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The role of marine biotoxins on the trophic transfer of Mn and Zn in fish

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ABSTRACT

Essential nutrients are critical for physiological processes of organisms. In fish, they are obtained primarily from the diet, and their transfer and accumulation are known to be impacted by environmental variables such as water temperature, pH and salinity, as well as by diet composition and matrices. Yet, prey items consumed by fish may also contain toxic compounds such as marine toxins associated with harmful algae. These biotoxins have the potential to affect essential trace element assimilation in fish through chemical interactions such as the formation of trace element-toxin complexes or by affecting general fish physiology as in the modification of ion-specific transport pathways. We assessed the influence of dietary exposure to brevetoxins (PbTxs), ichthyotoxic neurotoxins produced by the dinoflagellate *Karenia brevis*, on trophic transfer of two essential trace elements, Mn and Zn, in a fish model. Using ecologically relevant concentrations of PbTxs and trace elements in controlled laboratory conditions, juvenile turbots *Scophthalmus maximus* were given food containing PbTxs before or at the same time as a feeding with radiotracers of the chosen essential elements (^{54}Mn and ^{65}Zn). Treatments included simultaneous exposure (PbTxs + ^{54}Mn + ^{65}Zn) in a single-feeding, 3-week daily pre-exposure to dietary PbTx followed by a single feeding with ^{54}Mn and ^{65}Zn, and a control (^{54}Mn and ^{65}Zn only). After a 21-day depuration period, turbot tissue brevetoxin levels were quantified and assimilation efficiencies of ^{54}Mn and ^{65}Zn were assessed. PbTxs were found in turbot tissues in each exposure treatment, demonstrating dietary trophic transfer of these toxins; yet, no differences in assimilation efficiencies of Mn or Zn were found between treatments or the control ($p > 0.05$). These results indicate that, in our experimental conditions, PbTx exposure does not significantly affect the trophic transfer of Mn and Zn in fish.

1. Introduction

Some trace elements found in the marine environment are termed essential because marine organisms such as fish require them for fundamental physiological processes. For example, trace elements are part of the functional groups of various metabolic enzymes; as such, they play a structural role in respiratory pigments and metalloenzymes, and can act as co-factors for various proteins (see for example Simkiss, 1979; Watanabe et al., 1997; Williams, 1981). At either insufficient or excessive levels, these trace elements can have serious physiological effects (e.g. Förstner and Wittmann, 1981). In fish, trace elements are mainly acquired via the dietary pathway (Xu and Wang, 2002; Mathews and Fisher, 2009). Among the essential trace elements, manganese (Mn) and zinc (Zn) are particularly important due to their roles as components of or cofactors in key enzyme systems in lipid, protein and carbohydrate metabolism (Watanabe et al., 1997; Bury et al., 2003). As a result, these elements are directly involved in fish growth, bone formation, reproduction, development and immunity (Tacon, 1987; Watanabe et al., 1997). Although present in relatively high abundance in the environment, many mechanical or chemical barriers can impede their uptake or adequate distribution to ultimate target tissues, cells or molecules. Thus, the influence of factors on the trophic transfer of these essential trace elements in fish is an important focus of research.

In the marine environment, fluctuations in abiotic variables such as the temperature, pH or salinity of the surrounding seawater are known to influence the digestive physiology of fish, and thus, their ability to bioaccumulate essential trace elements from the diet. For example, laboratory experiments on various 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 can directly affect essential element absorption or assimilation through its influence on feeding behaviour and food conversion efficiency (reviewed by Bœuf and Payan, 2001). In addition to environmental

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factors, the composition of the food itself may affect the assimilation of essential elements in fish (Pouil et al., 2016), where they may be associated with diverse compounds, and these associations affect their bioavailability. These compounds may include toxicants such as natural biotoxins associated with blooms of toxic species of phytoplankton. Although biotoxins regularly cause mass mortalities of marine organisms, some species can accumulate these toxins without any signs of intoxication and, when these organisms represent a food source to other organisms, they may hence provide essential nutrients while also acting as a vector for biotoxin transfer (Landsberg, 2002; 2009 Naar et al., 2007). Moreover, with increasing number of reported harmful algal bloom events over the last decades (Hallegraeff, 1993; Smayda, 1990; Sournia, 1995; Van Dolah, 2000), a trend expected to continue with foreseen global change (Hallegraeff, 2010; Moore et al., 2008; Wells et al., 2015), the natural occurrence of toxin-containing marine organisms is likely to increase correspondingly. Ingested algal biotoxins impact species interactions, organismal physiology and health, and population dynamics (Anderson, 2009; Smith and Schindler, 2009; Van Den Bergh et al., 2002), particularly in fish species (Landsberg, 2002). Despite the fact that biotoxins may often be present in the tissues of fish prey, their effects on the uptake and the assimilation of key dietary nutrients by fish remain unknown.

