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Physiological effects of marine natural organic matter and metals in early life stages of the North Pacific native marine mussel *Mytilus trossulus*; a comparison with the invasive *Mytilus galloprovincialis*

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ABSTRACT

The role of seawater NOM in reducing metal toxicity for marine organisms is not well understood. We investigated the effects of five different marine NOMs (two autochthonous, one allochthonous, two of mixed origin, at 8 mg C/L), three metals (6 μg Cu/L; 20 μg Pb/L; 25 μg Zn/L), and combinations between them, to early life stages of *Mytilus trossulus* (a North Pacific native) in 48-h tests. Endpoints were whole body $Ca^{2+}+Mg^{2+}$-ATPase activity, carbonic anhydrase (CA) activity and lipid peroxidation. Comparisons were made with previously reported tests (identical conditions) on the invasive *M. galloprovincialis*. Unexposed *M. trossulus* had lower $Ca^{2+}+Mg^{2+}$-ATPase but similar baseline CA activity and lipid peroxidation to unexposed *M. galloprovincialis*. NOMs alone induced increased enzyme activities, and increased lipid peroxidation, but the latter did not occur with NOMs of mixed origin in *M. trossulus*. There was no clear difference in the sensitivity to various NOMs between species. In *M. trossulus*, all three metals by themselves caused increases in lipid peroxidation, as did many metal-NOM combinations. The origin of the NOMs influenced the nature of the responses to NOM-metal combinations in both species, but no clear relationship to NOM chemistry was apparent. Overall, *M. trossulus* was more sensitive to metals and NOM-metal combinations, with a greater number of significant responses (27 versus 22 treatment endpoints, out of a total of 72) and a greater proportion of negative effects (81% versus 50%) than in *M. galloprovincialis*. Therefore, marine NOMs by themselves, as well as metals by themselves and NOM-metal combinations, can induce both positive and negative physiological responses. Lipid peroxidation appears to be a particularly common negative response. In future studies, NOM quality and mussel species should be considered since native *M. trossulus* and invasive *M. galloprovincialis* exhibited markedly different responses after exposure to the same environmental conditions.

1. Introduction

Aquatic natural organic matter (NOM) originates from the decomposition of lignin-rich plant material and other dead organic biomass (allochthonous NOM), and may also be synthesized by aquatic microorganisms (autochthonous NOM) (Perdeu, 1998; Thurman, 1985). In marine waters, the former may dominate in inshore waters due to runoff from land, while the latter usually dominates in offshore waters. The major compounds of allochthonous NOMs are humic substances that generally represent 50–90% of total content (Benner, 2002). These compounds play important roles in physical, chemical and microbiological processes in aquatic ecosystems, as well in modulating the toxicity and bioavailability of metals. The free ionic forms of metals are responsible for most toxicity by binding to nutritive ion transport sites and key transport enzymes in the epithelial membranes of aquatic animals (Paquin et al., 2002; Wood, 2012). However, NOMs contain functional groups (e.g. carboxyl, amine, phenolic, thiol ligands) that will also bind metal ions, thereby reducing their bioavailability and toxicity to organisms (Al-Reasi et al., 2011; Baken et al., 2011; Perdeu, 1998; Tipping, 2002; Thurman, 1985).

In fresh water, the capacity of NOMs to protect against metal toxicity is clearly related to their chemical properties. NOMs of

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allochthonous origin appear to be more effective in this regard than the less aromatic NOMs of autochthonous origin, which tend to be richer in amino acid-like molecules such as tyrosine and tryptophan (Al-Reasi et al., 2011; Baken et al., 2011; De Schamphelaere and Janssen, 2004; Gheorghiu et al., 2010; Glover et al., 2005; Schwartz et al., 2004; Tipping, 2002; Xue and Sigg, 1999). However, in sea water, the situation is unclear. Some protective capacity against metal toxicity exists, and is variable in some studies according to the different sources of organic matter, but potential relationships between effects and the chemical properties of NOMs remain uncertain (DePalma et al., 2011; Nadella et al., 2009, 2013; Nogueira et al., 2017; Tait et al., 2016).

There is mounting evidence that NOMs alone, independent of their abilities to complex metals, may exert both positive and negative effects on the physiology of organisms (reviewed by Steinberg et al., 2003; Steinberg et al., 2006; Wood et al., 2011). For example, in freshwater animals, NOMs may bind to epithelial surfaces with subsequent alteration of their permeability, diffusive, active transport, and electrical properties (Campbell et al., 1997; Galvez et al., 2008; Glover et al., 2005; McGeer et al., 2002; Timofeyev et al., 2004; Vigneault et al., 2000). In seawater animals, there is little comparable information. However, our previous work (Nogueira et al., 2017) showed that natural marine NOMs from various sources differentially induced increases of enzyme activities and oxidative stress in early life stages of the mussel *Mytilus galloprovincialis*, and that these effects were more pronounced than those of metals (Cu, Pb, and Zn) alone. These results were surprising inasmuch as the early life stages of mussels are among the most sensitive organisms to metal toxicity in the marine environment (Arnold et al., 2010; US EPA, 2003).

Mytilus galloprovincialis originated in the Mediterranean, but is now an invasive species in the North Pacific, having been originally introduced by shipping, and now by extensive aquaculture; it appears to be displacing the native species *Mytilus trossulus* in some areas (Geller, 1999; Seed and Suchanek, 1992; Suchanek et al., 1997). Physiological differences between the species have been identified, such as greater tolerance of cold temperature and low salinity in *M. trossulus* and greater tolerance of high temperature and high salinity in *M. galloprovincialis*, so climate change may exacerbate the issue (Braby and Somero, 2006; Seed and Suchanek, 1992; Tomanek and Zuzow, 2010). In general, invasive species are often able to physiologically adapt to new habitats by being more tolerant of environmental stressors compared to a native species (Bax et al., 2003; Kelley, 2014; Sakai et al., 2001).

In the present study, we performed an identical set of experiments on early life stages of *M. trossulus* as in our previously published study on *M. galloprovincialis* (Nogueira et al., 2017). Both were conducted in the North Pacific at Bamfield Marine Sciences Centre with the offspring of locally collected parental animals. Our first goal was investigate the positive or negative effects caused by exposure to five different sources of marine NOM (two autochthonous, one allochthonous, and two mixed) and three different metals (Cu, Zn and Pb), both alone, and in all possible NOM-metal combinations, in *M. trossulus*. The endpoints analyzed were $Ca^{2+} + Mg^{2+}$-ATPase activity, carbonic anhydrase activity, and one oxidative stress parameter, lipid peroxidation. Our second goal was to directly compare the responses of the two species, an objective facilitated by the fact that the two investigations were performed simultaneously with the same experimental materials and conditions. Our general hypothesis was that the native *M. trossulus* would show a greater suite of physiological disturbances than the invasive *M. galloprovincialis*.

