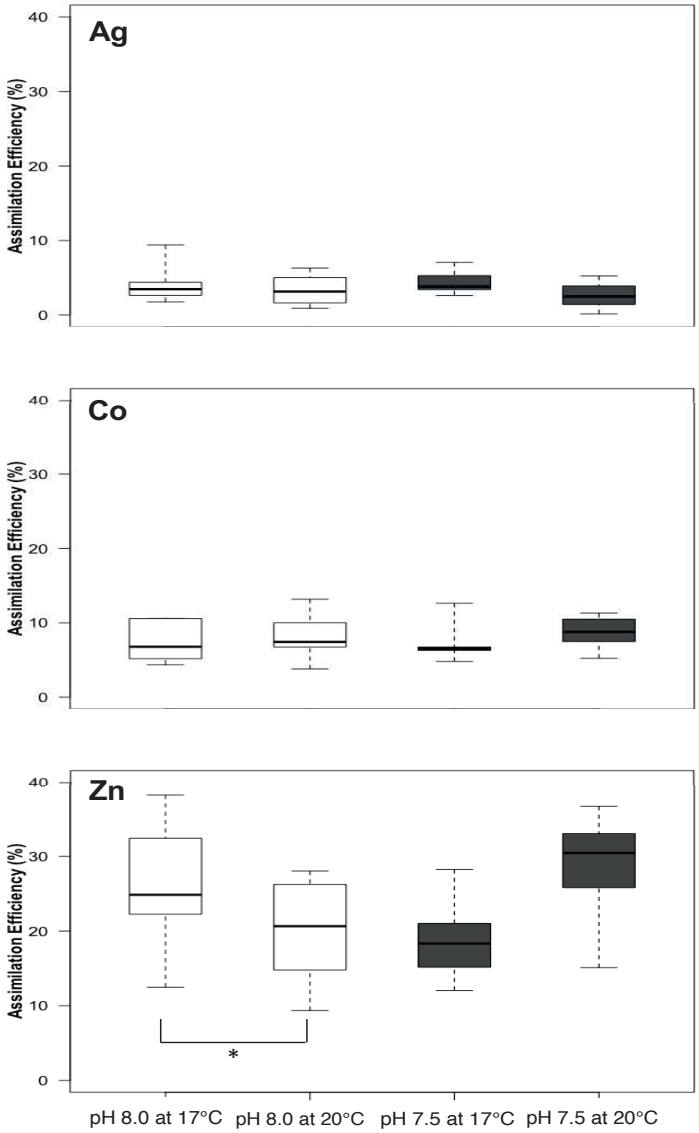

Figure 2. Comparison of assimilation efficiencies (AEs) calculated for each individual turbot from the four experimental treatments. The best fitting model obtained for the entire set of turbots (see Fig. 1) was applied to individuals.
*p < 0.05.

4. DISCUSSION

Scientists increasingly realize that single-stressor experiments may not be appropriate to assess the realistic effects of environmental variables in marine habitats (Wernberg et al. 2012). In this context, the present study analysed the combined effects of two abiotic factors on the assimilation efficiency of three metals in a coastal marine fish, the turbot. Temperature and pH are important drivers of fish physiology and are subject to important fluctuations at various temporal scales, especially in coastal environments, therefore it is important to better understand the influence of such environmental factors on the assimilation of metals in marine fish.

The main result of this study is that the temperature and pH together have limited influence on the AE of Ag and Co, while the Zn AE appears to be only influenced by temperature. At optimal pH for the turbot (pH = 8.0), increasing the seawater temperature resulted in a significantly increase of Zn AE, which could result either from: (1) the gut passage of Zn reduced at lower temperature and/or (2) less Zn was strongly retained by the body at lower temperature. In some flatfish species (i.e., the winter flounder Pseudopleuronectes americanus and the European plaice *Pleuronectes platessa*), anterior intestine is the most important body compartment involved in Zn assimilation (Pentreath 1976; Shears and Fletcher 1983). For this element, although the mechanisms of transfer from the gut lumen to the intern compartment (adsorption) are not completely elucidated yet, it seems dominated by active processes involving specific transporters (Bury et al. 2003). Temperature variations have been shown to provoke changes in the structure and the protein status of the gut cell membranes (Hazel 1995; Zehmer and Hazel 2005) or in digestive enzyme kinetics (Smit 1967; Brett and Higgs 1970) which can in turns possibly influence the active transport mechanisms of Zn and lead to the increase of Zn AE observed in this study at the highest temperature.

In the current experimental setup, AE of Zn was much higher (AE >19%) compared to the AEs for Ag and Co, both being poorly assimilated by the turbot (AE < 9 %). These results are in accordance with the literature (Zn AE: 17 - 32 %, Ag AE: 0.3 - 3 %, Co AE: 5 - 43 %; see Mathews et al. 2008; Pouil et al. 2015; Pouil et al. 2016) and could explain why temperature only influenced Zn AE. Indeed, for these other metals (Co and Ag), a poor assimilation makes difficult to highlight any significant effect.

A temperature-dependent effect on Zn assimilation has been already shown in a freshwater fish: the common carp *Cyprinus carpio* (fed with Zn contaminated prey; Van Campenhout et al. 2007). However, in marine fish, although temperature-dependent effect on metal assimilation was not identified yet, Pouil et al. (2017) have also shown, using the concentration index defined by Rouleau et al. (2000), that the intestine is involved in the absorption process of Zn in the silver moony *Monodactylus argenteus* (one of the two species studied in their work). As discussed by Van Campenhout et al. (2007), one of the possible explanations for the observed differences might be possibly explained by the higher concentration of Zn transporters in the intestine of fish exposed to higher temperatures.

In contrast to temperature, fewer studies investigated the influence of pH on the assimilation of metals by marine biota (Lacoue-Labarthe et al. 2011; Götze et al. 2014; Ivanina et al. 2015), and to the best of our knowledge, even none has investigating the influence of pH on metal trophic transfer in fish. However, in the context of the current ocean acidification, some authors have recently highlighted the effects of the partial pressure of CO_2 (pCO_2) on the digestion of fish (Pimentel et al. 2015; Rosa et al. 2016). Indeed, these authors have shown that the activity of the digestive enzymes in marine fish is dependent of the pCO_2. Usually, pH values were converted in pCO_2 from seawater carbonate chemistry. In the present study, in addition to the constant monitoring of pH, the total alkalinity has also been regularly monitored (see section Materials and Methods). Thus, pH values were converted in pCO_2. In the present paper, we have used an integrated approach for assessing the effect of pH on fish physiology using assimilation efficiency as end-point but no effect of the pH was found on the trophic transfer of the three studied metals in turbot.

Temperature and pH can interact in different ways on the physiology of marine organisms (Boyd and Hutchins 2012; Gunderson et al. 2016). In the present study, we did not find any combined effect of temperature and pH on metal assimilation. Contrasting responses regarding the bioaccumulation of metal in marine organisms have been reported in the scientific literature. Temperature can affect the bioconcentration of essential (Co, Mn, Se and Zn) and non-essential (Cd and Ag) metals with similar patterns at different pH (7.60, 7.85 and 8.10) as already demonstrated in cuttlefish eggs (Lacoue-Labarthe et al. 2009; Lacoue-Labarthe et al. 2012).

However, Belivermiş et al. (2015) have shown, in Pacific oyster *Crassostrea gigas*, that the effects of temperature on the bioaccumulation of Cd, Co and Mn were dependent of the pH conditions (7.5, 7.8 and 8.1). Even if the relations between temperature and pH effects can be complex to interpret, the absence of effect of the temperature at the lower pH (i.e. 7.5) observed in our study could be related to antagonistic effects of these abiotic factors.
Thus, further studies investigating a wider range of exposure of temperature and pH and based on a mechanistic approach will be needed to support this assumption.

5. CONCLUSIONS

This study provides new information on the assimilation efficiency of two essential (Co and Zn) and one non-essential (Ag) metals in marine fish (turbot). Our results suggest that two abiotic factors (temperature and pH) do not have a significant role in the assimilation efficiency of Co and Ag, however temperature have a slightly effect on Zn assimilation in the juvenile turbot *Scophthalmus maximus*. Based on these results, further studies should be carried out in order to cover a wider range of exposure of temperature and pH to assess precisely its effect on Zn assimilation in fish, taking into account the high variability of the responses between marine organism (Parker et al. 2011), and the adaptive capacities of organisms, especially in the context of global change where organisms are facing long-term modifications of the environmental conditions.

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ANNEXE 9

Contrasting effects of water salinity on essential metal assimilation efficiency in a euryhaline teleost, the turbot *Scophthalmus maximus*

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Contrasting effects of water salinity on essential metal assimilation efficiency in a euryhaline teleost, the turbot *Scophthalmus maximus*.

ABSTRACT: Studying trophic transfer of trace element in fish is important to understand the global trace element bioaccumulation of this taxa. While it is well known that various biotic factors can affect this transfer, recent studies start to show contrasting effect of abiotic factors on trace elements assimilation in teleost such as. Nevertheless, information regarding the influence of salinity among such factors is very limited. Therefore, the present laboratory study investigated the potential influence of salinity, a factor strongly affecting fish physiology, on the assimilation efficiency (AE) of two essential elements (Mn and Zn) in the turbot *Scophthalmus maximus* using radiotracer techniques. After a gradual acclimation of 3 weeks to 3 salinities (10, 25 and 38), 3 batches of juvenile turbots were fed with radiolabelled pellets (^{54}Mn, and ^{65}Zn). Assimilation efficiencies of these elements juvenile turbot were then determined after a 21-d depuration period. AE of Mn at the highest salinity was significantly lower than for the other conditions($p<0.05$) whereas salinity did not significantly influence of AEs of Zn ($p>0.05$). The specific physiological functions of each studied essential element and thus the distinct processes involved in their respective regulation (homeostasis) should explain the contrasting influence of water salinity for these essential elements.