The mechanisms controlling the uptake and distribution of metals such as Mn or Zn are not completely understood, but are known to be impacted by tissue and cellular chemistry (i.e. redox processes, hydrolysis, solubility, chelation of free ions or ligand exchange rates of metal ion chelates) and physiology (e.g. direct or indirect effects on metal transporters, digestion). Yet, despite well-established impacts of algal neurotoxins such as brevetoxins on the physiology of exposed organisms (Poli et al., 1990; Walsh et al., 2010), potential effects of these toxins on essential element bioavailability and whole body kinetics have never been studied. Moreover, because algal toxin molecules generally contain functional groups such as amino, carboxyl, phenol, sulfhydryl and hydroxyl groups, they have the potential to complex with trace element ions (Humble et al., 1997; Rue and Bruland, 2001) and thus affect their transfer in aquatic food chains. The common formation of a domoic acid-Cu complex provides such an example (Rue and Bruland, 2001). Finally, changes in membrane potential that occur with exposure to algal neurotoxins impact cellular ion transport (Berman and Murray, 2000; Liberona et al., 2008; Tian et al., 2011) and consequently may affect distribution of trace elements to target tissues and cells, and hence their kinetics in marine organisms.

The aim of the present study was to examine potential impacts of the occurrence of biotoxins in food items on the assimilation of essential elements in fish, using the impacts of brevetoxins (PbTxs) on Mn and Zn kinetics in the turbot, *Scophthalmus maximus*, as a model. More specifically, we assessed the effect of single or repeated exposures to PbTxs, a suite of neurotoxins produced by the red-tide forming dinoflagellate *Karenia brevis* (Landsberg et al., 2009), on trophic transfer of Mn and Zn in *S. maximus*, using assimilation efficiencies (AEs; details in Pouil et al., 2018) as end-points. Methodologically, this work was realized by feeding *S. maximus* a processed biological matrix with naturally-accumulated PbTxs (*viz.* mussels that were experimentally fed on toxic microalgae) and by utilizing highly sensitive radiotracer techniques. These techniques were used at 2 levels of the study: (1) to determine PbTx accumulation in juvenile turbots and (2) to determine the AEs of the essential trace elements.

2. Materials and methods

2.1. Origin, acclimation and maintenance of organisms

Juvenile turbots *S. maximus* were purchased from a fish farm (France Turbot, France). As an aquaculture species of economic interest that is easily accessible and has relatively low sensitivity to stress (Lepage et al., 2000), this model organism has been commonly used for trophic transfer study of trace elements (e.g. Mathews et al., 2008; Pouil et al., 2016; Pouil et al., 2017).

Fish were acclimated to laboratory conditions for at least 6 months (aerated, open-circuit 700-L 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. The daily dietary intake was determined during this period by the weight of the food rations (full ingestion confirmed by visual observation).

Blue mussels *M. edulis* (brevetoxin-free as assessed using a radioligand receptor binding assay, RBA) were purchased from a seafood seller (Les Halles du Midi, Monaco). They were transported to the IAEA-EL laboratory in Monaco and were acclimated in the same conditions as the turbot for 1 month prior to the experiment. During this period, mussels were fed daily with phytoplankton (*Isochrysis galbana*).

Cultures of *Karenia brevis* (NOAA-1 strain isolated from Charlotte Harbor, FL, USA; mean = 18.5 ± 2.7 pg PbTx-3 equiv $cell^{-1}$) were grown in a 20-L plastic container containing aerated seawater (0.45 µm filtered seawater aged in the dark for 1 month minimum; salinity = 38; temperature = 20 ± 1 °C; pH = 8.0 ± 0.1; bilateral luminosity 66–80 µE $m^{-2} s^{-1}$; light/dark: 12 h/12 h) spiked with an atypical f10k medium (Holmes et al., 1991). 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 obtain 20 L of culture in the stationary phase (approx. 10000 cells mL^{-1}), which was used for the experiment. Cell counting was performed in 10% Lugol's solution using a Sedgewick Rafter counting chamber (20 × 50 µL) under a light microscope.

2.2. Experimental procedures

To investigate the influence of dietary exposure to PbTx on the AEs of Mn and Zn in fish, juvenile turbots were fed a gel food diet prepared from brevetoxin-containing mussel tissue either prior to or concurrent with trace element exposure and compared with fish fed with trace elements alone. A schematic of experimental treatments is provided in Fig. 1. The first condition (Treatment 1) simulated a single, concurrent exposure event in which fish consume prey long after a *K. brevis* bloom and thus prey tissues contain only low concentrations of PbTx. The second condition (Treatment 2) investigated the effects of repeated dietary exposures to higher concentrations of PbTx in prey tissues as occur shortly after a bloom event. The last condition (Treatment 3) served as the experimental control. 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. Details of all gel food types used are provided in Table 1.