2. Material and methods

2.1. Collection of gametes and fertilization

Adults of the mussel *Mytilus trossulus* were collected from natural populations in the Broken Island Group, British Columbia (48.45N, 125.10W), where this is the only mussel species in the high intertidal zone (Akester and Martel, 2000). Adults were transferred to Bamfield Marine Sciences Centre (BMSC) and held in 30-L tanks at 10-15 °C in aerated seawater (30 ppt). To start the spawning, animals were exposed in groups to 22–25 °C until individuals started to release gametes, according to ASTM Standard Guide E-724-98 (2004). Spawning animals were immediately isolated into individual beakers and gamete viability checked. Eggs and sperm from several animals were pooled into a single beaker (~350 mL) containing 0.2-μm filtered 30-ppt sea water (constantly aerated) to initiate fertilization. Fertilization success was determined through a microscope (200× magnification; Olympus BH-2, Shinjuku, Japan) by identification of the cleavage initiation (2-cell or 4-cell stage). After 90% of the eggs were fertilized, the density of embryos per liter was counted in 1 mL under the microscope.

2.2. Experimental design

Embryos of *M. trossulus* were exposed to isolated natural organic matters (NOMs, separately, from five different sources), single metals (Cu, Pb, or Zn), and all possible combined treatments of single metals and single NOMs (summary details are given in Supplementary Material Table S1 of the present paper). The five different sources of NOMs were extracted followed the solid-phase method described by Rodriguez and Bianchini (2007). Full details on the NOM collection sites (four of which were from the local North Pacific area), isolation methods, and chemical characterization of the NOMs based on fluorescence properties are given in Nogueira et al. (2017). Two were characterized as clearly authochthonous (Offshore-CA and Offshore-BR), one as clearly allocthonous (Pachena), and two as "mixed" NOMs with intermediate properties (Bamfield and Port) (Nogueira et al., 2017; summary details are given in Supplementary Material Table S2 of the present paper). All treatments were performed in triplicate (i.e. N = 3 flasks of approximately 2500 embryos each) starting at about 1 h post-fertilization. All exposure tests were based on the acute toxicity test protocol for embryos of saltwater bivalve molluscs (ASTM Standard Guide E724-98, 2004). The isolated NOM exposures were conducted by adding either Port, Bamfield, Pachena, Offshore-CA or Offshore-BR NOM at a nominal concentration of 8 mg DOC/L to BMSC sea water. This NOM level was shown to induce physiological effects from NOM alone in our previous study (Nogueira et al., 2017). The combined NOM-metal exposures were performed by adding one of these NOMs (8 mg DOC/L) and either 6 μg Cu/L or 20 μg Pb/L or 25 μg Zn/L, added as $CuCl_2$ $2H_2O$, $Pb(NO_3)_2$ and $ZnSO_4$ These concentrations were chosen as measured EC_{20} values for Cu and Pb (Nadella et al., 2009, 2013) and EC_5 values for Zn in BMSC sea water for *M. trossulus* (Nadella et al., 2013). The latter differed because the concentration (25 μg Zn/L) was based on preliminary tests, whereas the true measured EC_{20} proved to be greater (69 μg Zn/L; Nadella et al., 2013). Embryos were also exposed in BMSC sea water without addition of NOMs or metals (control condition), and also exposed to single metals without addition of NOMs. All tests were run in 250-mL seawater exposure solutions, at a density of 10 individuals/mL and 15 °C for 48 h. According to the embryonic development of mussels, embryos should be in the D-veliger stage (larvae) after 48 h of fertilization. Thus, at the end of 48-h exposure, an aliquot was removed to check for mortalities and abnormalities (i.e. lack of proper development to the D-veliger stage) and the remaining volume was filtered using an 8-μm polycarbonate filter (Nucleopore Track-Etch Membrane PC MB 47 mm, Whatson PLC, Maidstone, U.K) under gentle vacuum. The filtered larvae were collected in vials and stored at −80 °C for later analyses.

2.3. $Ca^{2+} + Mg^{2+}$-ATPase activity

$Ca^{2+} + Mg^{2+}$-ATPase activity in the early life of *M. trossulus* was measured using an assay originally described by Vajreswari et al. (1983). Since the enzyme in *M. galloprovincialis* is rather unspecific in

its substrate specificity, being activated in the presence of either divalent cation (Nogueira et al., 2017), we have reported only the results obtained in the medium with both cations present (in mM: 80 NaCl; 5 $CaCl_2$; 5 $MgCl_2$; 20 Tris Base; 3 ATP; 1 ouabain; pH 7.6). The method is based on the amounts of inorganic phosphate (Pi) released after incubation (30 min) in the media. Samples were homogenized in a buffer solution (in mM: 150 sucrose; 50 imidazole; pH: 7.6). Pi concentration was determined using a commercial phosphate colorimetric assay kit (Fosfato; Doles, Goiânia, GO, Brazil). Protein concentrations in the embryo homogenates were determined using the method of Bradford (1976). The specific enzyme activities were expressed as μmol Pi/mg protein/h.

2.4. Carbonic anhydrase activity

Carbonic anhydrase (CA) activity was measured using a method based on the enzyme-catalyzed hydration of the carbon dioxide present in a CO_2-saturated solution, with subsequent release of H^+ and consequent reduction of the pH (Henry, 1991). The larvae were homogenized in the same buffer as used for $Ca^{2+} + Mg^{2+}$-ATPase measurements, and an aliquot was added to the reaction solution (in mM: 225 mannitol; 75 sucrose; 10 Tris Base; 10 Na_2HPO_4). The reaction was started by the addition of an aliquot of a CO_2-saturated solution, and the pH was measured every 5 s up to 30 s using a combination glass electrode (pH HI 1110B, Hanna, Rhode Island, USA) and a meter (pH 21, Hanna, Rhode Island, USA). To separate the rate of uncatalyzed CO_2 hydration from the CA-catalyzed hydration, the pH of the CO_2-saturated solution was monitored over time after the addition of an equal volume of homogenate-free buffer. The slope value of the linear regression for this blank was subtracted from that of our samples. The blank-corrected slope of the linear regression for the pH change over time in the homogenate sample yielded the enzyme activity which was normalized to the protein content in the cell homogenate, as determined by the method of Bradford (1976). The specific enzyme activity was expressed as an arbitrary unit of carbonic anhydrase (slope of the linear regression)/mg protein.