Keywords: Trophic transfer, Trace elements, Salinity, Teleost, Manganese, Zinc

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1. INTRODUCTION

Environmental conditions can affect metal bioaccumulation aquatic organisms such as fish (Phillips and Rainbow 1993; Luoma and Rainbow 2005). Among environmental factors, water salinity is known to influence metal accumulation by causing (1) changes in metal speciation and therefore on their bioavailability, and (2) modifications and fish physiology especially in the osmoregulation processes (Ni et al. 2005). Salinity affects biokinetic parameters, such as the uptake rate, the assimilation efficiency (AE) or the efflux rate of some metals (see Wang 2002). Nevertheless, most of the studies that looked at the potential effects of salinity on metal accumulation were focused on waterborne metals (e.g. Zhao et al. 2001) rather than dietary ones. Thus, limited information is available about the influence of salinity on metal trophic transfer while diet is recognized as the major pathway for most of the metals bioaccumulated by fish (e.g. Xu and Wang 2002; Mathews and Fisher 2009). The turbot, *Scophthalmus maximus* (Scophthalmidae), is a demersal fish widely distributed in Western European coastal waters. This species inhabits in presenting a wide range of water salinities with its breeding usually occurring in low-salinity waters (Kuhlmann and Quantz 1980). The euryhaline nature of this species has been confirmed by Waller (1992) who reported that osmoregulatory disturbances only occurred below 6. The turbot is wild-caught in and commonly farmed (jointly reaching 72 000 tonnes in 2014; FAO 2017). Consequently, many works to date have been done on the improvement of aquaculture productivity of this valued resource, including the investigation of the influence of water salinity on growth and nutrition enhancement (e.g. Imsland et al. 2001, 2003, 2007). Furthermore, turbot became few year ago a model species for trace element studies on fish. This species has been used for example to assess the influence of numerous parameters on trace element assimilation efficiency (AE) such as the food quality (Pouil et al. 2016) and the water pH and temperature (Pouil et al. in press) or to study the phylogenetical divergences between elasmobranchs and teleost (Jeffree et al. 2010). However, the influence of salinity on the AEs of essential trace elements in turbot remains unknown.

In this context, the present study investigates the potential effects of a range of salinities on the AE of 2 essential elements (Mn and Zn) in the turbot *Scophthalmus maximus*. Radiotracer techniques were used to determine these AE and the other kinetic parameters after a single-feeding exposure to radiolabelled pellets and under controlled conditions (3 distinct salinities; 10, 25 and 38).

2. MATERIALS AND METHODS

2.1. Origin and acclimation of fish

Juvenile turbot *Scophthalmus maximus* were purchased from a fish farm (France Turbot, www.france-turbot.com) and shipped to the International Atomic Energy Agency premises in the Principality of Monaco. The fish were set in a 700-L aquarium (open circuit, water renewal: 350 L h^{-1}; 0.45µm filtered seawater; salinity: 38; light/dark: 12h/12h). Then, three weeks before the experiment, the fish were randomly placed in four 20-L aquaria (n=8) and acclimated to the target salinities (10, 25, 38). During the first days of acclimation, salinities were gradually decreased and then stabilized for 10 days before experiment. During the whole acclimation period, the fish were fed a daily ration of 1.5% of their biomass with 1.1-mm pellets (proteins: 55% and lipids: 12%; Le Gouessant, www.legouessant.com).
Salinity was measured twice per day in each aquarium using a hand-held conductivity/salinity meter, which was calibrated using conductivity standards encompassing the range of the three selected experimental waters. Furthermore, in each aquarium, pH and temperature were monitored every 15 minutes using a continuous measurement system (IKS ComputerSysteme, www.iks-aqua.com). Mean and standard error values for physico-chemical measurements are given in Table 1.

Table 1. Seawater parameters during the experiment on the assimilation of essential metals in juvenile turbots exposed to different conditions of salinity. Values are Means ± SD.

Condition	Salinity measured	Conductivity (mS cm^{-1})	pH	Temperature (°C)
Low salinity (10)	10.05 ± 0.13	16.99 ± 0.09	8.04 ± 0.04	19.60 ± 0.08
Medium salinity (25)	24.94 ± 0.16	39.27 ± 0.15	7.95 ± 0.07	19.72 ± 0.05
High salinity (38)	37.8 ± 0.05	56.98 ± 0.04	7.93 ± 0.03	19.71 ± 0.12

2.2. Experimental procedures

2.2.1. Radiolabelling of pellets

Fifteen grams of 1.1-mm pellets were dipped for 1 h in 20 mL of seawater spiked with 1.5 kBq mL^{-1} of ^{54}Mn and ^{65}Zn. Pellets were then dried for 48 h at 50°C and kept in a dry environment in order to prevent mould growth.

Preliminary tests were performed to determine the possible leakage into the water of radioisotopes from the pellets during the feeding. When food was provided, acclimated fish consumed the pellets in less than 2 min.

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2.2.2. Exposure of turbot via radiolabelled pellets

A total of 8 acclimatized turbots were randomly selected for each experimental salinity (10: 44.0±2.9g; 25: 40.0±3.8g and 38: 42.6±3.4g). Slits cut into the fins were used to facilitate individual recognition. Each experiment consisted of a single feeding of fish with radiolabelled pellets. After the labelled feeding, an additional turbot was placed in each aquarium to assess any possible radiotracer recycling from seawater due to leaching from the radiolabelled food or, later on, from fish depuration. Two hours after the 15-min feeding, individual fish were whole-body γ-counted alive and then replaced in the same aquarium to follow subsequent metal depuration. All the fish (including control individual of each condition) were regularly radioanalysed to follow the radiotracer depuration kinetics over 21 days.

After the depuration period, 4 individuals per condition were dissected in 7 compartments: (1) the digestive tract, (2) the gall bladder, (3) the head (including gills), (4) the kidney, (5) the liver, (6) the 4 muscles (without dorsal skin) and (7) the remaining tissues (including ventral skin, skeleton, fins, heart and muscle residues) and were separated, weighed (wet wt) and radio-analysed to determine the radiotracer body distribution.

2.3. Radiotracers and Counting

Radiotracers of high specific activity were purchased from Polatom, Poland (^{54}Mn as $MnCl_2$ in 0.5M HCl, $t_{1/2}$ = 312 days; ^{65}Zn as $ZnCl_2$ in 0.1M HCl, $t_{1/2}$ = 244 days). The radioactivity of the tracers was measured using a high-resolution γ-spectrometer system composed of 5 Germanium - N or P type - detectors (EGNC 33-195-R, Canberra® and Eurysis®) connected to a multi-channel analyser and a computer equipped with a spectra analysis software (Interwinner 6, Intertechnique®). The radioactivity in living organisms and samples was determined by comparison with standards of known activity and of appropriate geometry (calibration and counting). Measurements were corrected for background and physical radioactive decay. Living organisms were placed in counting tubes (diameter: 160 mm, height: 80 mm) filled with 500 mL of clean seawater (at the appropriated conditions of salinity) during the counting period. The counting time was adjusted to obtain a propagated counting error less than 5% (e.g. Rodriguez y Baena et al. 2006) for a maximum of 20 min. As already described by (Pouil et al. 2017) tests were performed prior to the experiment, where fish were placed in similar counting conditions in order to observe their behaviour, i.e. in a counting box for 20 min in the dark. Dissolved O_2 concentration was monitored throughout these tests and was always > 3 mg L^{-1}. No alteration in organism health or behaviour was observed during the tests and then, the experiment.

2.4. Data treatment and statistical analysis

Depuration of radiotracers was expressed as the percentage of remaining radioactivity (radioactivity at time t divided by the initial radioactivity measured in the organism at the beginning of the depuration period; following methods developed in Warnau et al. (1996).

The depuration kinetics of Mn and Zn were best fitted using a two-component exponential model:

$$A_t = A_{0s} x\, e^{-k_{es}\, x\, t} + A_{0l} x\, e^{-k_{el}\, x\, t} \quad (1)$$

where A_t and A_0 are the remaining activities (%) at time t (d) and 0, respectively; k_e is the depuration rate constant (d^{-1}). "s" and "l" subscripts are related to the short- and long-lived component, respectively. The "s" component represents the depuration of the radiotracer fraction that is weakly associated with the organisms and rapidly eliminated (i.e. proportion associated with the faeces). The "l" component describes the depuration of the radiotracer fraction that is actually absorbed by the organism and eliminated slowly (Hubbell et al. 1965; Reichle 1967; Whicker and Schultz 1982). The long-lived component allows estimating the assimilation efficiency (AE) of the radiotracer ingested with food (AE = A_{0l}; see e.g. Warnau et al. 1996; Metian et al. 2010). For the two components, biological half-life can be calculated ($T_{b1/2}$) from the corresponding depuration rate constant according to the relation $T_{b1/2} = \ln 2/k_e$. Model constants and their statistics were estimated by iterative adjustment of the model and Hessian matrix computation, respectively, using the non-linear curve-fitting routines in the Statistica® software 7.0.

Statistical comparisons between the 3 different salinity experiments were conducted using individual depuration kinetics of each element: individual parameters (k_{es} and AE) were obtained using the best-fitting model at the global scale (Eq. 1) to the data of each individual. Then, differences between these parameters were tested using Kruskal-Wallis and Siegel and Castellan non-parametric tests (Zar 1996). The level of significance for statistical analyses was always set at α=0.05. All the statistical analyses were performed using R software 3.0.1 (R Core Team 2014).

3. RESULTS

In order to evaluate how salinity affects essential metal assimilation in the juvenile turbot *Scophthalmus maximus*, depuration kinetics of Mn and Zn were followed after a pulse-chase feeding, using radiolabelled pellets. During the whole experimental period (i.e. 3 weeks of acclimation to the targeted salinity values and 3 weeks of depuration) where the fish were exposed to a gradient of salinities (see the Material and methods section).