2.2.1. Preparation of the mussel-based gel foods

To prepare the different PbTx-containing and/or radiolabelled gel foods, *M. edulis* (33.1 ± 5.1 g wet wt) were randomly placed in three aerated, open-circuit 20-L aquaria (n = 40; salinity = 38; temperature = 20 ± 1 °C; pH = 8.0 ± 0.1; light/dark cycle: 12 h/12 h).

Mussels in two of the 20-L aquaria (aquaria 1&2) were exposed daily for four days to *K. brevis* at an environmentally relevant cell concentration of 980 ± 20 cells mL^{-1} (Gannon et al., 2009; Stumpf et al., 2003). Each day, *K. brevis* culture was carefully added to the aquaria to preserve *K. brevis* cell integrity and achieve the desired cell concentration. During 1 h of feeding, the aquaria were maintained in closed-circuit, after which mussels were fed *I. galbana* for 30 min. At the end of the exposure, a fraction of exposed mussels from each aquarium (n = 20) was used to prepare the non-radiolabelled high toxin content (NRHT) gel food (Table 1). Briefly, the soft tissues of the mussels were

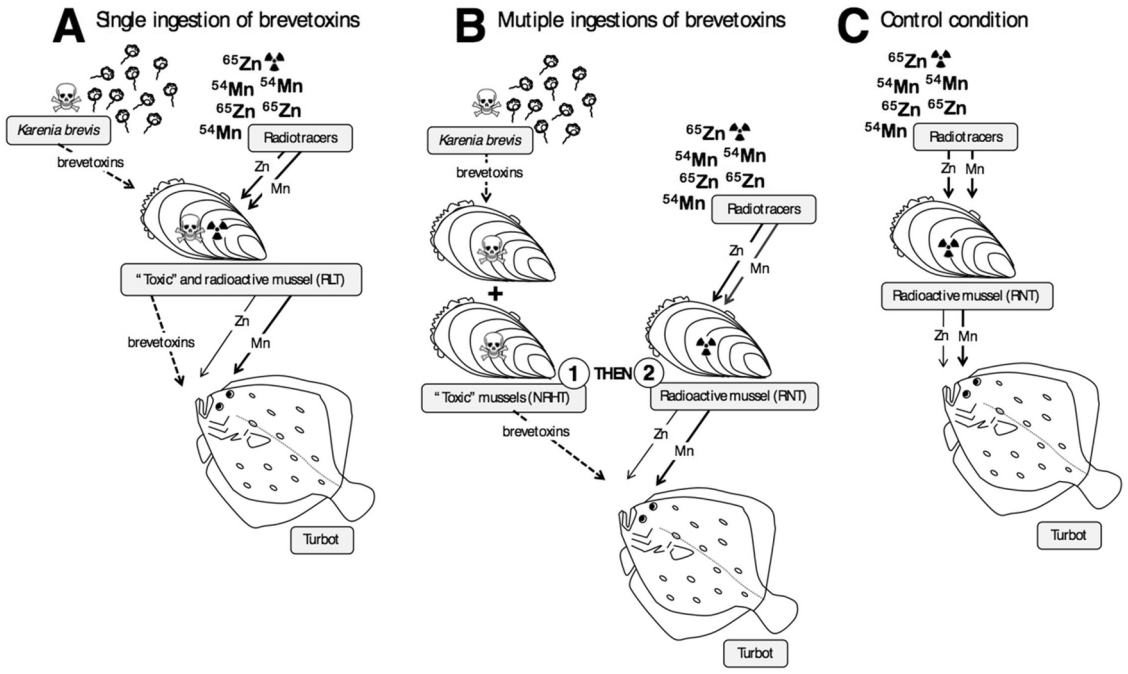

Fig. 1. Protocols where (A) turbots were fed a gel food prepared from radiolabelled mussels containing low brevetoxin (PbTx) concentrations (RLT) a single time (Treatment 1), (B) turbots were given a gel food prepared from mussels containing high PbTx concentrations (NRHT) five days per week for three weeks and then single-fed with radiolabelled gel food (RNT; Treatment 2) and (C) turbots were single-fed a radiolabelled gel food (RNT) to serve as the experimental control (Treatment 3).

collected, homogenized and mixed with food-grade leaf gelatin that had been dissolved in hot seawater (0.1 g mL^{-1}) and cooled to 30 °C to prepare a single batch of homogenous feed (ratio of 0.25 mL g^{-1}) with known toxicity.