2.5. Measurement of thiobarbituric acid-reactive substances (TBARS)

Lipid peroxidation was measured according to Oakes and Vander Kraak (2003). Embryo samples were homogenized in 1.15% KCl containing 35 mM butylated hydroxytoluene (BHT), then added to the reaction mixture containing sodium dodecyl sulfate (SDS, 8.1%; 20 μL), 20% acetic acid (pH 3.5; 150 μL), thiobarbituric acid 0.8% (TBA; 150 μL), and 67 mM BHT (20 μL). The mixture was incubated at 95 °C for 30 min. Ultrapure water (100 μL) and n-butanol (500 μL) were then added, followed by centrifugation at 3000 rpm for 10 min at 15 °C. The malondialdehyde (MDA) produced during the lipoperoxidation process with thiobarbituric acid was read in the organic phase which was placed in a fluorescence microplate reader (excitation: 515 nm; emission: 553 nm; Victor 2, Perkin Elmer, Waltham, MA, USA). The concentration of thiobarbituric acid reactive substances (TBARS) was expressed as nmol MDA/mg protein, based on protein concentration in the homogenate determined by the method of Bradford (1976).

2.6. Statistical analyses

Data have been expressed as means ± standard error (n = 3) where each sample (n) represents a pool of ~2500 embryos of *M. trossulus*. Effects of individual NOM exposures (without addition of metals) in larvae were calculated by one-way analysis of variance (ANOVA) followed by Tukey's test. ANOVA assumptions (data normality and homogeneity of variances) were checked and the data transformed as necessary to satisfy these assumptions. For simplicity, as there are so many possible comparisons, the differences among NOMs have not been marked. On the Figures, asterisks (*) have been used to indicate only significant differences between NOMs and the absolute control condition (BMSC sea water with no added NOM). Student's two tailed t-tests, were used to evaluate the effect of each particular metal in a particular NOM exposure, by comparing the presence and absence of metal in the exposure within the same source of NOM. Significant differences have been marked with # signs. The significance level adopted was 95% ($\alpha = 0.05$).

Table 1
Percentage of normal development (i.e. lack of dead or abnormally developed larvae) after the early life stages of *M. trossulus* were exposed for 48 h to five different sources of NOM (~8 mg C/L). No significant differences were observed between NOMs and the control treatment (no NOM). Means ± 1 SEM (N = 5).

	%
No NOM	84.1 ± 5.0
Port	82.8 ± 6.1
Bamfield	80.7 ± 3.5
Pachena	74.0 ± 11.8
Offshore-CA	82.5 ± 1.5
Offshore-BR	77.4 ± 6.4

3. Results

3.1. NOM toxicity

Early life stages of *M. trossulus* under control treatments (no NOMs added) had 84.1% ± 5.0 normal D-veliger larvae at the end of 48 h of exposure. The addition of five different sources of NOMs did not significantly change this parameter (Table 1).

3.2. Isolated effects of NOMs

NOM exposures by themselves, in the absence of added metals, had significant effects in 5 of the 15 treatment endpoints tested (marked with *) in the early life stages of *M. trossulus*. Both of the autochthonous NOMs (Offshore-BR and Offshore-CA) and the allochthonous NOM (Pachena) significantly increased the lipid peroxidation when compared to sea water lacking added NOM (Fig. 1C). $Ca^{2+} + Mg^{2+}$-ATPase activity and CA activity increased significantly after being exposed to Offshore-BR (Fig. 1A) and Bamfield NOM (Fig. 1B), respectively.

3.3. Isolated effects of metals

Isolated Cu, Zn and Pb exposures, in the absence of added NOMs, exerted significant effects in 4 of the 9 treatment endpoints tested (marked with # to the left of the dotted lines) in the early life stages of *M. trossulus*. None of these effects were inhibitory on enzyme activities, but Pb significantly increased CA activity (Fig. 2B). Additionally, all the metals significantly increased lipid peroxidation (Fig. 1C, Fig. 2C and Fig. 3C).

3.4. Combined effects of NOMs and metals

When Cu, Pb, or Zn were combined with the various NOMs, 18 of the 45 treatment endpoints (marked with # to the right of the dotted lines) were significantly affected. $Ca^{2+} + Mg^{2+}$-ATPase and CA activities decreased significantly when each of the metals was tested in combination with Offshore-BR (Fig. 1A, B, Fig. 2A, B and Fig. 3A), with exception of carbonic anhydrase and Zn (Fig. 3B). However, the only enzymatic response involving the other autochthonous NOM (Offshore-CA) was a significant reduction in $Ca^{2+} + Mg^{2+}$-ATPase in the presence of Pb (Fig. 2A). Besides that, $Ca^{2+} + Mg^{2+}$-ATPase activity significantly decreased when Zn was combined with Port (Fig. 3A), and CA activity also decreased significantly when Bamfield NOM was combined with