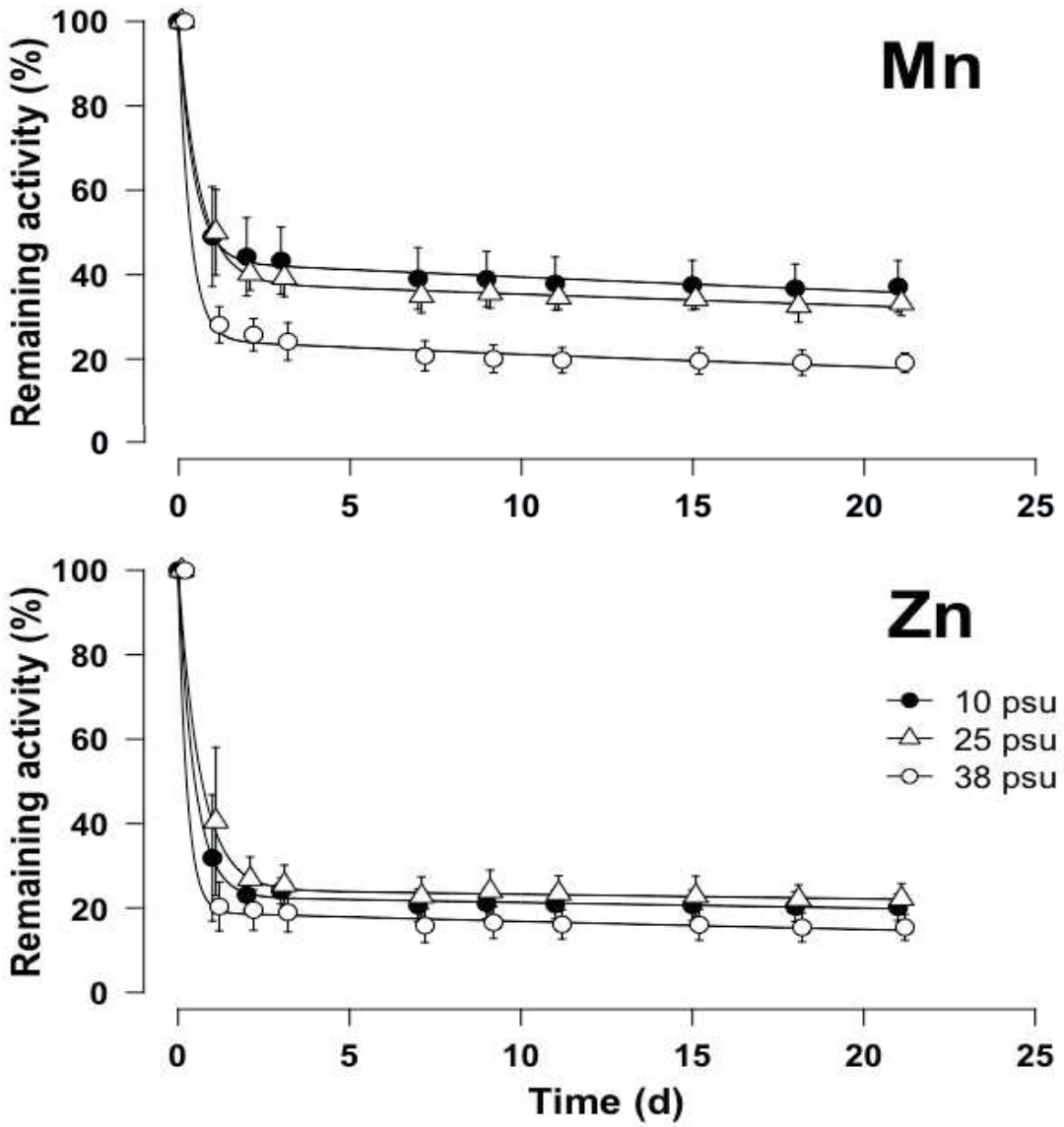
Table 2. Estimated depuration kinetic parameters of ^{54}Mn, and ^{65}Zn in turbot acclimated to three salinity conditions (10, 25 and 38; n = 7 per treatment) and exposed to the radiotracers during a single-feeding with radiolabelled pellets. After the radiolabelled feeding, turbot were maintained for 21d in unspiked seawater at the given salinity. Depuration parameters: A_{0s} and A_{0l} (=AE): activity (%) lost according to the short- and the long- lived exponential component, respectively; ke: depuration rate constant (d^{-1}); $T_{b1/2}$: biological half-life (d) [$T_{b1/2}=\ln 2/k_e$]; ASE: asymptotic standard error; R^2: determination coefficient. Probability of the model adjustment: $^{NS}p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Salinity	Short-term			Long-term			R^2
	$A_{0s} \pm ASE$	$k_{es} \pm ASE$	$T_{b1/2s} \pm ASE$	A_{0l} (=AE) $\pm$ ASE	$k_{el} \pm ASE$	$T_{b1/2l} \pm ASE$	
^{54}Mn							
10	56.93 ± 3.44***	2.14 ± 0.53***	0.32 ± 0.08	43.05 ± 2.24***	0.008 ± 0.004***	78.93 ± 37.75	0.88
25	61.63 ± 2.20***	1.62 ± 0.18***	0.43 ± 0.05	38.35 ± 1.51***	0.008 ± 0.003***	85.92 ± 32.86	0.95
38	75.44 ± 1.61***	2.91 ± 0.40***	0.24 ± 0.03	24.56 ± 1.03***	0.015 ± 0.004***	46.24 ± 11.17	0.98
^{65}Zn							
10	77.29 ± 2.64***	2.12 ± 0.28***	0.33 ± 0.04	22.71 ± 1.68***	0.006 ± 0.006^{NS}	+∞	0.95
25	75.63 ± 3.27***	1.55 ± 0.20***	0.47 ± 0.06	24.39 ± 2.20***	0.005 ± 0.007^{NS}	+∞	0.93
38	81.02 ± 1.82***	3.90 ± 1.12***	0.18 ± 0.05	18.98 ± 1.12***	0.012 ± 0.005***	57.64 ± 24.16	0.98

Only a limited growth of the individuals was recorded and no mortality occured. Before the single-feeding, the activity level of Mn and Zn was measured in the pellets: 2202 ± 158 Bq ^{54}Mn g^{-1} and 2394 ± 167 Bq ^{65}Zn g^{-1}. During the entire experiment, controls confirmed the exclusive foodborne exposure of the fish to radiotracers (no activity was recorded in the control turbot).

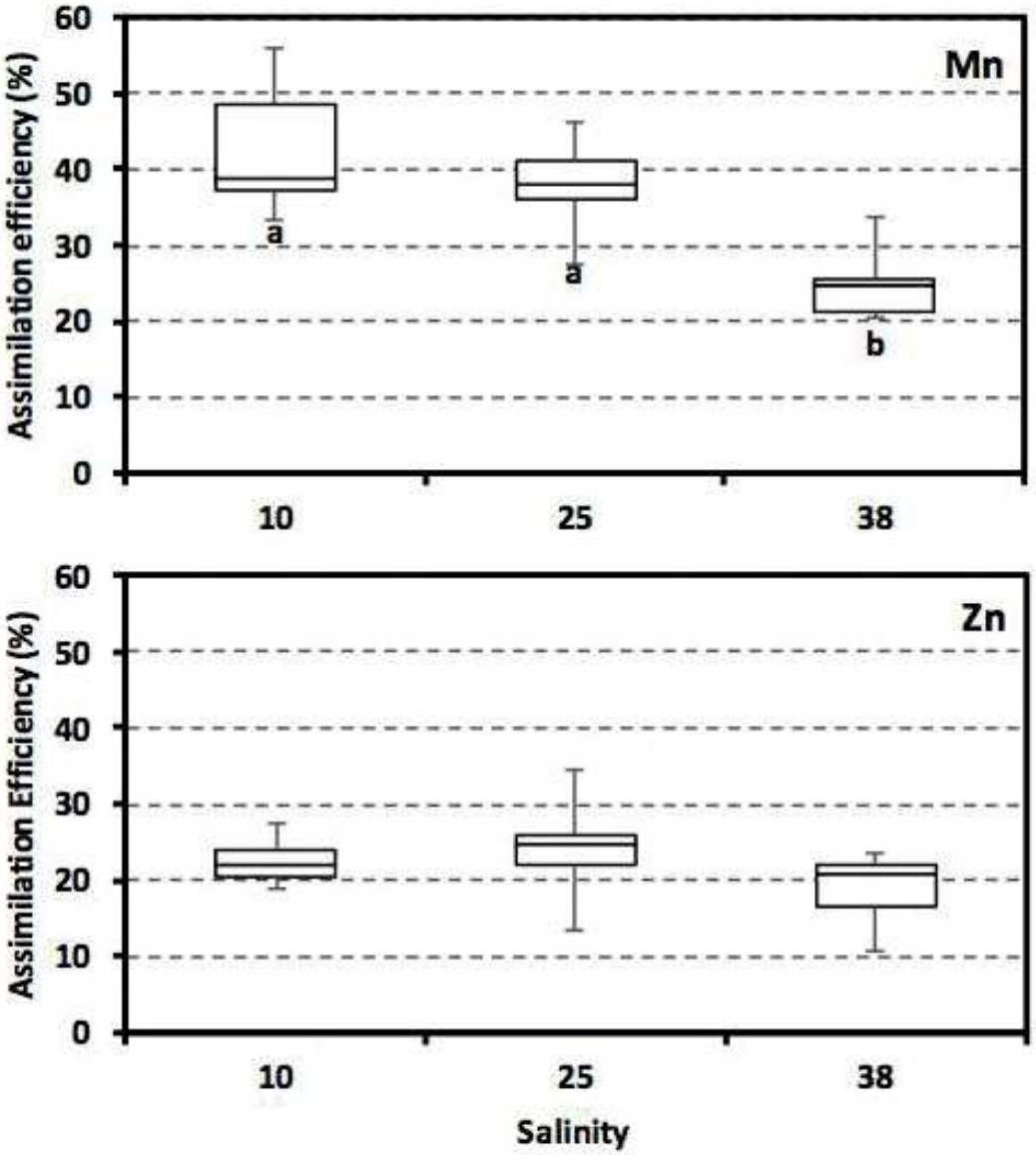
Whole-body depuration kinetics of ^{54}Mn and ^{65}Zn in turbot were best fitted by a two-phase model (Fig. 1; R^2: 0.88 - 0.98). A large proportion (57 - 81 %, Table 2) of the ingested radiotracers was associated with the short-term component for all the studied elements. This component was characterized by a very rapid loss ($T_{b1/2s}$ < 1 d, Table 2). Comparison of short-term depuration rate constants (k_{es}) determined for each individual turbot indicated that there was no significant difference for both studied elements (Mn and Zn), ($p > 0.05$, Fig. 2) independently of the salinity conditions.