After exposure to *K. brevis*, the remaining mussels from one of the 20-L aquaria (aquarium 1; n = 20) were then exposed for 23 days to dissolved radiotracers (^{54}Mn and ^{65}Zn) of known specific activity (^{54}Mn ($t/_{1/2}$ = 312.2 d) as $MnCl_2$ in 0.5 M HCl and ^{65}Zn ($t/_{1/2}$ = 243.9 d) as $ZnCl_2$ in 0.1 M HCl, Isotope Product Lab, USA). Levels of radioactivity in the seawater were kept constant at 0.40 ± 0.16 kBq ^{54}Mn L^{-1} and 0.80 ± 0.39 kBq ^{65}Zn L^{-1} with regular spiking using closed-circuit aquaria, where the frequency of spike injection was determined by measurements before and after each spike renewal (see Metian et al., 2009). In terms of stable trace element equivalents, each spike corresponded to an addition of 1 ng Mn L^{-1} and 27 ng Zn L^{-1} (i.e. concentrations that are substantially lower than the background concentrations of these trace element in open sea; Bruland, 1983). After each water renewal (i.e. before each spike renewal), mussels were fed 30 min with *I. galbana*. Mussels were collected at different time intervals and were γ-counted alive and returned to the aquarium. After the 23 d of exposure to the radiotracers, the radiolabelled, PbTx-containing mussels from aquarium 1 were processed to prepare the gel food with radiotracers and low toxin content (RLT; Table 1) using the mussel soft tissues and gelatin as described above.

During the same period, remaining mussels from the second *Karenia*-exposed 20-L aquarium (aquarium 2; n = 20) were kept in flow-through seawater and fed daily with *I. galbana*. Twenty-three days after the last exposure to *K. brevis*, mussels were dissected and soft tissues processed for toxin analysis as proxy for toxin content in mussels exposed to radiotracers (aquarium 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 days as described above without previous exposure to *K. brevis*. Soft tissues were used to prepare the radiolabelled gel food without toxin content (RNT).

All the five experimental gel foods (NRHT, RLT, RNT, NRLT and NRNT; Table 1) were stored at –4 °C until use, and radioactivity and PbTx concentrations were measured as described in Section 2.3. Furthermore, stable Mn and Zn were measured in the gels NRHT and NRNT. Briefly, samples (n = 3 for each gel) of 800–1000 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, involving a gradual increase to 190 °C over 20 min, followed by 20 min at 190 °C (1600 W). After the mineralisation

Table 1
PbTx and radiotracer contents (all values are given relative to fresh weight) in the different gel foods prepared from homogenized mussel soft tissues mixed with gelatin (see details in Section 2.2.1). Data are means ± SD (n = 3 per gel with triplicate assays for each sample).

Gels	Characteristics	Brevetoxin concentration (ng g^{-1})	^{54}Mn concentration (Bq g^{-1})	^{65}Zn concentration (Bq g^{-1})	Use
NRNT	No Radiotracers, No Toxins	–	–	–	To acclimate all experimental turbot to the gel food matrix
RNT	Radiotracers, No Toxins	–	48 ± 1	1059 ± 30	For the single-feeding of turbots (Treatments 2 & 3)
NRHT	No Radiotracers, High Toxins	525 ± 88	–	–	For multiple brevetoxin feedings by turbots (Treatment 2)
RLT	Radiotracers, Low Toxins	188 ± 38	37 ± 2	1012 ± 6	For single ingestion by turbots (Treatment 1)
NRLT	No Radiotracers, Low Toxins	188 ± 38	–	–	As proxy to estimate the brevetoxin content in the gel RLT

process, each sample was diluted to 50 mL with Milli-Q water, and an extra 1:5 dilution was prepared. Analyses were performed 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 (recovery over 80% for Mn and 97% for Zn). For each set of analyses, blanks were included in the analytical batch. These analyses confirmed the homogeneity of the stable trace element concentrations in the gels with values of < 0.8 and $< 0.6\ \mu g\ g^{-1}$ wet wt of Mn (below the detection limit) and 7.8 ± 0.4 and 6.3 ± 0.5 (Mean ± SD) μg^{-1} wet wt of Zn for the NRHT and the NRHT gels, respectively. All the gels were thawed and cut into small, homogenous pieces (size < 2 mm) just before being given to the turbot.

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

Prior to the experiment, seven juvenile turbots (18.2 ± 2.0 g wet wt) 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: 12 h/12 h). Turbots were fed once with the RLT gel food (see Section 2.2.1 and Table 1) for a maximum of 15 min and AEs of Mn and Zn were followed over 21 d. Water flow conditions and feeding duration were selected in order to avoid any risk of that fish were exposed to dissolved radiotracer leaching from the food. One additional turbot was placed within a net in the same aquarium to restrict access to food and serve as a control for possible radiotracer leaching from the contaminated food or from fish depuration. Two hours after the feeding period, all turbots were whole-body γ-counted alive (Section 2.3.2) and replaced in clean, flow-through seawater. Radio-isotopic analyses were then regularly performed over a 21-d period to follow the depuration kinetics of the radiotracers.

