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Indirect effects of climate changes on cadmium bioavailability and biological effects in the Mediterranean mussel *Mytilus galloprovincialis*

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HIGHLIGHTS

- Effects of multiple stressors were analyzed in tissues of *M. galloprovincialis*.
- Temperature and acidification did not affect cadmium accumulation.
- Synergistic effects of multiple stressors occurred at cellular level.
- Mechanisms of action can modulate tissue-specific metabolic functions.
- Variations at cellular level may indicate species responsiveness to multiple stressors.

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ABSTRACT

Despite the great interest in the consequences of climate change on the physiological functioning of marine organisms, indirect and interactive effects of rising temperature and pCO_2 on bioaccumulation and responsiveness to environmental pollutants are still poorly explored, particularly in terms of cellular mechanisms. According to future projections of temperature and pH/pCO_2, this study investigated the main cellular pathways involved in metal detoxification and oxidative homeostasis in Mediterranean mussels, *Mytilus galloprovincialis*, exposed for 4 weeks to various combinations of two levels of pH/pCO_2 (8.2/~400 μatm and 7.4/~3000 μatm), temperature (20 and 25 °C), and cadmium addition (0 and 20 μg/L). Bioaccumulation was increased in metal exposed organisms but it was not further modulated by different temperature and pH/pCO_2 combinations. However, interactions between temperature, pH and cadmium had significant effects on induction of metallothioneins, responses of the antioxidant system and the onset of oxidative damages, which was tissue dependent. Multiple stressors increased metallothioneins concentrations in the digestive gland revealing different oxidative effects: while temperature and cadmium enhanced glutathione-dependent antioxidant protection and capability to neutralize peroxyl radicals, the metal increased the accumulation of lipid peroxidation products under acidified conditions. Gills did not reveal specific effects for different combinations of factors, but a general stress condition was observed in this tissue after various treatments. Significant variations of immune system were mainly caused by increased temperature and low pH, while co-exposure to acidification and cadmium enhanced metal genotoxicity and the onset of permanent DNA damage in haemocytes. Elaboration of the whole biomarker data in a cellular hazard index, corroborated the synergistic effects of temperature and acidification which increased the toxicological effects of cadmium. The overall results confirmed that climate change could influence ecotoxicological effects of environmental contaminants, highlighting the importance of a better knowledge of cellular mechanisms to understand and predict responsiveness of marine organisms to such multiple stressors.

1. Introduction

Since the industrial revolution, human activities have caused a

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relevant enhancement of atmospheric concentration of carbon dioxide, raised from ~280 ppm in 1780 to 391 ppm in 2011 (IPCC, 2013). As a consequence of greenhouse effect, oceans have warmed by 0.7 °C and the absorption of about 30% of the anthropogenic carbon dioxide caused a pH reduction of 0.1 units (IPCC, 2013). During the 21st century, warming and acidification will continue and, on a global scale, ocean mean temperature is predicted to rise by 0.6–2 °C; while ocean mean pH will further decrease by 0.3–0.5 units (Rodolfo-Metalpa et al., 2011; IPCC, 2013; Hoegh-Guldberg et al., 2014).

Global warming and ocean acidification are considered major threats to marine biodiversity and a high priority for science, management and policy (Byrne and Przeslawski, 2013; Kroeker et al., 2013). Adverse biological effects have been widely documented in several species in terms of reduced calcification rates (Martin and Gattuso, 2009; Rodolfo-Metalpa et al., 2011, 2015; Cerrano et al., 2013), impaired energy metabolism (Ivanina et al., 2013; Rodolfo-Metalpa et al., 2014), altered immune response (Bibby et al., 2008; Hernroth et al., 2011; Mackenzie et al., 2014), decreased reproduction success and larval development (Dorey et al., 2013; Hardy and Byrne, 2014; Byrne et al., 2016; Chan et al., 2016; Ventura et al., 2016), enhanced production of reactive oxygen species (ROS) and oxidative stress (Tomanek et al., 2011; Rosa et al., 2012).

The effects of climate changes on marine ecosystems are also expected to interact with other environmental stressors, including the high levels of anthropogenic contamination in coastal areas where future shifts in ambient temperature and pH could be more frequent and pronounced than in the open ocean. In these areas, large CO_2 and pH fluctuations can be due to the influence of riverine waters on carbonate chemistry, inputs of nutrients, organic matter and consequently higher microbial degradation (Nikinmaa, 2013; Wallace et al., 2014; Wong et al., 2014). Although the possibilities for interactions of global change on ecotoxicological responses to pollutants have been addressed (Byrne, 2012), mechanistic pathways of interactions between such multiple stressors have been scarcely investigated in experimental conditions.