Figure 1. Influence of salinity on whole-body depuration of ^{54}Mn and ^{65}Zn in juvenile turbot (n = 7; % remaining activities, means ± SD). Parameters and statistics of depuration kinetics are given in Table 2.



Estimated AEs in turbot ranged from 25 % to 43 % for Mn whereas Zn was less assimilated by turbot (AE < 25 %, Fig. 1). Statistical analyses carried out on individual estimated AEs revealed that salinity affected the trophic transfer of Mn with a significantly lower AE at the highest salinity ($p<0.05$; Fig. 2). In contrast, no significant effect of the salinity was observed for AEs of Zn ($p>0.05$; Fig. 2)

Figure 2. Comparison of assimilation efficiencies (AEs) of Mn and Zn calculated for each individual turbot acclimated to three salinity conditions. The best fitting model obtained for the entire set of turbots (see Fig. 1 and Table 2) was applied to individuals. Letters denote significant differences between the salinity conditions.



Post-feeding distribution of Mn and Zn in turbots at the end of the 21-d depuration period is shown in Table 3, for the gradient of salinity. Similar patterns of Mn and Zn distribution among compartments were observed in turbots exposed to the three salinities ($p > 0.05$): distribution among the body compartments systematically ranked according to the following decreasing order (Table 3): remaining tissues (i.e. remaining skin, skeleton, fins, heart and muscle residues; 44-50%) > head (33-45%) >> muscles (4-10%) > digestive tract (<1-9%) >> liver (<2%) > kidney (<1.4%) >> gall bladder (< 0.2%).

Concentration Index (I_C) among body compartments did not differ in juvenile turbots exposed to the three different salinities ($p > 0.05$). For Mn, highest I_C were found in the head (1.3-1.5) whereas highest Zn I_C were calculated in the liver and the kidney (2.7-3.6) and, in a lesser extent in the digestive tract (2.1-2.4).

Table 3. Distribution (%) and Concentration Index (I_C) of ^{54}Mn, and ^{65}Zn in juvenile turbots acclimated to three salinity conditions (10, 25 and 38), exposed to the radiotracers during a single-feeding with radiolabelled pellets and then maintained for a 21-d depuration period in unspiked seawater at the given salinity. Values are means ± SD (n=4).

Compartment	Low salinity (10)		Medium salinity (25)		High salinity (38)	
	Mn	Zn	Mn	Zn	Mn	Zn
Distribution (%)						
Digestive tract	0.59 ± 0.17	6.93 ± 0.44	0.52 ± 0.13	6.53 ± 0.58	1.02 ± 0.25	8.76 ± 0.94
Gall bladder	0.01 ± 0.01	0.15 ± 0.07	0.02 ± 0.00	0.15 ± 0.07	0.03 ± 0.02	0.13 ± 0.04
Head	45.39 ± 2.14	33.42 ± 1.45	46.38 ± 0.52	34.12 ± 3.06	43.79 ± 2.61	33.72 ± 0.80
Kidney	0.08 ± 0.03	1.35 ± 0.32	0.07 ± 0.02	1.20 ± 0.41	0.13 ± 0.05	1.23 ± 0.30
Liver	0.23 ± 0.18	1.73 ± 0.56	0.18 ± 0.03	1.92 ± 0.23	0.30 ± 0.08	1.88 ± 0.39
Muscles	4.00 ± 0.58	10.37 ± 1.91	4.11 ± 1.06	10.78 ± 0.85	4.71 ± 0.54	10.14 ± 1.04
Remaining tissues*	49.70 ± 1.78	46.05 ± 2.50	48.72 ± 1.11	45.31 ± 3.51	50.01 ± 2.24	44.14 ± 2.14
Concentration Index (I_C)						
Digestive tract	0.19 ± 0.06	2.16 ± 0.23	0.16 ± 0.04	1.98 ± 0.18	0.28 ± 0.07	2.39 ± 0.37
Gall bladder	0.06 ± 0.03	0.67 ± 0.34	0.10 ± 0.04	0.92 ± 0.47	0.17 ± 0.08	0.96 ± 0.41
Head	1.46 ± 0.16	1.07 ± 0.09	1.49 ± 0.12	1.09 ± 0.05	1.31 ± 0.09	1.01 ± 0.08
Kidney	0.22 ± 0.11	3.63 ± 0.62	0.19 ± 0.05	3.22 ± 0.36	0.37 ± 0.11	3.45 ± 0.65
Liver	0.34 ± 0.19	2.74 ± 0.28	0.25 ± 0.06	2.66 ± 0.50	0.51 ± 0.12	3.21 ± 0.61
Muscles	0.19 ± 0.03	0.50 ± 0.06	0.19 ± 0.04	0.50 ± 0.02	0.25 ± 0.04	0.53 ± 0.02
Remaining tissues*	1.14 ± 0.05	1.06 ± 0.05	1.14 ± 0.07	1.06 ± 0.07	1.17 ± 0.03	1.03 ± 0.04

*The remaining tissues included remaining skin, skeleton, fins, heart and muscle residues

4. DISCUSSION

Salinity is one of the main important environmental variable in coastal marine ecosystems. Fish have thus to deal to these potential hypo- and hypertonic environments in order to maintain their blood osmolality constant. To do this, their main osmoregulatory adaptations include modifications of (1) their of drinking rate (digestive tract) (2) the production of urine (excretory system) (3) an active excretion or retention of ions. All these active mechanisms compensate for example the diffusive ion invasion and osmotic water loss in high salinity waters (e.g. Lorin-Nebel et al. 2006). With the occurrence of such physiological regulation processes, past studies have already shown that salinity influences the bioaccumulation in fish exposed for a short period of time (i.e. 48h) to dissolved trace elements such as Cd, Cs, Se and Zn (e.g. Zhao et al. 2001; Ni et al. 2005).

To the best of our knowledge, only one study investigated the effect of salinity on trace elements trophic transfer in fish (Cd, Se and Zn; Ni et al. 2005). Although AEs of the three trace elements (Cd, Se and Zn) in the mudskipper *Periophthalmus modestus* were not influenced by different water salinities (Ni et al. 2005), our study demonstrated it is not always true in turbot. Indeed, AEs of Zn estimated in juveniles turbot at the three different salinities were similar but Mn AEs in juveniles turbot were affected at the highest salinity (38) showing that the effects of salinity are metal dependent.

The physiology adaptation of the fish to a gradient of salinity may explain the different AE Mn at high salinity and the constant Zn AE observed in the experiments. Again, fish have to osmoregulate in order to maintain their internal ion concentrations when exposed to higher salinity. They thus can minimize the ion entrance and/or maximize their subsequent elimination. Biokinetic data, especially depuration rate constants, allow getting a better insight of these osmoregulation processes and are likely to explain differences between AE for the same element at different salinity or between the element. Indeed, for a same element (i.e. Mn or Zn) the slope of long-term component curves for each salinity condition was similar. We can thus assumed that the contrasting results observed between AE of Mn and Zn with salinity conditions are related to the occurrence of differences between the two studied elements within the first days of depuration, which are mainly characterized by the absorption (viz. process during which ions can pass from the intestine lumen to the internal body compartment; Wang and Fisher 1999).

Zn is one of the main important essential trace elements for fish due to its structural and catalytic role in more than 300 proteins and it serves as a cofactor in many enzyme systems playing a vital role in lipid, protein, and carbohydrate metabolism (Watanabe et al. 1997; Bury et al. 2003). Thus, this element is directly involves in growth, reproduction, development and immunity in fish (Tacon 1987; Watanabe et al. 1997). Although the mechanisms of Zn transfer from the gut lumen to the internal compartment (adsorption) are not fully elucidated yet, it seems to be dominated by active processes involving specific transporters (Bury et al. 2003). This element is, among other things, accumulated into cells through specific channels (ZIP family ; Bury et al. 2003; Hogstrand 2011). However, as concentrations can easily be toxic, steady-state cytosolic Zn concentration is controlled by efflux transporter of the ZnT family that transport Zn from the cytosol outside the cells (Bury et al. 2003; Hogstrand 2011). An excess of Zn can be excreted mainly via the bile, intestinal sloughing (Handy 1996) or the gills (Hardy et al. 1987). Thus, at both organismal and cellular levels Zn status in fish is tightly controlled by active mechanisms. Conversely to short-term exposure experiments (e.g. 48h ; Ni et al. 2005), where salinity can slightly affect the bioaccumulation of dissolved Zn, we assumed that, in a long-term experiments (i.e. several weeks), this strong Zn homeostasis can easily takes places. This mechanisms could explain the consistency observed in this study in the Zn AEs (i.e. 19-24%) despite the variations of salinity. It is not rare to see the absence of effect of abiotic factors on Zn AE in fish. For example, AE of Zn in the clownfish *Amphiprion ocellaris* and the turbot *S. maximus* did not vary when maintained at two different pH (7.5 and 8.0). At present, only temperature appear to influence AE of Zn: its transport across the apical membrane of the intestine is temperature-dependent in the rainbow trout *Oncorhynchus mykiss* (Glover et al. 2003) and this can explained the variability of Zn AE observed in other fish species exposed to different temperatures (e.g. Pouil et al. in 2017; Van Campenhout et al. 2007).

Mn is necessary for the normal functioning of brain and for lipid and carbohydrate metabolism. This element has key role as cofactor for enzymes and as structural element of metalloenzymes. As a cofactor or component of several key enzyme systems, manganese is also directly involved in bone formation, regeneration of red blood cells and reproduction (Tacon 1987; Watanabe et al. 1997).

Although the mechanisms of transport and absorption of Mn from food in fish are poorly reported, we can assumed the transport of Mn, at realistic concentrations, is dominated by passive ways as it has been demonstrated for other taxa with Mn entry via routes serving for the uptake of major ions such as Ca channels (Fukuda and Kawa 1977; Anderson 1979; Fasolato et al. 1993). Lowering the salinity and thus the competition of free Ca2+ and Mg2+ ions for Ca channels may enhance the influx and bioaccumulation of trace elements such as Mn (Langston and Bryan 1984). Therefore we can assumed that (1) the relative importance of the passive way for Mn transportation and (2) the presumably less tight homeostasis for Mn compared to Zn can explain the significant differences of Mn AEs in juvenile turbot at the different salinity conditions. Nevertheless, further studies based on a mechanistic approach will be needed to support this assumption.