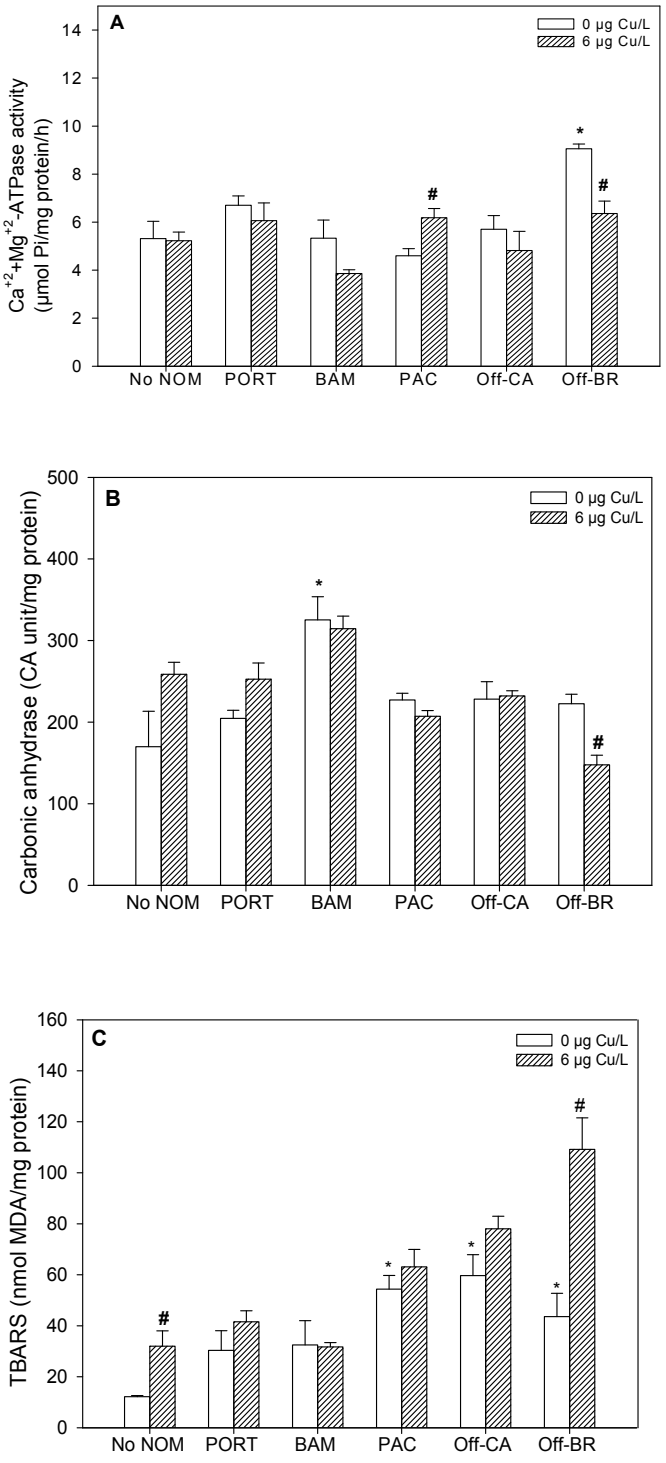

Fig. 1. Effects of copper (6 µg/L) in presence or absence of different seawater NOMs on (A) $Ca^{2+} + Mg^{2+}$-ATPase activity, (B) carbonic anhydrase activity, and (C) lipid peroxidation, quantified as TBARS, in the early life stages of *M. trossulus* exposed for 48 h from the beginning of development. The control condition (Bamfield sea water with no added NOM or metal) and isolated NOM exposures alone (no added metal) are represented by open bars. Treatments in which copper was added are represented by hatched bars. Mean values which are significantly different from their respective NOM alone controls are marked with #; mean values for isolated NOM exposures alone which are significantly different from the absolute control condition are represented by an asterisk. PORT = Port; BAM = Bamfield; PAC = Pachena; Off-CA = Offshore Canada; Off-BR = Offshore Brazil. Data are means ± 1 standard error (N = 3 replicates of 2500 larvae each).

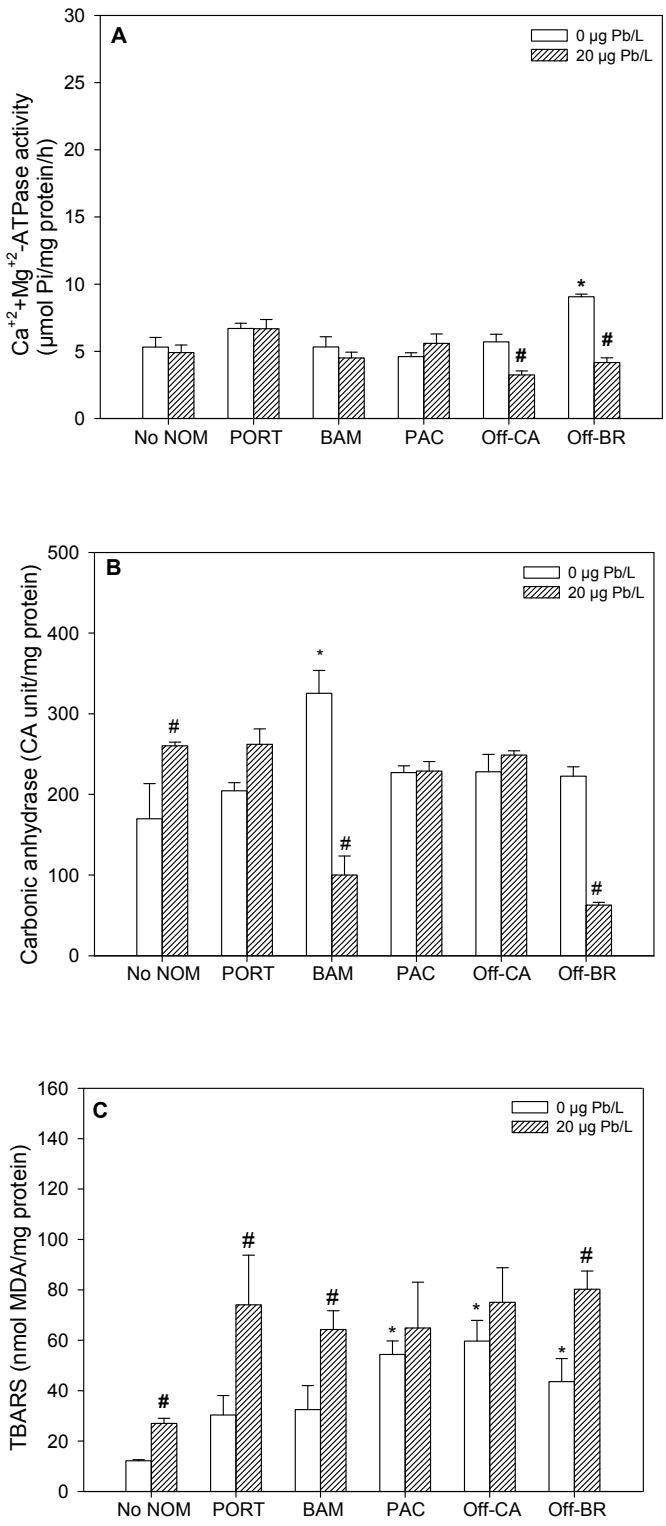

Fig. 2. Effects of lead (20 µg/L) in presence or absence of different seawater NOMs on (A) $Ca^{2+} + Mg^{2+}$-ATPase activity, (B) carbonic anhydrase activity, and (C) lipid peroxidation, quantified as TBARS, in the early life stages of *M. trossulus* exposed for 48 h from the beginning of development. The control condition (Bamfield sea water with no added NOM or metal) and isolated NOM exposures alone (no added metal) are represented by open bars. Treatments in which copper was added are represented by hatched bars. Mean values which are significantly different from their respective NOM alone controls are marked with #; mean values for isolated NOM exposures alone which are significantly different from the absolute control condition are represented by an asterisk. PORT = Port; BAM = Bamfield; PAC = Pachena; Off-CA = Offshore Canada; Off-BR = Offshore Brazil. Data are means ± 1 standard error (N = 3 replicates of 2500 larvae each).