There are evidences that high CO_2 partial pressure (pCO_2) and low pH may influence solubility and speciation of metals in seawater, with increased release from polluted sediments of those elements forming complexes with carbonate and hydroxide ions (Millero et al., 2009; Millero and DiTrolio, 2010; Hoffmann et al., 2012). Additive and synergistic effects of high CO_2/low pH and metal exposure have been demonstrated in various invertebrates (Ivanina et al., 2013, 2014; Lewis et al., 2013; Campbell et al., 2014; Götze et al., 2014; Ricevuto et al., 2016), with enhanced accumulation in bivalves, *Crassostrea virginica*, *Mercenaria mercenaria* and *Ruditapes philippinarum* (López et al., 2010; Ivanina et al., 2014; Götze et al., 2014), polychaetes, *Hediste diversicolor* (Rodríguez-Romero et al., 2014), eggs and embryos of the squid *Loligo vulgaris* and of the cuttlefish *Sepia officinalis* (Lacoue-Labarthe et al., 2009, 2011).

In addition, temperature was shown to modulate uptake and toxicity of metals, i.e. through accelerated metabolic rates, impairment of mitochondrial function, oxidative stress, accumulation of lipid peroxidation products, damages to lysosomal system and DNA (Sokolova, 2004; Baines et al., 2005; Cherkasov et al., 2006, 2007; Mubiana and Blust, 2007; Ivanina et al., 2008; Sokolova and Lannig, 2008; Guinot et al., 2012; Izagirre et al., 2014; Múgica et al., 2015). Reciprocal interactions between temperature, pH/pCO_2 and cadmium have been recently described in the Antarctic scallop *Adamussium colbecki* exposed for 14 days to moderate warming and hypercapnia (Benedetti et al., 2016). Due to the naturally elevated basal levels of cadmium in this area, digestive glands possess specific cellular adaptations to this metal, appearing more tolerant toward additional prooxidant factors.

Considering the growing interest on the interactive effects between climate changes and environmental chemicals, this study investigated the influence of various combinations of temperature and pH on bioaccumulation and sub-lethal effects of cadmium in the Mediterranean mussel *Mytilus galloprovincialis*. Mussels are typical bioindicator organisms for their ability to accumulate pollutants and the wide knowledge on the influence of both abiotic and biological factors (Fattorini et al., 2008; Regoli et al., 2014; Avio et al., 2015). In addition, cellular responses of *M. galloprovincialis* are widely used as biomarkers of environmental disturbance, and various studies indicated this species as potentially susceptible to the effects of ocean warming and acidification (Rodolfo-Metalpa et al., 2011; Range et al., 2014; Gazeau et al., 2014). Selected biomarkers reflected the main components of the sophisticated cellular network modulating responsiveness to pollutants, oxyradical metabolism and occurrence of early cellular damages (Regoli and Giuliani, 2014). Such responses were investigated in both digestive gland and gills in terms of metallothioneins induction, variations of individual antioxidant defenses, total antioxidant capacity and onset of lipid peroxidation processes; immunological parameters and genotoxic damages were evaluated on haemocytes. The overall significance of biomarkers results has been summarized through a quantitative hazard model (Sediqualsoft) which provides a cellular hazard index by giving a different weight to various biological endpoints and magnitude of observed variations (Piva et al., 2011; Benedetti et al., 2012). Results of the present study were expected to provide new insights on the combined effects of climate change and environmental pollutants focusing on the different tissue sensitivity, and the main cellular pathways responsible of metal detoxification and cellular homeostasis; these results will allow a better understanding of *Mytilus galloprovincialis* responsiveness towards multiple environmental stressors.

2. Materials and methods

2.1. Animal collection and experimental design

Mussels, *M. galloprovincialis* (6.0 ± 0.5 cm shell length), were obtained in June 2014 from a shellfish farm in an unpolluted area of Central Adriatic Sea (Regoli et al., 2014) and acclimatized for 7 days in aquaria with aerated artificial seawater (ASW; Instant Ocean®) at local environmental conditions of salinity (37 practical salinity units), temperature (20 °C) and pH (8.20): pH was calibrated against the National Bureau of Standards (NBS) standard solutions.