2.2.3. Treatment 2: influence of multiple PbTx exposures on AE of Mn and Zn in fish

Seven juvenile turbots (22.1 ± 3.7 g wet wt), kept in the same conditions as described in Section 2.2.2, were pre-exposed to NRHT gel food for 3 weeks (5 feedings/week) and then given a single-feeding with RNT gel food (Table 1). One additional, non-exposed turbot served as a control (see Section 2.2.2). Two hours after the 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.

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

To describe the depuration kinetics of Mn and Zn without PbTx exposure, juvenile turbots (n = 7, 21.1 ± 4.3 g wet wt) were given a single-feeding of RNT gel food. Fish were maintained and γ-counted as previously described.

2.2.5. Accumulation of PbTx in fish

To quantify the accumulated PbTx in fish tissue after both single and multiple exposures, treatments 1 and 2 were repeated in the absence of radiotracers. Quantification of PbTx concentrations cannot be performed on samples with radiotracers because γ-emitters (here ^{54}Mn and ^{65}Zn) can cause inference in β-detection with liquid scintillation counting (here tritiated PbTx: [^{3}H]PbTx-3; see Section 2.3.1). Each time, 8 turbots were single-fed or multi-fed (3 weeks) with NRLT gel food or NRHT gel food, 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.

2.3. PbTx and radiotracers quantification

2.3.1. PbTx quantification using β-spectrometry

PbTxs were quantified in the three mussel-based gel foods (RLT, NRLT and NRHT; Table 1, n = 3 per food type) and tissue samples from eight turbots. Due to low sample size of gall bladder tissue (mg range) and the limit of quantification of brevetoxin detection methods, samples were pooled (n = 1) for each body compartment within each treatment. Pooled homogenized samples provided sufficient quantity for a minimum of three analytical replicates, each run in triplicate. For each pooled sample, PbTxs were then extracted as previously described (Poli et al., 2000) with minor modifications and tested (recovery: 90–108%; Dickey et al., 1999). Briefly, samples were homogenized in 3 volumes of acetone, sonicated (minimum 1 min in ultrasonic water bath) and centrifuged at 3000*g* for 10 min. Each supernatant was collected in 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 to which 6 mL of n-hexane was added. Samples were mixed by inversion and centrifuged at 3000*g* for 1 min. The methanol phase was collected in glass tubes and evaporated under a stream of nitrogen. Extracts were resuspended in 100% methanol and stored at –18 °C until analysis.

Composite PbTx concentrations in the different tissue extracts were determined using an activity-based radioligand receptor-binding assay (RBA) (following Dechraoui Bottein and Clausing, 2017). 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 a standard or sample extract. The reduction in [^{3}H]PbTx-3 binding is directly proportional to the quantity of PbTx-3 standard or of unlabelled toxin present in the sample (Dechraoui Bottein, 2014; Poli et al., 1986). In each assay, samples were analysed in triplicate at one (liver, gall bladder and digestive tract) or three (gel foods, muscle) dilutions in a 96-well filter-plate format (MSFB N6 B 50 MultiscreenHTS), and radioactivity was counted in a liquid scintillation beta counter (MicroBeta2 Microplate Counter, PerkinElmer) after a 2-h period of incubation. Each sample was run in three independent assays. Toxin quantification of samples was calculated from standard curves of PbTx-3 (4-parameter logistic regressions) determined using GraphPad Prism software version 6.0 (San Diego, USA).

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 gel-food preparations was determined by comparison with standards of known activity and appropriate geometry (calibration and counting). Measurements were corrected for background and physical radioactive decay (Cresswell et al., 2017). Both radiolabelled experimental gel foods (gel RNT and gel RLT) were weighed (wet wt) and placed in plastic tubes (diameter: 42 mm, height: 65 mm) in triplicate. Then, 25 mL of 2 M HCl was added to each tube to get an appropriate geometry, and samples were stored overnight before radioanalyses. Living organisms were counted in circular plastic boxes (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) while maintaining fish health and normal behaviour, and generally varied between 15 and 60 min (Fig. 1).

2.4. Data treatment and statistical analyses

Depuration of the 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 Warnau et al., 1996). The depuration kinetics of Mn and Zn were best fitted using a two-component exponential model Eq. (1):

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

where A_t and A_0 are the remaining activities (%) at time t (in days) and time 0, respectively. Here, time 0 refers to the initial γ-counting of the fish performed two hours after the radiolabelled single-feeding. k_e is the depuration rate constant (d^{-1}); and "s" and "l" represent the short- and long-lived radiotracer component during depuration, respectively. The "s" component is the radiotracer fraction that is weakly associated with the organism and rapidly excreted out of the body (i.e. mainly the proportion not absorbed but associated with the faeces; Whicker and Schultz, 1982; Warnau et al., 1996). The "l" component describes the fraction of radiotracer that is actually absorbed by the organism and excreted more slowly. The long-lived component allows estimation of the AE of the radiotracer ingested with food (AE = A_{0l}; Fowler and Guary, 1977; Miramand et al., 1982).