5. CONCLUSION

In summary, our study showed that salinity differently impacted the AE of Mn and Zn, two essential elements in the juveniles turbots, although this species is euryhaline (i.e. species with a large osmoregulation capacity). Indeed, Mn AE was higher at lower salinities (10 and 25) than at high salinity (38) while Zn AE was not affected by the salinity conditions. These differences were likely caused by the physiological changes instead of the change in metal speciation. After the 21-d depuration period, organotropism was the same for Mn and Zn in turbot acclimated to the three salinities. Given the evidence that food is the major pathway of trace element bioaccumulation in marine fish, salinity would be one of the most important environmental variable driven the trophic transfer of trace elements in coastal aquatic ecosystems.

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ANNEXE 10

Investigations of temperature and pH variations on metal trophic transfer in turbot (*Scophthalmus maximus*) fish

Pouil S[1,2], Oberhänsli F[1], Bustamante P[2], Metian M[1] (2017) Dietary Zn and the subsequent organotropism in fish: no influence of food quality and environmental conditions (pH and temperature). *Chemosphere*, 183C: 503-509.

ABSTRACT: Studying dietary metal transfer kinetics is essential to gain a better understanding in global metal accumulation rates and its impacts in marine fish. While there exists a solid understanding on the influence of various biotic factors on this transfer, metal assimilation in fish might be also affected by abiotic factors, as has been observed in marine invertebrates. The present study therefore aim to understand the potential effects of two climate-related master variables, temperature and pH, on the assimilation efficiency (AE) of essential (Co and Zn) and non-essential (Ag) metals in the turbot *Scophthalmus maximus* using radiotracer tools. Juvenile turbots were acclimated for 8 weeks at two and two temperatures (17° C and 20° C) and pH (7.5 and 8.0) regimes, under controlled laboratory conditions and then fed with radio-labelled shrimp (^{57}Co, ^{65}Zn and ^{110m}Ag). Assimilation efficiencies of Co and Ag in juvenile turbot, determined after a 21-d depuration period, were not affected by pre-exposition to the different environmental conditions. In contrast, temperature did significantly influence Zn AE ($p<0.05$), while pH variations did not affect the assimilation of any of the metals studied. In fact, temperature is known to affect gut physiology, specifically the membrane properties of anterior intestine cells where Zn is adsorbed and assimilated from the ingested food. These results are relevant to accurately assess the influence of abiotic factors in AEs of metals in fish as they are highly element-dependant and also modulated by metabolic processes.

Keywords: Metal trophic transfer, Trace elements, Teleost, Ocean acidification, Global warming

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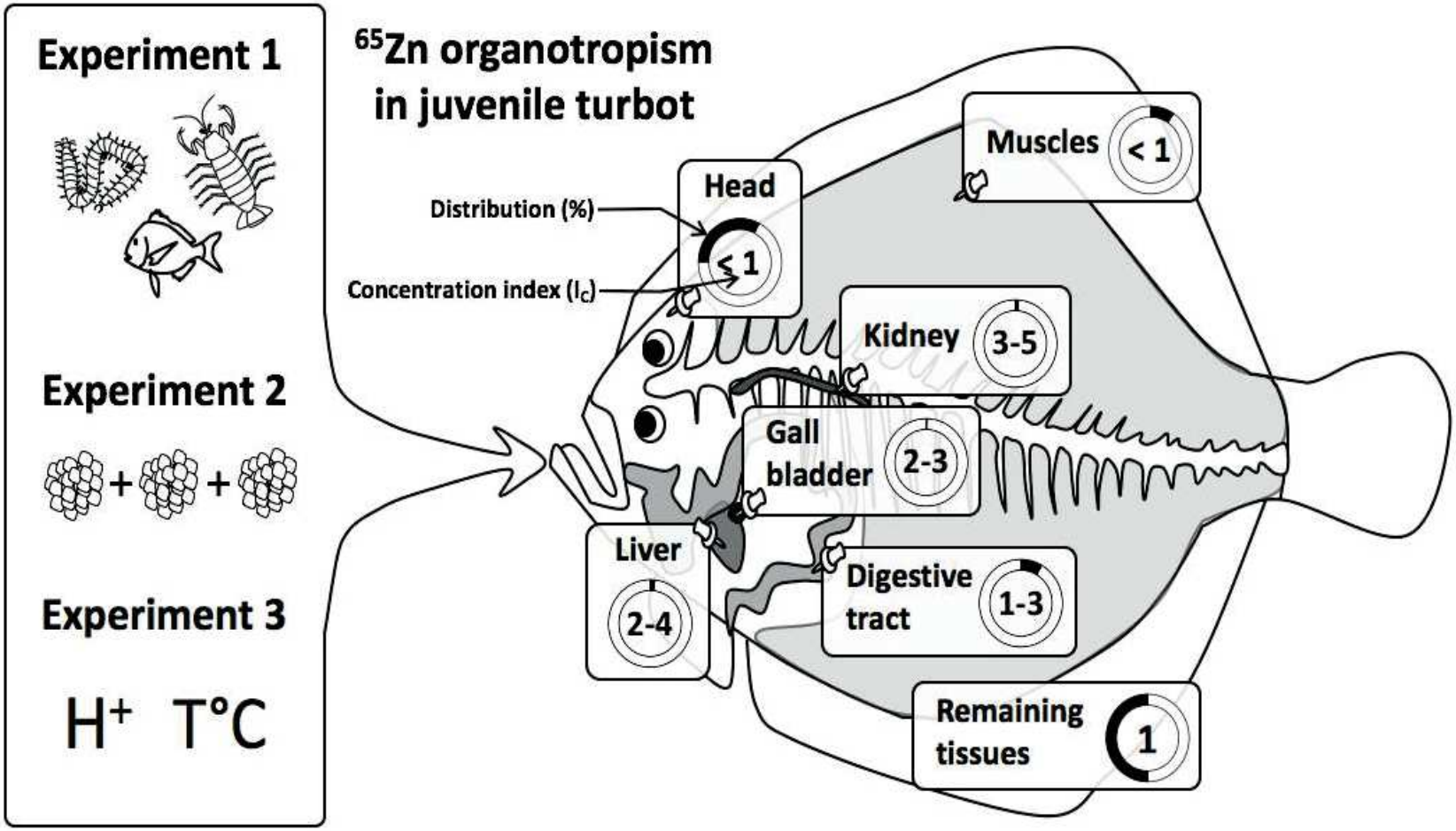
GRAPHICAL ABSTRACT:



1. INTRODUCTION

Zinc (Zn) is a crucial microelement for living organisms, including fish (Watanabe et al., 1997). Indeed, it is an essential element for fish playing a vital role in lipid, protein, and carbohydrate metabolism but it also can be potentially toxic at higher concentrations in the environment (Spry and Wood, 1985; Eisler, 2009; Hogstrand, 2011). Due to these two opposite aspects, the accumulation of Zn by fish has been extensively studied (e.g. Spry et al., 1988; Clearwater et al., 2002; Van Campenhout et al., 2007). Fish have mainly two sources of Zn uptake: the surrounded water and their diet (Bury et al., 2003) and it is now well-identified that food is the major pathway of Zn intake in fish especially at low ambient Zn concentrations (Spry et al., 1988). Indeed, as early as the 1980s, Willis and Sunda (1984) have highlighted that food ingestion represented up to 82% of total Zn accumulation for two species of fish *Gambusia affinis* and *Leiostomus xanthurus* fed with radiolabelled brine shrimp. More recently, Xu and Wang (2002) and Mathews and Fisher (2009) have confirmed using biokinetic models that Zn in teleosts and elasmobranchs was predominantly bioaccumulated from the dietary source. Numerous experimental studies have focused on the study of Zn trophic transfer in fish (e.g. Pentreath, 1976; Milner, 1982; Zhang and Wang, 2007; Pouil et al., 2016) but the understanding of the physiological mechanisms governing the assimilation of this element is still very limited. It is known that Zn trophic transfer may, in some cases, be affected by the type of dietary supply or by the environmental conditions (e.g. Van Campenhout et al., 2007). The observed effects are usually related to the differential Zn subcellular fractionation in the food items or the chemical forms of this element that can be affected the bioavailability of dietary Zn and, by extension the ways of it storage in the organisms (e.g. Zhang and Wang, 2007; Pouil et al., 2016). At present, however, there is little known about how Zn is stored in fish.

The organotropism of Zn (viz. whole-body distribution of Zn) can provide a better understanding of the variability observed in the Zn trophic transfer under different conditions. Measurements carried out in the field highlighted that a series of factors (trophic habits, gender and season) can affect Zn body burden and its distribution among organs and tissues (Andres et al. 2000; Kojadinovic et al. 2007; Dural et al. 2007). It is, however, always complex with an in-situ approach to establish unambiguous trends because origins of Zn intakes cannot be properly identified and the life-history traits of the organisms, that may play a role in its body distribution are usually unknown (Gray, 2002).

The experimental approach, in controlled conditions, is a relevant option to unequivocally assess Zn organotropism, especially using radiotracer techniques (Warnau and Bustamante, 2007). In the present work, we experimentally assessed the dietary Zn organotropism in juvenile turbot *Scophthalmus maximus* single-fed with ^{65}Zn radiolabelled food and exposed to different experimental conditions. Thus, after a 21-d depuration period, the fish from the different experimental were dissected in order to (1) understand the long-term storage of dietary Zn in fish through the measurement of the body concentration and distribution of this element and (2) investigate potential effects of some environmentally relevant factors: food quality, feeding frequency, seawater pH and temperature on Zn organotropism. These factors were selected for their previously-shown potential to affect the trophic transfer of Zn in fish and/or their physiology (e.g. Van Campenhout et al., 2007; Pouil et al. 2016, Pouil et al. in press). The food items used were natural prey of the juvenile turbot (i.e. crustaceans, fish and polychaetes; Florin and Lavados, 2010; Sparrevohn and Støttrup, 2008) and compounded pellets used in aquaculture for this species. The values of pH (7.5 and 8.0) and temperature (17°C and 20°C) were chosen based on the optimal temperature for food conversion efficiency in juvenile turbot (Imsland et al., 2001) and the current projections provided by the literature for the next two centuries (ΔT°C: +3° C and ΔpH: -0.5; IPCC 2013; Orr et al. 2005).