Mussels were then exposed to one of the following treatments, each containing 36 organisms in 20 L: 1) control condition (CTRL), at environmental temperature of 20 °C, normocapnia with pH = 8.20/pCO_2 = ~400 μatm; 2) cadmium exposure (Cd), 20 °C, pH = 8.20/pCO_2 = ~400 μatm and 20 μg/L cadmium; 3) acidification (A), 20 °C, hypercapnia with pH = 7.40/pCO_2 = ~3000 μatm; 4) warming (W), 25 °C and pH = 8.20/pCO_2 = ~400 μatm; 5) acidification + Cd (A-Cd), 20 °C, pH = 7.40/pCO_2 = ~3000 μatm and 20 μg/L cadmium; 6) warming + Cd (W-Cd), 25 °C, pH = 8.20/pCO_2 = ~400 μatm and 20 μg/L cadmium; 7) acidification + warming (A-W), 25 °C and pH = 7.40/pCO_2 = ~3000 μatm; 8) acidification + warming + Cd (A-W-Cd), 25 °C, pH = 7.40/pCO_2 = ~3000 μatm and 20 μg/L cadmium. Cadmium concentration was chosen as representative of a polluted but environmentally realistic scenario in coastal waters (Neff, 2002), while the temperature of 25 °C is typically experienced by mussels during the warmer period of Mediterranean summer season. Selected target pH was adapted from scenario RCP 8.5 and IPCC WGII AR5 (IPCC, 2014; Wong et al., 2014) reporting a mean pH value of 7.7 for open oceans, but predicting more pronounced variations

of pH/pCO_2 in coastal areas. The latter condition was reached by mixing ASW (pH = 8.2) with small amounts of CO_2-saturated ASW, obtained by bubbling pure CO_2 in ASW for at least 24 h (Schulz et al., 2013). For each experimental condition temperature, pH and salinity were measured daily, while total alkalinity (A_T) was measured twice per week according to Dickson et al., 2007. Seawater carbonate parameters (pCO_2, and saturation state (Ω) for calcite and aragonite) were calculated in CO2SYS (Pierrot et al., 2006) using barometric pressure values, as well as A_T, pH, temperature and salinity values for the respective samples (full seawater chemistry is provided in Table 1). For calculations, we used NBS scale for seawater pH, constants from Millero, 2010, KSO_4^- constant from Dickson et al., 2007, and concentration for silicate and phosphate for Instant Ocean® seawater (0.21 μmoL/kg and 0.05 μmoL/kg, respectively). Water was changed every other day, and mussels fed 12 h prior the water change with a commercial mixture of zooplankton (50–300 μm) for filter-feeding organisms.

After four weeks, animals were sampled from each tank and tissues collected for chemical and biological analyses. Gills and digestive glands were excised, pooled in 12 samples, each constituted by tissues of 3 individuals, rapidly frozen in liquid nitrogen and maintained at −80 °C: these samples were shared for analyses of cadmium content or biomarker responses, to guarantee a n value = 5 for each measurement. Haemolymph was withdrawn from the adductor muscle of 5 specimens and immediately used for immunity parameters and measurement of genotoxic damage.

2.2. Cadmium determination

Cadmium (Cd) concentration in mussels tissues was analyzed according to previously described methods (Regoli et al., 2005). For each treatment, digestive glands and gills were dried at 60 °C overnight, digested in a microwave system (Mars V, CEM) and analyzed by atomic absorption spectrophotometry with graphite furnace atomization and Zeeman effect (Regoli et al., 2005). Quality assurance and quality control was assessed by processing blank samples and reference standard material (Mussel Tissue Standard Reference Material SRM NIST-2977, National Institute of Standards and Technology Gaithersburg, MD, USA), which always resulted within the 95% confidence interval of certified values. Data are expressed as μg/g dry weight (mean values ± standard error, n = 5).

2.3. Biomarkers responses

Biomarkers in mussels tissues were analyzed through standardized methods which are detailed in Supplementary Material 1, SM1 (Avio et al., 2015). Metallothioneins (MTs), single antioxidant defenses (catalase, glutathione S-transferases, glutathione peroxidases, glutathione reductase, total glutathione), total oxyradical scavenging capacity toward peroxyl radicals (TOSC ROO•) and hydroxyl radicals (TOSC HO•), malondialdehyde content (MDA) were evaluated in both digestive gland and gills of exposed mussels; cryostat sections of digestive glands were further analyzed for lipofuscin and neutral lipids content. Immunological alterations in haemocytes were evaluated in terms of lysosomal membrane stability by neutral red retention time (NRRT), phagocytosis activity and granulocytes versus hyalinocytes ratio; onset of genotoxic effects in haemocytes were assessed in terms of DNA strand breaks (Comet assay) and micronuclei frequency (MN).