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

Comparison of assimilation of essential elements among the different experimental conditions was performed using Kruskal-Wallis and Siegel and Castellan non-parametric tests on the AEs calculated for each individual turbot, where the best-fit model obtained for the entire set of turbot was applied to each individual (Zar, 1996). For Mn, two individuals per condition had insufficient initial activity (i.e. < 1.5 Bq of ^{54}Mn, activity measured 2 h after the radiolabelled feeding) and were excluded from statistical analysis. Toxin accumulation data were not statistically analysed as replicate samples within each treatment/tissue type combination were pooled for obtaining sufficient quantity for rigorous toxin determination. Thus, the given values represent the overall trends between treatments, as each sample is a homogenate of 8 individuals. Moreover, each sample was analysed over at least 3 independent assays, giving a high level of confidence to the accuracy of the reported values. The level of significance was $\alpha = 0.05$. All 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 PbTx-containing gel foods without radiotracers (i.e. NRLT and NRHT) confirmed that after 4 exposures to *K. brevis*, the mussels used to prepare the gel food had accumulated PbTx in their tissues. Low variance among PbTx concentrations among the 3 replicate samples for each food type indicated that the gel foods were homogenous. PbTx concentrations were 188 ± 38 and 525 ± 88 (Mean ± SD) ng PbTx-3 equiv g^{-1} food for the NRLT and the NRHT gel foods, respectively (Table 1). Thus, the average quantities of PbTx given to each turbot during the single (Treatment 1) and the multiple (Treament 2) dietary exposures were 4 ng PbTx-3 equiv g^{-1} fish and 222 ng PbTx-3 equiv g^{-1} fish, respectively (Table 2).

PbTxs were measured in the pooled samples from the four body compartments of juvenile turbots (the digestive tract, the gall bladder, the liver and the muscles). In both treatments and all tissues types, turbot accumulated measurable quantities of PbTx-3, demonstrating effective dietary exposure to the toxin. Concentrations appeared roughly similar in all tissues, but as composite tissue measurements,

Table 2
Quantities of ingested PbTx for the single (Treatment 1) and multiple (Treatment 2) feeding treatments and the corresponding concentrations subsequently measured in selected body compartments. Data are expressed as averaged concentrations in ng g^{-1} wet weight (enclosed in square brackets) and total quantities in ng. Body compartment means represent replicate analyses on one pooled extract comprised of 8 replicate samples.

Experimental conditions	PbTx given per feeding and per turbot	Total PbTX given 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 given	[ng g^{-1}] and ng	% of given	[ng g^{-1}] and ng	% of given	[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	

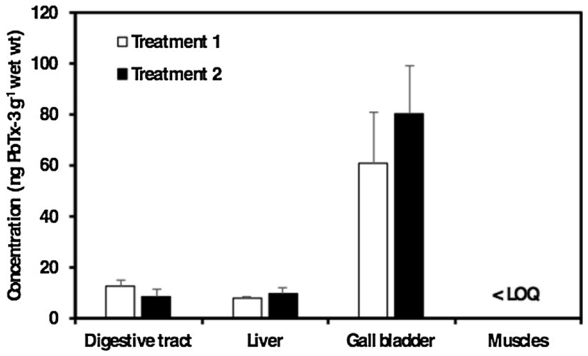

Fig. 2. Concentration of PbTx (expressed as PbTx-3 equiv.) in four body compartments (digestive tract, liver, gall bladder and muscles) after single (Treatment 1) or repeated exposure (Treatment 2) to the brevetoxin-containing gel food. Replicate samples (tissue types) were pooled within each treatment (n = 8) before extraction and analysis as explained in Section 2.3.1. Values are means ± SD calculated from replicate assays (n = 3).

these values do not indicate the potential intra-group variability among fish and thus cannot be statistically compared. In general, concentrations in composite samples ranged from ~70 ng PbTx-3 equiv g^{-1} (gall bladder) to 10 ng PbTx-3 equiv g^{-1} (liver and digestive tract) to values below the limit of quantification (i.e. 0.3 ng PbTx-3 equiv g^{-1}; muscle samples; Fig. 2, Table 2).