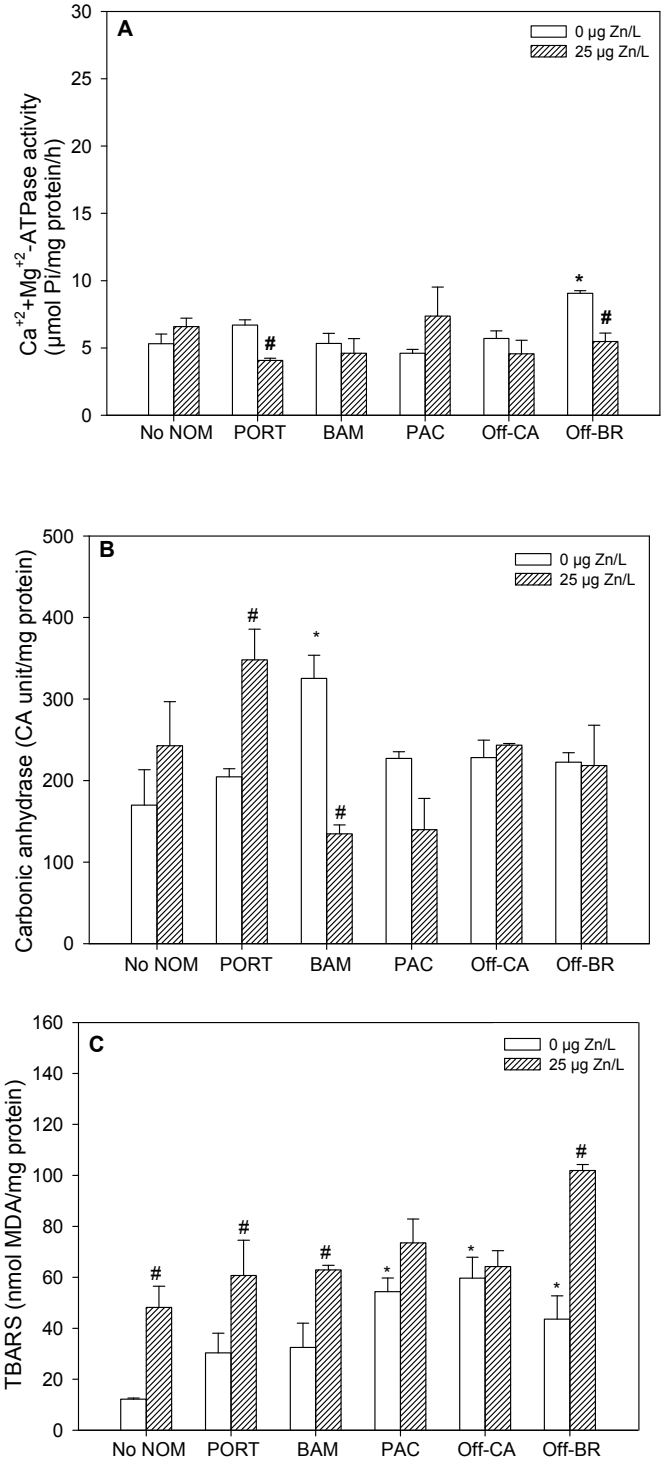

Fig. 3. Effects of zinc (25 µg/L) in presence or absence of different seawater NOMs on (A) $Ca^{2+}+Mg^{2+}$-ATPase activity, (B) carbonic anhydrase activity, and (C) lipid peroxidation, quantified as TBARS, in the early life stages of *M. trossulus* exposed to for 48 h from the beginning of development. The control condition (Bamfield sea water with no added NOM or metal) and isolated NOM exposures alone (no added metal) are represented by open bars. Treatments in which copper was added are represented by hatched bars. Mean values which are significantly different from their respective NOM alone controls are marked with #; mean values for isolated NOM exposures alone which are significantly different from the absolute control condition are represented by an asterisk. PORT = Port; BAM = Bamfield; PAC = Pachena; Off-CA = Offshore Canada; Off-BR = Offshore Brazil. Data are means ± 1 standard error (N = 3 replicates of 2500 larvae each).

either Pb (Fig. 2B) or Zn (Fig. 3B). The addition of Cu and either of these two "mixed" NOMs did not affect the enzymes tested (Fig. 1A and B).

As already mentioned, exposure to each of the three metals alone increased lipid peroxidation in the early life stages of *M. trossulus*. Therefore, possible protective effects of different sources of NOMs could be evaluated against Cu, Zn and Pb. There were none. "Mixed" NOMs (Bamfield and Port) and Offshore-BR did not protect against the effects of either Pb (Fig. 2C) or Zn (Fig. 3C); indeed the extent of lipid peroxidation increased significantly. The combination of Offshore-BR with either Cu (Fig. 1C), Pb (Fig. 2C), or Zn (Fig. 3C) also further increased lipid peroxidation.

4. Discussion

4.1. Overview

The five different sources of NOMs, three metals, and combinations between them altered the different physiological endpoints analyzed in the early life stages of *M. trossulus*. The effects were interpreted using the same criteria as used previously for the early life stages of *M. galloprovincialis* (Nogueira et al., 2017). A stimulation of either $Ca^{2+}+Mg^{2+}$-ATPase or CA activity was defined as a positive effect, while the stimulation of lipid peroxidation was defined as a negative effect. In numbers, the overall significant effects represented 27 of 72 total treatment endpoints, only 5 of which were positive and 22 were negative (Table 2A, B, C). Compared to *M. galloprovincialis* where 22 of 72 total treatment endpoints exhibited significant changes (11 positive, 11 negative; Nogueira et al., 2017), there were more significant effects, and a much greater proportion of negative effects in *M. trossulus* (Table 2A, B, C). Therefore our original hypothesis that the native *M. trossulus* would show a greater suite of physiological disturbances than the invasive *M. galloprovincialis* was supported. On closer examination, all three metals by themselves caused significant increases in lipid peroxidation in *M. trossulus* (Table 2B), effects which were not seen in *M. galloprovincialis,* and these were exacerbated to a greater extent by the presence of NOMs in *M. trossulus* (Table 2C) so the hypothesis was strongly supported for the impact of metals, both alone and in combination with NOMs, on the lipid peroxidation endpoint. In addition to the differences in the number of treatment endpoints affected, there was also a much lower baseline level of Ca^{2+} + Mg^{2+}-ATPase activity in *M. trossulus* (Table 3), and in the details of the responses (Table 2A, Table 3B and 3C), as will be discussed subsequently.