2. MATERIALS AND METHODS

2.1. Origin and acclimation of organisms

Juvenile turbots *Scophthalmus maximus* were purchased from a fish farm (France Turbot, France) and shipped to the International Atomic Energy Agency premises in the Principality of Monaco. Fish were acclimated to laboratory conditions for a minimum of 3 weeks (open circuit; 700-L tank, water renewal: 300 L h^{-1}; 0.45µm filtered seawater; salinity: 38.; temperature: 17 ± 2 °C; pH: 8.0 ± 0.1; light/dark: 12 h/12 h). During the acclimation period, the fish were fed a daily ration of 2% of their biomass with 1.1-mm pellets (proteins: 55% and lipids: 12%; Le Gouessant, France). Natural prey of juvenile turbots i.e., crustacean (common prawn Palaemon serratus), fish (seabream Sparus aurata), and ragworm (estuary ragworm Hediste diversicolor), were respectively purchased from French suppliers (Poissons du Soleil, Poissons Vivants and Normandie Appâts). All prey were acclimated to the same laboratory conditions as the turbots for a minimum of 2 weeks prior to experiments.

2.2. Radiolabelling and counting

For Zn radiolabelling, radiotracer of high specific activity was purchased from Polatom, Poland (^{65}Zn as $ZnCl_2$ in 0.1M HCl, $t_{1/2}$ = 244 d). The use of gamma-emitting isotope allowed accurate measurements using environmentally realistic zinc concentrations. All the natural prey (crustacean, fish and polychaete) were exposed to dissolved ^{65}Zn for 1 to 3 weeks following the protocol described by Pouil et al. (2016). For radiolabelling, pellets were dipped in ^{65}Zn radiolabelled seawater for 1 h (ratio: 0.32 g dry wt mL^{-1}). Then, radioactive pellets were dried and stored in dry conditions (for more details see Pouil et al., in press).

The radioactivity in prey, fish and dissected samples was measured using a high-resolution γ-spectrometer system composed of 4 Germanium - N or P type - detectors (EGNC 33-195-R, Canberra® and Eurysis®) connected to a multi-channel analyser and a computer equipped with a spectra analysis software (Interwinner 6, Intertechnique®). The radioactivity in living turbot and samples from dissections was determined by comparison with standard of known activity and of appropriate geometry (calibration and counting; see Pouil et al. in press; Pouil et al. 2016). The counting time was adjusted to obtain a propagated counting error less than 5% (e.g. Rodriguez y Baena et al., 2006).

2.3. Zn organotropism in juvenile turbots

2.3.1. General experimental approach

The following conditions were applied to all the Zn organotropism experiments, unless stated otherwise. The experiments were performed in 20-L or 70-L aquaria (open-circuit, same conditions that during acclimation, see section 2.1). Two weeks before the start of the experiment, juvenile turbots were randomly transferred from the acclimation tank to the aquaria used for experiments and kept 2 d without food until the beginning of the experiment. Slits cut into the fins were used to facilitate individual recognition. Two hours after the unique (Experiments 1 and 3) and the last (Experiment 2) radiolabelled feeding, all the turbots from the different experiments were counted to determine the ingested ^{65}Zn activity, estimated the ingestion rate for each individual and the stable Zn quantity eaten (see Pouil et al., 2016 for Zn stable analysis methodology for the different food items).

After a 21-d depuration period, when the whole-body Zn activity as reached a stable level in fish (i.e., when Zn distribution among tissues has been achieved and depuration is low, see Pouil et al., 2016), fish were sampled, were anaesthetized euthanized by exposure to high concentrations of anaesthetic (Eugenol) and dissected: (1) muscles (the 4 fillets without dorsal skin), (2) the kidney, (3) the liver, (4) the gall bladder, (5) the digestive tract, (6) the head (including gills) and (7) the remaining tissues (including remaining skin, skeleton, fins, heart and muscle residues) were separated, weighed (wet wt) and placed in plastic tubes (diameter: 42mm, height: 65mm) for further radioactivity counting. Then, 20 mL of 2M HCl were added in each tube to digest the tissues in order to get an appropriate geometry and samples were stored overnight before radioanalyses. During all the experiments, no mortality was recorded.

2.3.2. Experiment 1: Influence of food items

For this experiment, natural prey of the juvenile turbots (common prawn, seabream and ragworm) were exposed to dissolved ^{65}Zn as described in section 2.3.1. The average activities in the prey at the end of the exposure period were 144 Bq g^{-1} wet wt, 67 Bq g^{-1} wet wt and 250 Bq g^{-1} wet wt, respectively for common prawn, seabream and ragworm. The stable Zn concentrations were 56, 110 and 127 µg g^{-1} dry wt, respectively for common prawn, seabream and ragworm. Then, three batch of turbot (n=5, 12.1 ± 4.9 g wet wt) were fed *ad libitum* with the three different freshly killed radiolabelled prey. After the depuration period following the single feeding, fish were dissected as described in section 2.3.1.

2.3.3. Experiment 2: Influence of feeding frequency

In order to understand the effect of feeding frequency on Zn organotropism in fish, 5 juvenile turbots were single-fed (23.9 ± 6.0 g wet wt) using radiolabelled pellets (2350 Bq g^{-1} in average) and another batch of turbots (n=5, 23.7 ± 3.1 g wet wt) were pre-exposed to the radiolabelled pellets during a 12-day period (one-labelled pellet feeding every 4 d; Pouil et al. 2017b). Between each labelled-pellet feeding, turbot were fed daily with non-labelled pellets. The stable Zn concentrations in the pellets was 148 µg g^{-1} dry wt (see Pouil et al., 2016 for Zn stable analysis methodology). After the last radiolabelled feeding, depuration of the single-fed and multi-fed turbot was followed for 21 d and all the fish were dissected (see details in section 2.3.1).

2.3.4. Experiment 3: Influence of pH and temperature

In order to study the effects of pH and temperature on Zn organotropism, 4 batches of juvenile turbots (n=4, 23.7 ± 5.3 g wet wt) were acclimated for 2 months to the target experimental conditions (pH 8.0 at 17 °C, pH 8.0 at 20 °C, pH 7.5 at 17 °C and pH 7.5 at 20 °C) controlled using an IKS system (Pouil et al., in press). Then, all the turbot were fed with radiolabelled common prawn (213 Bq g^{-1} wet wt in average). After the 21-d depuration period, fish were dissected as detailed in section 2.3.1.

2.4. Data analysis

Data obtained by radioanalyses of dissected samples (viz. fish compartments) were used to calculate the distribution (expressed in %) of the Zn in fish whole body. The concentration index (I_C) was also calculated as defined by Rouleau et al. (2000) using the following equation (Eq. (1)):

$$I_C = [^{65}Zn] \text{ in tissue} / [^{65}Zn] \text{ in whole body} \quad (1)$$

Values of $I_C > 1$ indicate that the considered tissue is enriched in Zn compared to the whole-body average Zn concentration. Distribution and concentration (i.e. I_C values) of Zn in the body compartments of fish exposed to the different experimental conditions (type of food, frequency of feeding, pH and temperature), were compared using the Kruskal-Wallis non-parametric test, followed by a multiple-comparison test of Siegel and Castellan (Zar, 1996). The level of significance for all statistical analyses was always set at $\alpha = 0.05$. All the statistical analyses were performed using either the Statistica® software 7.0 or R freeware 3.0.1 (R Development Core Team, 2014).

3. RESULTS AND DISCUSSION

It is already known that the digestive physiology of fish and especially their ability to assimilate dietary Zn can be influenced both by biotic factors such as the food items ingested (e.g. Ni et al., 2000; Pouil et al., 2016) or abiotic factors such as the water temperature (e.g. Pouil et al., in press). Information related on the long-term storage of dietary Zn is nevertheless still limited.

To fill this gap, dietary Zn distribution in the body compartments of juvenile turbots exposed to several environmentally relevant factors (food quality, feeding frequency, seawater pH and temperature) was investigated after a 21-d depuration period.

Our results indicated that the proportion of Zn in the remaining tissues (including remaining skin, skeleton, fins, heart and muscle residues), in each of the tested conditions, was always the highest with approx. 50% of the Zn total body burden (Fig. 1), which can be related to the high weight of this compartment (always over 45% of the total body weight). The high proportion of dietary Zn found in the remaining tissues indicates that there is no specialized storage organ for Zn presumably because of it essentiality for the normal physiological functions of the fish (Hogstrand, 2011). Nevertheless, the Zn distribution among the other body compartments (viz. excluding remaining tissues) systematically ranked according to the following decreasing order (Fig. 1, Table 1): Head (28-36%) >> Muscles (8-11%) > Digestive tract (7-10%) >> Liver (1-2%) > Kidney (1-1.5%) > Gall bladder (< 0.5%). Although the weight of the head and the muscles are comparable (20-30% of the total body weight), a much higher proportion of Zn is stored in the head and Zn concentration IC is always > 1 in the head (Table 1). We assume two reasons to understand this observation. Indeed, field investigations have already shown that the highest concentration of Zn can be found in the eyes of fish (Bowness and Morton, 1952; Eckhert, 1983) although the exact function(s) of Zn in the eyes remain(s) unclear. Furthermore, Pouil et al., (2017a; supplementary material) have shown, experimentally, a high concentration index, I_C (up to 2), of Zn in the eyes of silver moony *Monodactylus argenteus* and spotted scat *Scatophagus argus*. Another reason to explain the high Zn concentration in the head is the presence gills in this compartment. Indeed, branchial excretion that has been suggested as the major Zn excretory route in euryhaline fish exposed through dietary Zn (Hardy et al., 1987). The high concentration index, I_C (1-4) found by Pouil et al. (2017a) in the gills of *M. argenteus* and *S. argus*, indicate that this organ plays an important role in Zn in fish. Although branchial excretion dominates, Zn can be also excreted, in a lesser extent, via the bile (Hardy et al., 1987; produced by the gall bladder) and urine (Spry and Wood, 1985). It is thus not surprising to observe the highest IC values in gall bladder and kidney, ranking from 2 to 5 for these organs (Table 1). In the same time, it is important to note that these organs separately only represent less than 1% of the total body weight which also can explain a higher I_C.