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

After a single (Treatment 1) and multiple (Treatment 2) dietary exposures to PbTx, the depuration kinetics of ^{54}Mn and ^{65}Zn were followed in juvenile turbot for 21 d. No growth or mortality was recorded and rates and quantity of food consumption remained constant throughout the experiment. The measured activity levels of each tracer in both radiolabelled gel foods (RLT and RNT) were 48 ± 1 and 37 ± 2 Bq g^{-1} for ^{54}Mn and 1059 ± 30 and 1012 ± 63 Bq g^{-1} for ^{65}Zn, respectively (Table 1). No activity was measured in the control turbot at any time confirming that the exposure to ^{54}Mn and ^{65}Zn was strictly through food and not through radiotracer recycling from seawater due to radiotracer leaching from the contaminated food or from fish depuration.

Depuration kinetics of ^{54}Mn and ^{65}Zn were most accurately described by 2-component exponential models (Fig. 3, Table 3; R^2 = 0.51 - 0.96). For both Mn and Zn, a large proportion (42–83%) of the ingested radiotracer was short-lived. The short-lived component was characterized by a very rapid loss with $T_{b1/2s}$ ranging from 0.27 to 0.35 d for Mn and 0.23–0.28 d for Zn. Examination of the long-lived component revealed that Zn (AEs: 16–18%) was less assimilated than Mn (AEs: 56–60%). For both elements, AEs calculated from the best fit model for each individual turbot revealed no significant effect of PbTx, whether for single or multiple exposures ($p > 0.05$, Fig. 3). For Mn, $T_{b1/2l}$ average values ranged from 23 to 73 d, suggesting long-term

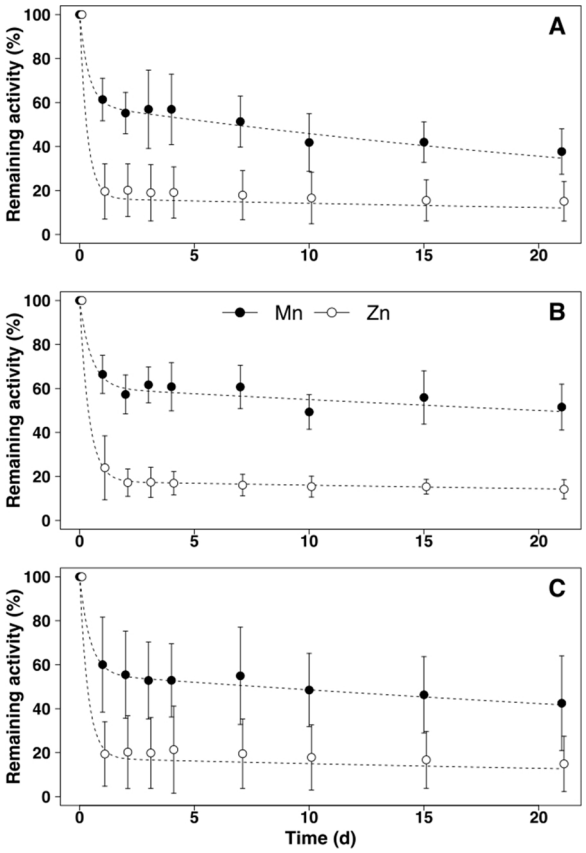

Fig. 3. Whole-body depuration of ^{54}Mn and ^{65}Zn in juvenile turbots (A) given a single feeding with PbTx, (B) given multiple feedings with PbTx and (C) non-exposed to toxins, which served as the control (n = 5-7). Parameters and statistics of depuration kinetics are given in Table 3.

retention of this trace element 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 $+\infty$.

4. Discussion

The most common routes of PbTx 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 subsequent toxin absorption across the gastrointestinal epithelia (Kimm-Brinson and Ramsdell, 2001). Although blooms of *K. brevis* are often associated with massive fish mortalities (Landsberg, 2002), PbTx accumulation and toxicological effects in this taxon have not been well characterized. PbTxs bind and activate voltage gated sodium channels (Na_v) that are present in most excitable cells, causing neurotoxic shellfish poisoning (NSP) in

Table 3
Depuration kinetic parameters of ^{54}Mn and ^{65}Zn in juvenile turbot (n = 5-7) (A) given a single feeding of algal toxins, (B) given multiple toxin feedings and (C) non-exposed to toxins (n = 5-7 per treatment). All fish were maintained for 21d in normal seawater after the radiotracer feeding. k_e: depuration rate constant (d^{-1}); $T_{b½}$: 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 (d^{-1})	$T_{b1/2s}$ ± ASE (d)	A_{0l} (=AE) ± ASE (%)	k_{el} ± ASE (d^{-1})	$T_{b1/2l}$ ± ASE (d)	
^{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}	+∞	0.94
	B	82.22 ± 3.31***	2.48 ± 0.47***	0.28 ± 0.05	17.79 ± 1.95***	0.011 ± 0.011^{NS}	+∞	0.94
	C	82.52 ± 3.27***	2.74 ± 0.60***	0.25 ± 0.06	17.48 ± 2.05***	0.015 ± 0.012^{NS}	+∞	0.96

mammals exposed through the diet (Flewelling et al., 2005; Landsberg et al., 2009). PbTxs are also potent ichthyotoxins targeting Na_v on fish tissues (Dechraoui et al., 2006). Binding of PbTx to Na_v induces cell Na^+ influx, altering cellular regulation through activation of ion pumps and/or channels (Baden, 1983), including Ca^{2+} channels. As suggested by previous works in fish, Ca^{2+} channel states are also involved in trace element membrane flux (Spry and Wood, 1989; Bury et al., 2003; Hogstrand 2011). Changes in such ion channels have therefore the potential to affect the distribution of trace elements such as Mn and Zn to their ultimate target tissue.