4.2. Isolated effects of NOMs

In the present study, NOMs induced only two positive physiological effects in larvae of *M. trossulus*: increased $Ca^{2+}+Mg^{2+}$-ATPase activity with Offshore-BR NOM and increased CA activity with Bamfield NOM. Qualitatively similar physiological responses were seen with these same two NOMs in the congeneric *M. galloprovincialis* (Table 2A) and could be attributed to the nutritive function of organic matter. It is well known that larvae of bivalves are able to take up dissolved organic matters such as glycine and other amino acids (Pütter, 1909; Manahan and Crisp, 1983), though the quantitative importance as a source of nutrition to larval and early settlement stages has been controversial (Jorgensen, 1976).

Increased lipid peroxidation was observed when *M. trossulus* early life stages were exposed to both autochthonous (optically light NOMs: Offshore-CA and Offshore-BR) and allochthonous (optically dark NOM: Pachena), and again this response pattern was the same as in *M. galloprovincialis* (Table 2A). Potentially these could reflect either external or internal effects of NOMs. NOMs are well known to catalyze the formation of H_2O_2 in water (Scully et al., 1996; Johannsson et al., 2016) and H_2O_2 can be taken up across epithelia into organisms, causing oxidative damage (Abele et al., 1998; Da Rosa et al., 2008).

Table 2
Comparison of significant (P < .05) changes in treatment endpoints induced by various NOMs by themselves (A), by metals by themselves (B), and by combination among them (C) in the early life stages of *M. trossulus* (*Mt*) *versus M. galloprovincialis* (*Mg*). Experiments with the two species were performed under identical conditions. Arrow up indicates a significant increase in the endpoint; arrow down means a significant decrease in the endpoint. Increases in Ca^{2+} + Mg^{2+}-ATPase and carbonic anhydrase activities are interpreted as positive effects, and increases in lipid peroxidation as negative effects (and *vice versa*). All *M. galloprovincialis* data are from Nogueira et al. (2017).

(A)

	Port		Bamfield		Pachena		Offshore-CA		Offshore-BR	
	Mt	*Mg*	*Mt*	*Mg*	*Mt*	*Mg*	*Mt*	*Mg*	*Mt*	*Mg*
Ca^{2+} + Mg^{2+}-ATPase									↑	↑
Carbonic anhydrase			↑	↑		↑		↑		
Lipid peroxidation					↑	↑	↑	↑	↑	↑

(B)

	Cu		Pb		Zn	
	Mt	*Mg*	*Mt*	*Mg*	*Mt*	*Mg*
Ca^{2+} + Mg^{2+}-ATPase						
Carbonic anhydrase		↑	↑	↑		
Lipid peroxidation	↑		↑		↑	

(C)

	Port						Bamfield					
	Cu		Pb		Zn		Cu		Pb		Zn	
	Mt	*Mg*	*Mt*	*Mg*	*Mt*	*Mg*	*Mt*	*Mg*	*Mt*	*Mg*	*Mt*	*Mg*
Ca^{2+} + Mg^{2+}-ATPase					↓			↓				
Carbonic anhydrase		↑		↑	↑	↑		↑	↓		↓	↑
Lipid peroxidation			↑		↑			↑	↑	↑	↑	↑

	Pachena						Offshore-CA					
	Cu		Pb		Zn		Cu		Pb		Zn	
	Mt	*Mg*	*Mt*	*Mg*	*Mt*	*Mg*	*Mt*	*Mg*	*Mt*	*Mg*	*Mt*	*Mg*
Ca^{2+} + Mg^{2+}-ATPase	↑								↓			↓
Carbonic anhydrase												↓
Lipid peroxidation						↓						

	Offshore-BR					
	Cu		Pb		Zn	
	Mt	*Mg*	*Mt*	*Mg*	*Mt*	*Mg*
Ca^{2+} + Mg^{2+}-ATPase	↓		↓		↓	
Carbonic anhydrase	↓	↓	↓			
Lipid peroxidation	↑		↑		↑	

Alternately or additionally, NOMs themselves may be taken up by the organisms (Steinberg et al., 2003; Timofeyev et al., 2004) and these compounds or their metabolites could produce reactive oxygen species internally. Some earlier studies have shown alterations in oxidative equilibrium in freshwater amphipods exposed to different sources of freshwater NOMs (Timofeyev et al., 2004, 2006; Timofeyev and Steinberg, 2006). However, a pattern of NOM effects on marine mussel larvae begins to be delineated from the results of our present and previous studies. Both *M. trossulus* and *M. galloprovincialis* presented an increase of lipid peroxidation in response to the same sources of NOM exposure (Table 2A), indicating the quality of NOM as an important aspect in causing negative effects. It is important to note that the two NOMs of intermediate properties (Port and Bamfield) did not affect lipid peroxidation in either congener, so it may be that autochthonous and allochthonous properties tend to cancel each other out in the mixed inshore waters where mussels are most abundant. Overall, there appeared to be no difference in the sensitivity to various NOMs between the native *M. trossulus* and the invasive *M. galloprovincialis*.

Table 3
Comparison of the background levels of Ca^{2+} + Mg^{2+}-ATPase activity, carbonic anhydrase activity and lipid peroxidation between *M. trossulus* and *M. galloprovincialis*. Mean values (± standard error) represent larvae maintained in Bamfield seawater with no added NOM (N = 3 replicates of 2500 larvae each). Asterisk indicates significant difference (P < .05) between the two species.