Some field investigations have shown that, in some species the Zn concentration in the kidney can be incredibly high (e.g. 23 500 µg g^{-1} in the yellowfin tuna *Thunnus albacares*, Kojadinovic et al., 2007) suggesting an important role of this organ in the Zn storage in fish. In our study, we found that a non-negligible part of the Zn is distributed in the digestive tract (7-10%, Fig. 1 and Table 1). Furthermore, we calculated relatively high I_c values observed (up to 3) for this body compartment. These findings are in accordance with the literature.

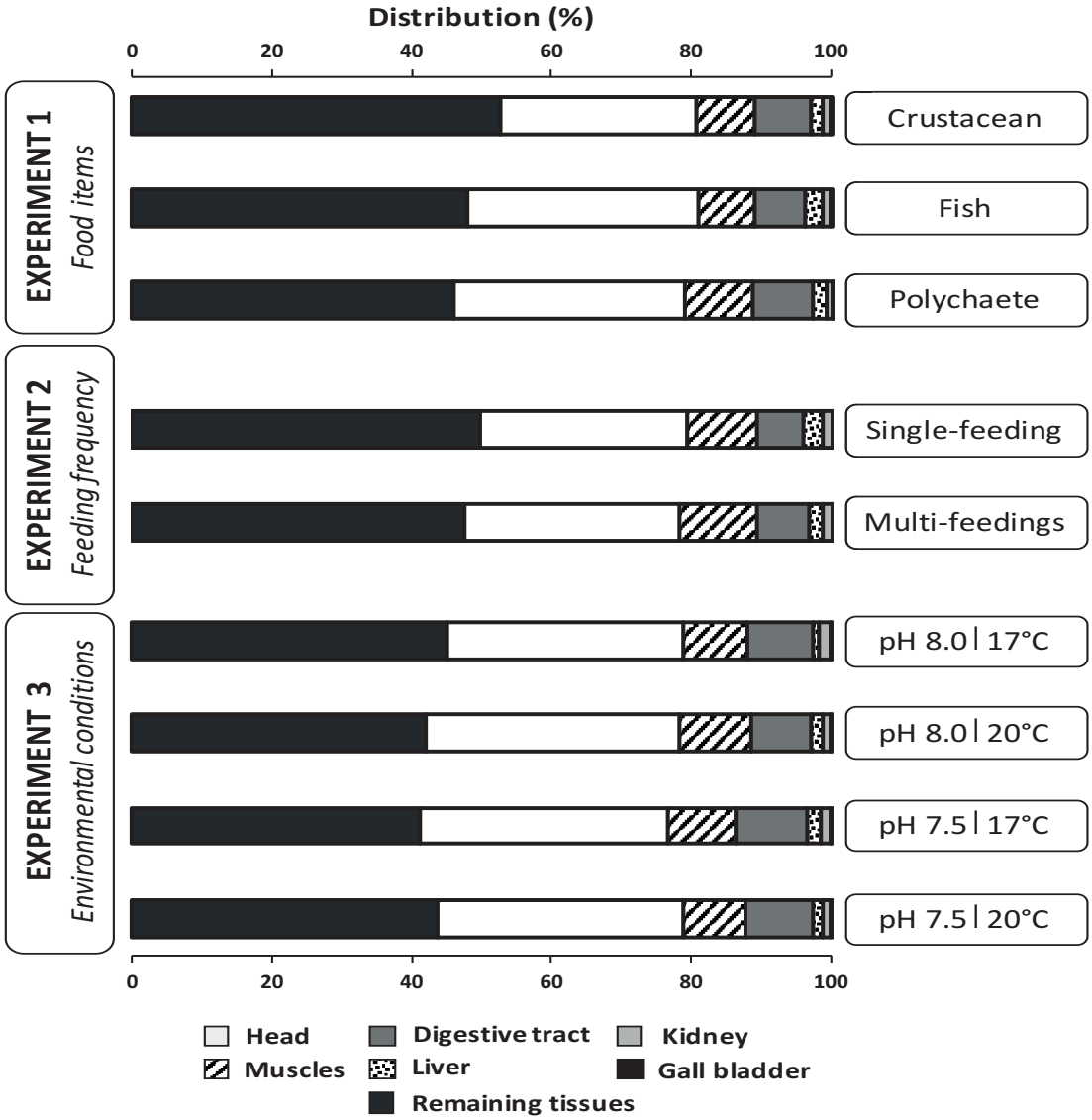


Figure 1. Distribution patterns of Zn (%) in the 7 body compartments of turbots exposed single time to different food items (Experiment 1), fed one and multiple times with compounded pellets (Experiment 2) or maintained under different pH and temperature conditions and single fed with shrimp (Experiment 3). Values are means (n=4-5). Details are provided in Table 1. The remaining tissues included remaining skin, skeleton, fins, heart and muscle residues.

Table 1. Distribution (%) and concentration index (IC) of Zn calculated from the 7 body compartments of turbot exposed single time to different food items (Experiment 1) or fed one and multiple times with compounded pellets (Experiment 2) or maintained under different pH and temperature conditions and single fed with shrimp (Experiment 3). Values are means ± SD (n=4-5).

Compartments	Experiment 1 (n=5 for each condition)			Experiment 2 (n=5 for each condition)		Experiment 3 (n=4 for each condition)			
	Crustacean	Fish	Polychaete	Single-fed	Multi-fed	pH 7.5 at 17 °C	pH 7.5 at 20 °C	pH 8.0 at 17 °C	pH 8.0 at 20 °C
Distribution (%)									
Digestive tract	7.97 ± 0.78	6.98 ± 0.71	8.78 ± 1.68	7.34 ± 1.20	6.67 ± 1.41	10.16 ± 2.02	9.62 ± 1.42	9.22 ± 1.23	8.80 ± 0.46
Head	28.12 ± 0.99	32.74 ± 3.94	32.95 ± 1.50	30.64 ± 2.59	29.70 ± 1.10	35.34 ± 2.48	35.11 ± 2.09	33.78 ± 5.83	36.23 ± 2.06
Gall bladder	0.40 ± 0.24	0.34 ± 0.13	0.10 ± 0.05	0.13 ± 0.07	0.82 ± 0.28	0.35 ± 0.24	0.21 ± 0.07	0.36 ± 0.24	0.11 ± 0.00
Liver	1.70 ± 0.73	2.51 ± 0.71	1.75 ± 0.67	2.01 ± 0.41	2.76 ± 0.87	2.03 ± 0.24	1.33 ± 0.86	0.95 ± 0.28	1.55 ± 0.75
Kidney	1.14 ± 0.44	1.27 ± 0.65	0.96 ± 0.36	1.26 ± 0.15	1.31 ± 0.12	1.31 ± 0.58	1.26 ± 0.65	1.56 ± 0.24	1.26 ± 0.69
Muscles	8.19 ± 1.33	8.16 ± 0.97	9.54 ± 0.88	11.24 ± 1.90	9.87 ± 1.29	9.75 ± 0.59	8.94 ± 2.08	9.22 ± 1.30	10.13 ± 0.95
Remaining tissues*	52.48 ± 1.22	48.00 ± 3.65	45.92 ± 3.39	47.44 ± 4.37	49.62 ± 2.22	41.15 ± 3.47	43.59 ± 1.99	45 ± 6.27	41.96 ± 1.80
Concentration index (Ic)									
Digestive tract	1.35 ± 0.22	1.75 ± 0.16	1.67 ± 0.38	2.34 ± 0.62	2.36 ± 0.42	2.87 ± 0.66	2.76 ± 0.23	2.82 ± 0.56	2.82 ± 0.09
Head	1.40 ± 0.06	1.39 ± 0.08	1.25 ± 0.05	1.27 ± 0.04	1.30 ± 0.08	1.26 ± 0.06	1.27 ± 0.12	1.15 ± 0.20	1.19 ± 0.07
Gall bladder	3.01 ± 2.20	2.82 ± 1.43	2.03 ± 0.44	1.70 ± 0.37	1.82 ± 0.28	1.93 ± 1.15	1.87 ± 0.22	2.22 ± 2.03	1.90 ± 0.10
Liver	1.79 ± 0.78	2.13 ± 0.56	2.80 ± 0.27	2.59 ± 0.33	2.44 ± 0.33	3.48 ± 1.05	2.18 ± 0.86	2.66 ± 1.46	3.62 ± 1.11
Kidney	3.70 ± 1.02	3.7 ± 1.44	3.78 ± 0.67	2.98 ± 0.52	2.87 ± 0.47	4.64 ± 1.04	3.96 ± 1.69	4.26 ± 0.63	3.61 ± 1.06
Muscles	0.39 ± 0.06	0.44 ± 0.06	0.42 ± 0.03	0.39 ± 0.04	0.48 ± 0.04	0.49 ± 0.02	0.45 ± 0.09	0.44 ± 0.04	0.51 ± 0.04
Remaining tissues*	1.02 ± 0.03	1.04 ± 0.05	1.04 ± 0.06	1.08 ± 0.04	0.98 ± 0.05	0.87 ± 0.06	0.91 ± 0.05	1.00 ± 0.20	0.92 ± 0.05

*The remaining tissues included remaining skin, skeleton, fins, heart and muscle residues

Indeed, very high Zn concentration (up to 500 µg Zn g^{-1} wet wt) have been measured in the digestive tract of common carp Cyprinus carpio (Sun and Jeng, 1999; Reynders et al., 2006). Thus, the digestive tract is not only involved in the absorption of the dietary Zn but is also an important sink for long-term storage of this element. This fact could be due to the presence in high quantity of specific low molecular weight Zn-binding membrane proteins (Jeng et al., 1999).

