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

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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 aims 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 temperatures (17 and 20 °C) and pH (7.5 and 8.0) regimes, under controlled laboratory conditions, and then fed with radiolabelled shrimp (^{57}Co, ^{65}Zn and ^{110m}Ag). Assimilation efficiencies of Co and Ag in juvenile turbot, determined after a 21-day 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-dependent and also modulated by metabolic processes.

Keywords Metal trophic transfer · Trace elements · Teleost · Ocean acidification · Global warming

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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 nanoparticles 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 and Wang 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 has 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 is poorly 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 drivers 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 seem to be, on 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 and 20 °C) and pH (7.5 and 8.0) after a single feeding with radiolabelled shrimp.

Materials and methods

Origin and acclimation of fish

Juvenile turbot *S. 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 12/12 h; 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 "Exposure of turbot via radiolabelled shrimp" section).

Juveniles were exposed under controlled temperature and pH conditions in a crossed experimental design (two temperatures × two pH levels). The two temperatures were 17 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 (FCE) ratio of juvenile turbot at 17.4 ± 0.5 °C at normal pH (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; 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 calibrated weekly using

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})	pCO_2 (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

Tris-HCl and NBS buffer solutions (Dickson et al. 2007). Total alkalinity was measured by titration using Metrohm 809 Titrando calibrated with NBS buffers, Tris-HCl (Batch 150, Dickson 2016) and reference materials (Batch 137, Dickson 2016). pCO_2 was determined from pH, temperature and total alkalinity measurements using the R package seacarb (Lavigne et al. 2011).

Experimental procedures

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 12/12 h; 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 0.1 M 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 four 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.

Exposure of turbot via radiolabelled shrimp

A total of eight acclimatized turbot were randomly selected for each experimental treatment (viz. 4 × 20-L tanks with each time eight organisms; wet weights were 22.4 ± 3.4, 22.1 ± 3.8, 22.3 ± 5.4 and 23.7 ± 4.2 g 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 anaesthetised 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, 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 radioanalysed 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.

Radioanalysis

The radioactivity of the tracers was measured using a high-resolution γ-spectrometer system composed of four 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 at the last days of depuration; at the beginning, average increase temperature and decrease of pH were negligible.

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 of the three studied elements were best fitted using a two-component exponential model:

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

where A_t and A_0 are the remaining activities (%) at time t (days) and 0 respectively; k_e is the depuration rate constant

(day^{-1}). "*s*" and "*l*" subscripts are related to the short-lived and long-lived components 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}). Because depuration of the assimilated fraction of the three studied elements was extremely slow, the long-term depuration rate constant (k_{el}) might not be significantly different from 0, then $T_{b1/2l}$ tends towards $+\infty$ 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

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

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 h 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).

Results

In order to evaluate whether different abiotic factors (i.e. temperature and pH) affect metal assimilation in the juvenile turbot *S. 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 "Materials 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 (Bq g^{-1} wwt) 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 days). Comparison of k_{es} determined for each individual turbot indicated that, for all the elements (Co, Zn and Ag), there is no significant difference (p_{ANOVA} >0.05, Fig. 2) independently of the pH and temperature conditions.

Estimated AEs in turbot ranged from 19 to 29% for Zn whereas Co and Ag were very poorly assimilated by turbot