	M. trossulus	*M. galloprovincialis*
Ca^{+2} + Mg^{+2}-ATPase (μmolPi/mgprotein/h)	4.4 ± 0.8	10.3 ± 1.9*
Carbonic anhydrase (CA unit/mg protein)	169.8 ± 43.5	108.8 ± 12.8
Lipid peroxidation (nmolMDA/mg protein)	12.2 ± 0.4	15.9 ± 3.3

4.3. Isolated metal effects

Our initial expectations were inhibitory effects of metals on Ca^{2+} + Mg^{2+}-ATPase and CA activities, and stimulatory effects on lipid peroxidation. Only the latter expectations were fulfilled. Metals are well known to cause ionoregulatory disturbances due the competition for binding sites on cell membranes and inhibition of transport enzymes related to ionoregulation (e.g. carbonic anhydrase, Na^+,K^+-ATPase, various Ca^{2+} activated ATPases etc.; Dudev and Lim, 2014; Paquin et al., 2002; Wood, 2012). While these effects are best documented in freshwater animals, they have also been seen in seawater organisms (e.g. Grosell et al., 2007; Nogueira et al., 2013; Tellis et al., 2014a, 2014b; Zimmer et al., 2012). However, the only observed enzymatic alteration caused by a metal alone in the present study was stimulation of CA activity after *M. trossulus* larvae were exposed to Pb. Similar CA stimulation after metal exposure was seen in *M. galloprovincialis* larvae exposed to Pb (and also Cu; Table 2B), while Ca^{2+} + Mg^{2+}-ATPase activity was stimulated at some times (and inhibited at others) in purple sea urchin larvae exposed to various metals (Tellis et al., 2014a, 2014b). In these circumstances, enhanced enzymatic activity may be a compensatory response to earlier enzyme inhibition or developmental delay. Due to damage-repair and acclimation processes, metal effects on enzyme activities are generally both time- and concentration-dependent (Jorge et al., 2013; Lionetto et al., 2006, 2012; McDonald and Wood, 1993), and this is probably one of the explanations for the lack of negative effects observed.

Despite the absence of inhibition of either Ca^{2+} + Mg^{2+}-ATPase or CA activities, the early life stages of *M. trossulus* exhibited a significant increase of lipid peroxidation after exposure to all three metals tested. These alterations in oxidative stress response represent a key differentiation among the congeners, because *M. galloprovincialis* did not show this response (Table 2B).

4.4. Combined NOM-metal effects

In *M. trossulus*, there were only two positive effects of NOM-metal combinations on enzyme activity (Cu *plus* Pachena on Ca^{2+} + Mg^{2+}-ATPase, Zn *plus* Port on CA), while the majority of the significant effects from the combinations between NOMs from different sources and metals were negative – i.e. decreases in Ca^{2+} + Mg^{2+}-ATPase and CA activities back to basal levels and exacerbation of lipid peroxidation. In general these results differ from those in *M. galloprovincialis* where there were fewer significant effects, more of which were positive, such as further stimulation of enzyme activity and reduction of lipid peroxidation (Table 2C). Certainly, there was less exacerbation of lipid

peroxidation by combinations of metals and NOM in *M. galloprovincialis*, though in both species, Bamfield NOM seemed to be most potent in this regard (Table 2C).

The reduced enzyme activities caused by the combined treatments in *M. trossulus* could be explained in two ways. Firstly, they may be direct negative effects of NOMs, but this seems most unlikely as they were not seen when these same NOMs were tested alone. Secondly, NOMs, may have reduced the bioavailability of metals by binding them, such that the compensatory increase in enzyme activity in response to metals never occurred in the first place. While this seems a more probable explanation, we would have expected the most allochthonous and aromatic NOM (Pachena) to have the greatest attenuating effects, and the most autochthonous, least aromatic NOMs (Off-CA, Off-BR) to have the least attenuating effects, as explained in the Introduction. This was not seen, though an important caveat is that this explanation depends on the metal binding properties of NOMs in fresh water, whereas the situation in sea water remains uncertain.

With respect to the exacerbation of lipid peroxidation by combinations of metals and NOM in *M. trossulus,* the first explanation offerred above may apply. In all cases, the metals alone caused oxidative damage, and in many cases, the NOMs alone caused oxidative damage, so the combined effects could be additive or even synergistic direct effects. These effects were not seen in all combinations (e.g. Pachena NOM did not increase the peroxidative effect of any metal; Port, Bamfield and Offshore-Ca NOMs did not increase the peroxidative effects of Cu; and Offshore-Ca did not increase the peroxidative effects of Pb and Zn), but it is difficult to see any patterns related to the chemistry of the NOMs (Supplementary Table S1). Further studies are needed on the chemical behaviour of different NOMs in sea water.

4.5. Native versus invasive species, and regulatory perspectives

Based on their different geographic origins (*M. trossulus* from the North Pacific, *M. galloprovincialis* from the Mediterranean; Geller, 1999; Seed and Suchanek, 1992; Suchanek et al., 1997), these congeners would be expected to differ in their sensitivities to habitat temperature and salinity, and this has been confirmed by experimental tests (Braby and Somero, 2006; Seed and Suchanek, 1992; Tomanek and Zuzow, 2010). The present data demonstrate that they also differ in their baseline levels of Ca^{2+} + Mg^{2+}-ATPase activity (Table 3). More importantly, the current results suggest that they also differ in their sensitivity to toxic metals, both alone and in combination with NOMs, as demonstrated by a greater incidence of negative physiological responses, especially lipid peroxidation in the native species, *M. trossulus* (Table 2A,B,C). It is important to point out that the analyses were performed together by the same researchers, using the same equipment, batch of reagents, and period of analysis, which excludes any possibility of methodological differences. Both species were harvested from the same general area, and the levels of Cu, Pb, and Zn tested were well above the normal concentrations expected in such environments (Grosell, 2012; Hogstrand, 2012; Mager, 2012). The greater sensitivity of the native species to environmental stressors (in this case metals, and metal-NOM combinations) compared to the invasive species, may be an important factor which favours the range extension of the latter (Bax et al., 2003; Kelley, 2014; Sakai et al., 2001). Note however that this conclusion is based on sublethal physiological responses, not mortality. Nevertheless, current environmental policy in most jurisdictions would be to protect both the native *M. trossulus* and the invasive *M. galloprovincialis*, because the latter is of great importance in aquaculture.