In this study, several biotic factors were examined to understand the Zn organotropism in juvenile turbot: the food quality (different prey items with different Zn bioavailability) and the feeding frequency. Our results did not show any significant effect of these parameters in Zn distribution and concentration in body compartments of juvenile turbots were found after a 21-d depuration period ($p>0.05$; Fig. 1, Table 1). The absence of changes in distribution and concentration of this element in the body compartments could be related to the fact that the experimental context is reflecting non-polluted conditions (i.e., no excess of Zn in the diet) and rather reflects normal physiological processes. The stable Zn concentrations in the different types of food used were ranging between 56 to 148 µg g-1 dry wt for all the food items used which represent concentration of prey living in non-polluted environments (Eisler, 2009). In addition, during the depuration period, we estimated an average dietary Zn input of approx. 15-20 µg g^{-1} dry wt per fish based on the stable Zn concentration in pellet and daily food eaten by each fish. These values presumably satisfied the daily Zn requirements for fish (Antony Jesu Prabhu et al., 2016). Since the Zn is not in excess, we therefore assumed that it is stored in the tissues without activation of any abnormal excretion mechanisms. This assumption is supported by the results of Experiment 2 where fish were single-fed or multi-fed with ^{65}Zn radiolabeled pellets without any effect on Zn long-term storage in fish. Furthermore, a preliminary assessment of the relationship between stable Zn ingested dose and the ^{65}Zn stored in the different body compartments was therefore done for single-fed turbots from the Experiment 2 (Fig. 2) to determine if we were reached a tipping point from physiological perspectives. The linear relation observed indicates that, even if the ingested dose of Zn can be multiplied by 3 for juvenile turbots, there is no visible saturation of the ^{65}Zn burden in the body compartments.It is however important to keep in mind that, at higher Zn concentrations in food, the uptake and excretion pathways of Zn may be impacted (Bury et al., 2003). Furthermore, at high Zn concentrations, an increase in synthesis of metallothioneins, active metal transporters, may be observed (Zhang and Wang, 2005).

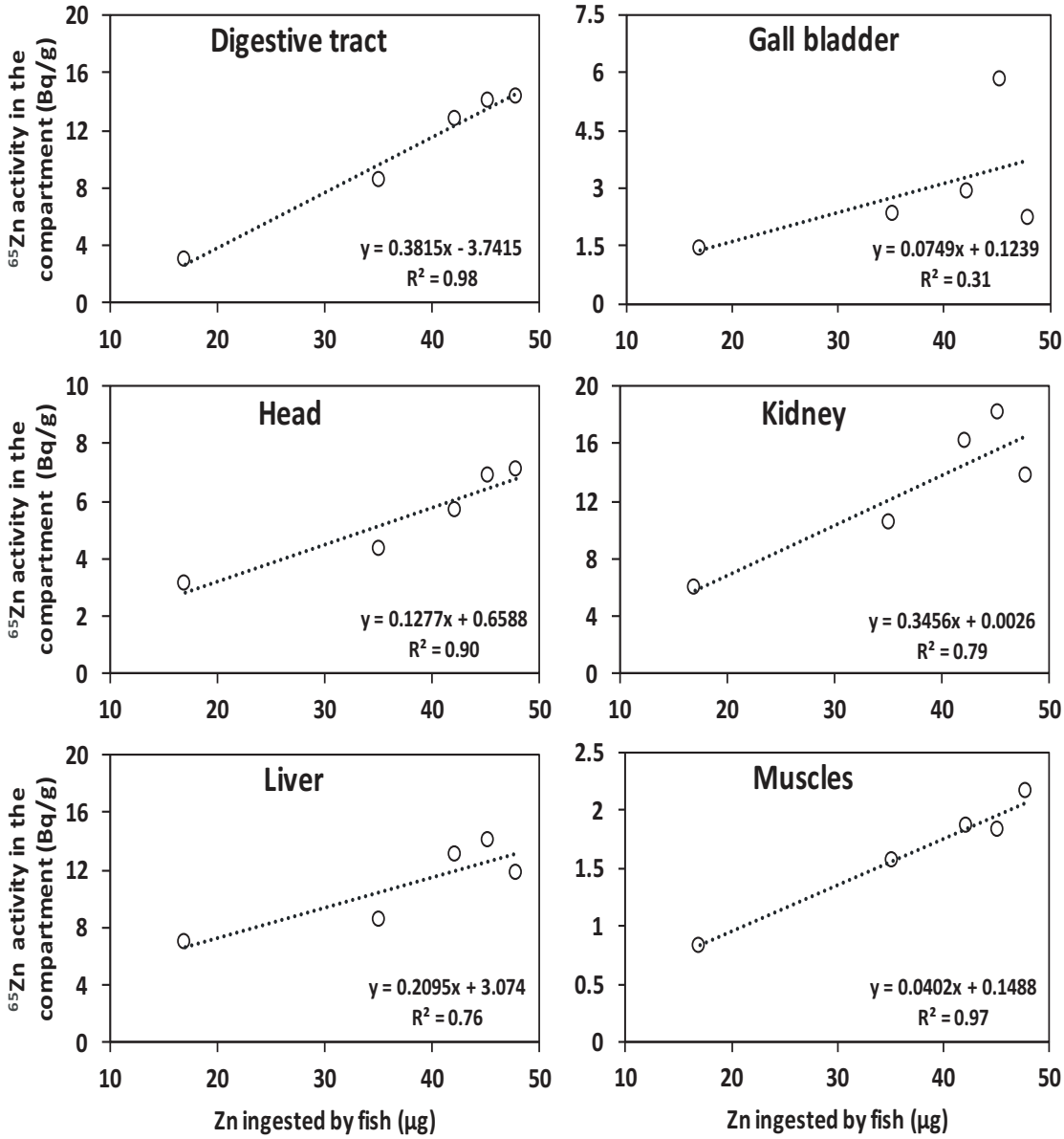


Figure 2. Relationship between the estimated Zn ingested by fish (µg) and ^{65}Zn stored in each body compartments (Bq g^{-1} wet wt) for the five single-fed turbot from the Experiment 2.

These physiological changes may affect the distribution and concentration of Zn in body compartments and further investigations are needed to clarify this point. Concerning the potential effects of abiotic factors on Zn trophic transfer in fish, very few studies have been done to date with salinity (Ni et al., 2005), pH (Jacob et al., 2017), temperature (Van Campenhout et al., 2007) pH and temperature (Pouil et al., in press) as influencing factors. mudskipper *Periophthalmus modestus* was exposed to dietary Zn through a single-feeding at different salinities, Zn body distribution after a 2-d depuration period was not affected.

Although some of these studies have highlighted significant effetcs of these parameters on Zn trophic transfer (Van Campenhout et al., 2007; Pouil et al., in press), to the best of our knowledge, only one study considered the potential effects of such parameters (i.e. salinity, Ni et al., 2005) on dietary Zn organotropism. These authors showed that when the intertidal. In the present study, we showed that water pH and temperature, either considered separately or in combination, have no significant effect on Zn organotropism (Table 1). The body distribution of this essential element is governed in fish by homeostatic regulation (Hogstrand, 2011). The ability of fish to regulate themselves their acid-base balance and their osmolality (Brauner et al., 2004; Kültz, 2015) can explained, that in the range of pH and temperature values presently used, there is no disturbance of Zn homeostasis, and by extension, no change in the Zn organotropism. Nevertheless, in the context of global change, local important variations of such abiotic stressors can occur, especially in coastal areas and further investigations are needed to explore the dietary trace element organotropism in fish under a larger range of pH and temperature values.

In summary, this study revealed no statistically significant effect of food quality, feeding frequency, and water pH and temperature in the Zn organotropism of juvenile turbot. The concentration index (I_C) values, indicate that, when Zn was provided by food, digestive tract played a major role in the long-term storage of this element as well as kidney and gall bladder. We also suspected a non-negligible role of gills for Zn excretion as shown by others (Hardy et al., 1987) but we were not able to prove it. Because metal concentrations in surrounded environment are likely to affect their body concentration (Le Pabic et al. 2015), further investigations are needed to study the influence of this parameter on the Zn organotropism.

4. ACKNOWLEDGMENTS

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Résumé : Rôles de différents facteurs écologiques sur le transfert trophique des éléments traces chez des téléostéens marins

Les poissons téléostéens accumulent les métaux au travers de différentes voies et il est actuellement bien établi que la nourriture joue un rôle majeur sur cette accumulation. Néanmoins de nombreuses lacunes persistent sur la variabilité du transfert trophique des métaux chez les poissons en fonction de leur contexte de vie. La présente recherche doctorale vise à caractériser l'influence de facteurs biologiques et environnementaux sur l'efficacité d'assimilation (AE) de métaux essentiels et non-essentiels chez les poissons. La détermination des AEs lors d'une série d'expériences en milieu controlé révèle que les facteurs biologiques, et notamment le type de nourriture ingéré, jouent un role prépondérant sur l'AE des métaux chez les poissons alors que les facteurs environnementaux (pH, température, salinité …) semblent n'avoir qu'une influence plus limitée. L'ensemble des travaux réalisés lors cette thèse permet une meilleure compréhension du transfert trophique des métaux chez les poissons, et apporte ainsi de nouvelles connaissances dans les domaines de la nutrition et de l'écotoxicologie.

Mot-clés : Nourriture, Poisson, Métaux, Efficacité d'assimilation, Facteurs biotiques, Facteurs abiotiques, Radiotraceurs

Abstract: Roles of several ecological factors on the trophic transfer of trace elements in marine teleosts

Teleost fish accumulate metals through different pathways and It is now well established that food plays a major role in this accumulation. Nevertheless, there is still lack of knowledge on the variability of metal trophic transfer in fish in connection with their life context. The present doctoral research aims at characterizing the influence of biological and environmental factors on the assimilation efficiency (AE) of essential and non-essential metals in fish. AE determination during a series of experiment under controlled condition reveals that biological factors, in particular the type of food ingested, play a predominant role in metal AE in fish while environmental factors (pH, temperature, salinity ...) seem to have a limited influence. All the work carried out during this thesis allows a better understanding of the trophic transfer of metals in fish and, thus bring new knowledge in the field of fish nutrition and ecotoxicology.

Keywords: Food, Fish, Metals, Assimilation efficiency, Biotic factors, Abiotic factors, Radiotracers