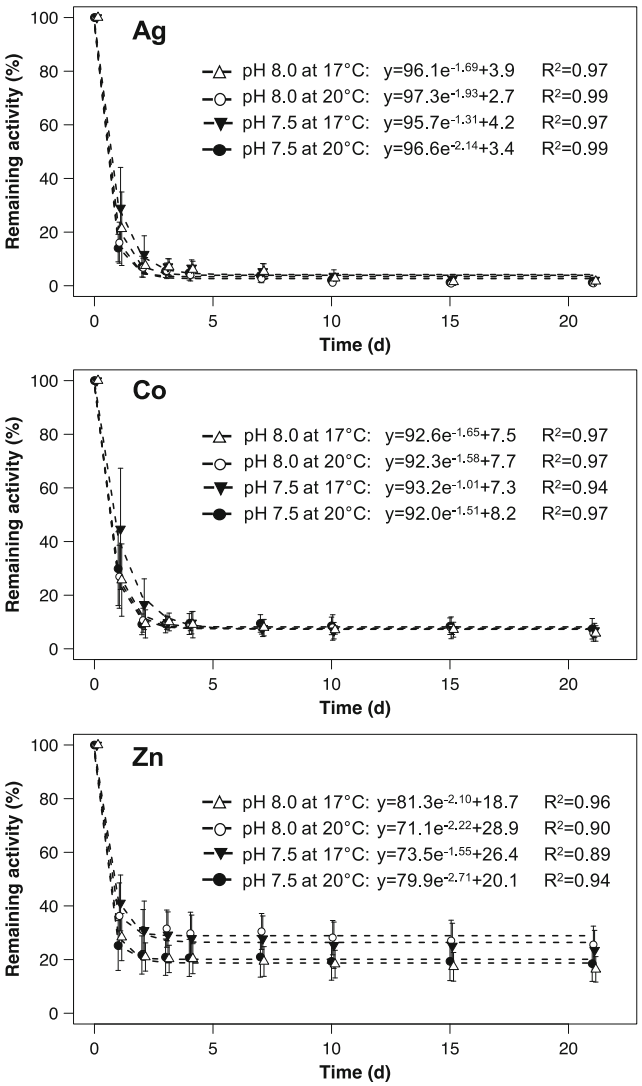

Fig. 1 Influence of temperature and pH (see details of experimental conditions in Table 1) on whole-body depuration of ^{110m}Ag, ^{57}Co and ^{65}Zn in juvenile turbot (n = 6–8; percent remaining activities, means ± SD)

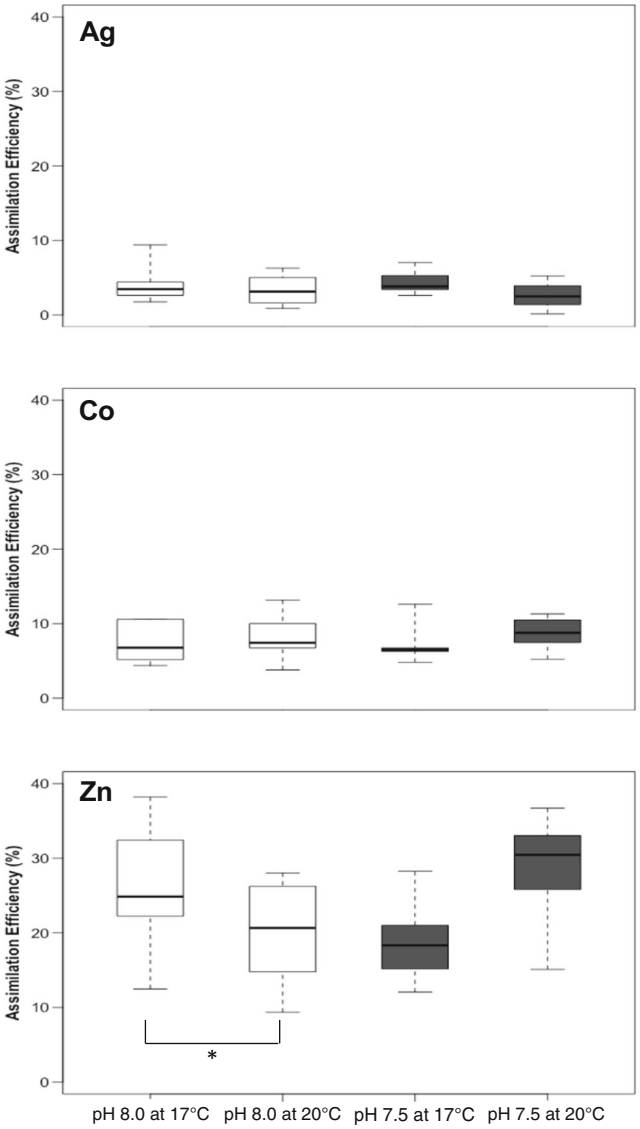

Fig. 2 Comparison of assimilation efficiencies (AEs) calculated for each individual turbot from the four experimental treatments. The best fitting model obtained for the entire set of turbots (see Fig. 1) was applied to individuals. *$p < 0.05$

(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 ($p_{ANOVA} = 0.03$; Fig. 2) but not at the lower pH.

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, 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 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 be due to either from the following: (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 turn, 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 freshwater fish: the common carp *Cyprinus carpio* (fed with Zn-contaminated prey; Van Campenhout et al. 2007). However, in marine fish, although a 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*. 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 investigated 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 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 the "Materials and methods" section). 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 an endpoint, but no effect of 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.

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 has a slight effect on Zn assimilation in the juvenile turbot *S. 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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