The metal levels tested (Cu at 6 µg/L, Pb at 20 µg/L and Zn at 25 µg/L) were fairly close to regulatory guidelines in many jurisdictions (see Grosell, 2012; Hogstrand, 2012; Mager, 2012; US EPA, 1996; US EPA, 2009). The present results suggest that regulatory guidelines for these metals, which are based on either acute or chronic toxicity, may not protect against sublethal effects in molluscan larvae, and that NOM (DOC) may not protect against and indeed may exacerbate such

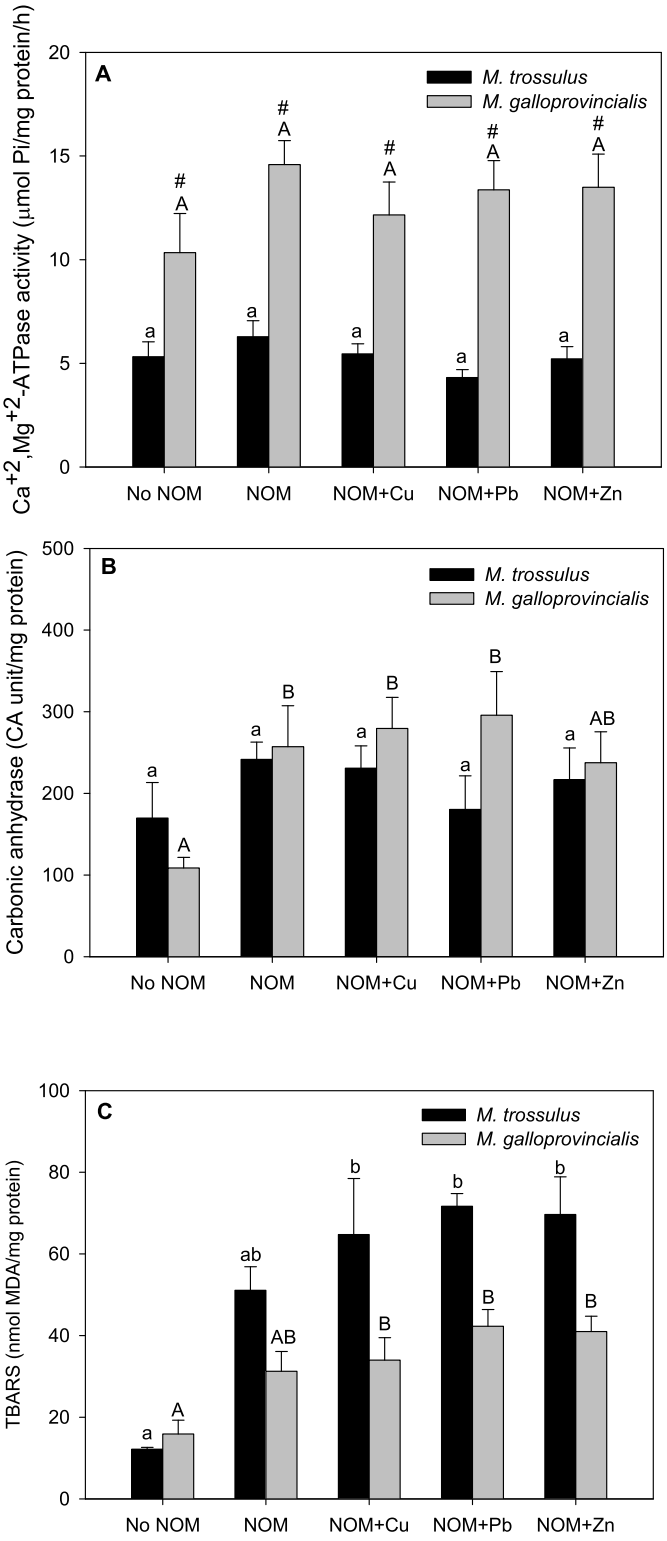

Fig. 4. Comparison between *M. trossulus* and *M. galloprovincialis* where all NOMs were considered the same and pooled. (A) $Ca^{2+}+Mg^{2+}$-ATPase activity, (B) carbonic anhydrase activity, and (C) lipid peroxidation of *M. trossulus* (black bars) and *M. galloprovincialis* (grey bars) larvae exposed to copper (6 µg/L), lead (20 µg/L), or zinc (25 µg/L) for 48 h from the beginning of development. No NOM, control condition (Bamfield sea water with no added NOM); NOM, average of all NOM exposures; NOM + Cu, natural organic matter plus copper; NOM + Pb, natural organic matter plus lead; NOM + Zn, natural organic matter plus zinc. Small letters indicate statistical differences between treatments in *M. trossulus*; Capital letters indicate statistical differences between treatments in *M. galloprovincialis*; # represent significant difference ($P < .05$) between species in the same treatment. Data are means ± standard error (N = 15 replicates of 2500 larvae each). All *M. galloprovincialis* data are from Nogueira et al. (2017).

sublethal effects At present, computational models such as the Biotic Ligand Model (DiToro et al., 2001; Niyogi and Wood, 2004; Paquin et al., 2002; Santore et al., 2001) which are used to predict the site-specific toxicity of metals or to derive ambient water quality criteria (e.g. US EPA, 2009) treat all NOMs as the same. Organic matter is simply represented as a dissolved organic carbon (DOC), and the chemical composition of the NOM is not taken into account. Therefore we grouped the data considering all NOMs as a single source, resulting in five different treatments: No NOM, NOMs, NOMs *plus* Cu, NOMs *plus* Pb, NOMs *plus* Zn. The comparison was performed among treatments and between *M. trossulus* and *M. galloprovincialis* (Fig. 4). The pooled data could not be normalized by standard transformations, so a one-way non-parametric ANOVA was used for each species, followed by multiple comparisons tests in all endpoints among treatments, whereas differences between species for common treatments were evaluated with Student's t-tests. Overall, $Ca^{2+}+Mg^{2+}$-ATPase activities (Fig. 4A) were unaffected by the presence or absence of either NOM or metals in both species, but were significantly higher in all treatments in *M. galloprovincialis* relative to *M. trossulus*, consistent with the difference in baseline levels of Table 3. CA activities (Fig. 4B) were unaffected by the presence or absence of either NOMs or metals in *M. trossulus*, but in *M. galloprovincialis* were significantly elevated by the presence of NOM, independent of the presence or absence of metals. There were no interspecies differences. Lipid peroxidation (Fig. 4C) showed a marked increase in the presence of NOMs in both species, regardless of the presence or absence of metals, with overall higher levels in *M. trossulus*. Thus, many variations in physiological effects were lost when the different origins of the NOMs were not considered, but important overall differences between the species were reinforced.

5. Conclusion

Results from the present and previous studies (Nogueira et al., 2017) allow a better understanding of differences in physiological responses of the two congeners. The native species (*M. trossulus*) was clearly more sensitive than the invasive species *M. galloprovicialis*. For the first time in the literature, a pattern of negative effects caused by two specific sources of seawater NOMs (allochthonous and autochthonous) occurred in two different species of marine animals. Lipid peroxidation proved to be the best biomarker to represent these effects. Besides that, the sensitivity of the native species (*M. trossulus*) to metal exposure allowed us to observe the non-protection by “mixed” NOMs. Thus, our results reinforce the importance of both the quality of seawater NOMs and the target species (native or invasive) in assessing the impacts of metals and NOMs, both alone and in combination, on the early life stages of marine mussels.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.marenvres.2017.12.009.

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