In this study, for all treatments, the observed assimilation efficiencies (AEs) of Mn and Zn were in accordance with values previously reported in the literature on turbots (Pouil et al., 2016, 2017; Mathews et al., 2008) and another flatfish species, the European plaice *Pleuronectes platessa* (Pentreath, 1976) fed with other species of prey. This comparison indicates the relative consistency in AEs, although the bioavailability likely varies between elements in prey tissues or subcellular fractions (Reinfelder and Fisher, 1994: Reinfelder et al., 1998; Zhang and Wang, 2006). Such results suggest, in the context of this study, that homogenization of the mussels and the use of gelatin do not appear to have an effect on trace element assimilation in turbot, as already demonstrated for an invertebrate predator (Wallace et al., 1998).

Contrary to expectations, we found that the assimilation of trace element in fish from food was not affected by either single or multiple dietary exposures to PbTx. Our toxin analysis data confirmed a dietary transfer of toxins from phytoplankton to mussels and from mussels to fish. The PbTx levels in tissues of the turbot (pooled samples for each tissue/treatment combination) were low for both treatments (consumption of mussel gel matrix with high: 525 ng g^{-1} or low: 188 ng g^{-1} PbTx-3 concentrations), suggesting low retention or rapid turnover in fish compartments after ingestion of the brevetoxins. Indeed, we found a relatively limited trophic transfer of PbTx from the gel food to juvenile turbots, where PbTx in fish 2 h after a single-feeding (188 ng g^{-1}) corresponded to 37% of the given dose (Table 2, sum of all body compartments). These low rates of toxin transfer to fish tissues in both treatments may in part explain the absence of differences in the AEs of Mn and Zn in turbots between the experimental treatments. However, a portion of the gel food and thus toxin dose given to the turbots was likely lost during the feeding process (small particles released in the water were observed during the feeding), potentially affecting the trophic transfer of PbTx.

Despite different toxin concentrations in the mussel-based gel foods and different numbers of feedings (single *vs.* 15 repeated exposures), PbTx concentrations in fish tissues appeared similar between fish with single or repeated exposure to brevetoxin-containing food. As composite measures in pooled tissues, however, significant differences cannot be assessed. These results suggest: (1) ingested PbTx is rapidly turned over, where the toxin is absorbed in the digestive tract and then quickly eliminated by the hepatobiliary system, a pathway that has been recognized as the key route for the metabolization and excretion of PbTx in fish (Landsberg, 2002); (2) PbTxs are not significantly re-distributed to organs that are not involved in digestion and excretion processes, such as muscle tissue. Although these pathways remain to be confirmed experimentally, Naar et al. (2007) found a similar pattern in field measurements on 42 species of fish exposed to *K. brevis* blooms in the field. PbTx concentrations in these fish were generally low or below the LOQ in the muscle (11–414 ng g^{-1}), but concentrations reached μg^{-1} levels in the liver and digestive tract (levels 10 and 30 times higher than in muscle tissue, respectively).

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, which are not natural prey of turbot (Florin and Lavados, 2010; Sparrevohn and Støttrup, 2008), were used for the preparation of the gel food. Therefore, the AEs observed in this study might not reflect actual assimilation of these trace elements in natural conditions. Furthermore, the PbTx concentrations used in the present study are relatively low (i.e. < 600 ng g^{-1}). Higher concentrations of PbTxs can be found in bivalves during *K. brevis* blooms in the natural environment (Landsberg et al., 2009), which could lead to greater physiological effects on their consumers including fish.

5. Conclusion

This study revealed no statistically significant differences in the trace element assimilation efficiencies of juvenile turbots after a single or multiple feedings of gel food containing natural levels of PbTx in comparison to control fish. Similarities observed indicate that the occurrence of PbTx in the food does not appear to affect the trophic transfer of either Mn or Zn, as essential elements, in juvenile fish. Nevertheless, since harmful algae can produce a variety of toxins with diverse modes of actions, further investigations are needed to better understand the influence of (1) higher dietary doses of PbTx, and (2) the possible synergistic effects of other biotoxins on essential element assimilation in fish.

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