2.4. Statistical analyses

Analysis of variance (One-way ANOVA) was used to evaluate the effects of the treatments for all investigated parameters, after checking the normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene's Test). Level of significance was set to $p < 0.05$; *post-hoc* tests, Student − Newman − Keuls (SNK), were used to compare group of means. All statistical analyses were performed using RStudio (version 0.99.491).

The overall significance of biomarkers results was summarized in a cellular hazard index elaborated through a previously developed quantitative model which applies weighted criteria to discriminate different endpoints and the magnitude of effects (Sediqualsoft, Piva et al., 2011). Despite whole calculations and assumptions have been fully given elsewhere (Piva et al., 2011; Benedetti et al., 2012), the general rationale of the model is to compare variations of biomarkers to a specific threshold, which consider the possibility of biphasic responses and the different responsiveness among various species and tissues. The calculated Hazard Quotient (HQ) does not include biomarkers with variations lower or equal to their threshold, averages or adds the summation (Σ) respectively for those biomarkers with variations up to 2-fold or more than 2-fold greater than the specific threshold (Piva et al., 2011; Benedetti et al., 2012, 2014; Regoli et al., 2014; Avio et al., 2015). The model finally assigns the elaborated HQ in one of five classes of hazard, from Absent to Severe (Piva et al., 2011).

3. Results

Exposure to Cd caused a significant accumulation of this element in both digestive gland and gills, without any additional modulation when mussels were co-exposed to high temperature and/or low pH (Fig. 1a and b).

Metallothioneins in digestive gland were significantly induced by cadmium in all experimental conditions and a synergistic effect was measured in organisms co-exposed to the metal, elevated temperature and reduced pH condition (A-W-Cd) (Fig. 1c). On the other hand, levels of metallothioneins in gills were more enhanced in organisms exposed to cadmium at control temperature and normocapnia, while a lower rate of induction was observed during

Table 1
Summary of water chemistry parameters during experimental exposure. S (salinity), T (temperature), pH_{NBS} (pH calibrated with National Bureau of Standard scale), A_T (total alkalinity), pCO_2 (partial pressure of CO_2), Ωc and Ωa (saturation state of respectively calcite and aragonite). Data are presented as means ± standard deviations.

Treatment	Measured parameters				Calculated parameters		
	S	T (°C)	pH_{NBS}	A_T (μmol/kg)	pCO_2 (μatm)	Ωc	Ωa
CTRL	37 ± 0.5	19.95 ± 0.10	8.21 ± 0.04	2453.6 ± 251.5	380.8 ± 25.8	5.3 ± 0.4	3.5 ± 0.2
Cd	37 ± 0.5	20.00 ± 0.10	8.19 ± 0.04	2390.5 ± 354.1	410.6 ± 30.9	5.1 ± 0.4	3.3 ± 0.3
A	37 ± 0.5	19.98 ± 0.06	7.42 ± 0.04	2557.3 ± 183.7	2897.6 ± 183.8	1.0 ± 0.1	0.7 ± 0.1
W	37 ± 0.5	24.80 ± 0.13	8.15 ± 0.06	2325.4 ± 267.7	468.1 ± 47.9	5.4 ± 0.4	3.6 ± 0.3
A-Cd	37 ± 0.5	19.95 ± 0.06	7.41 ± 0.04	2556.7 ± 479.0	2928.2 ± 144.4	1.0 ± 0.1	0.7 ± 0.1
W-Cd	37 ± 0.5	24.83 ± 0.08	8.14 ± 0.04	2517.9 ± 206.9	477.4 ± 44.6	5.2 ± 0.4	3.5 ± 0.2
A-W	37 ± 0.5	24.76 ± 0.18	7.42 ± 0.03	2721.4 ± 215.7	3100.1 ± 241.7	1.2 ± 0.1	0.8 ± 0.1
A-W-Cd	37 ± 0.5	24.87 ± 0.16	7.43 ± 0.04	2504.2 ± 182	2993.7 ± 186.7	1.3 ± 0.1	0.9 ± 0.1

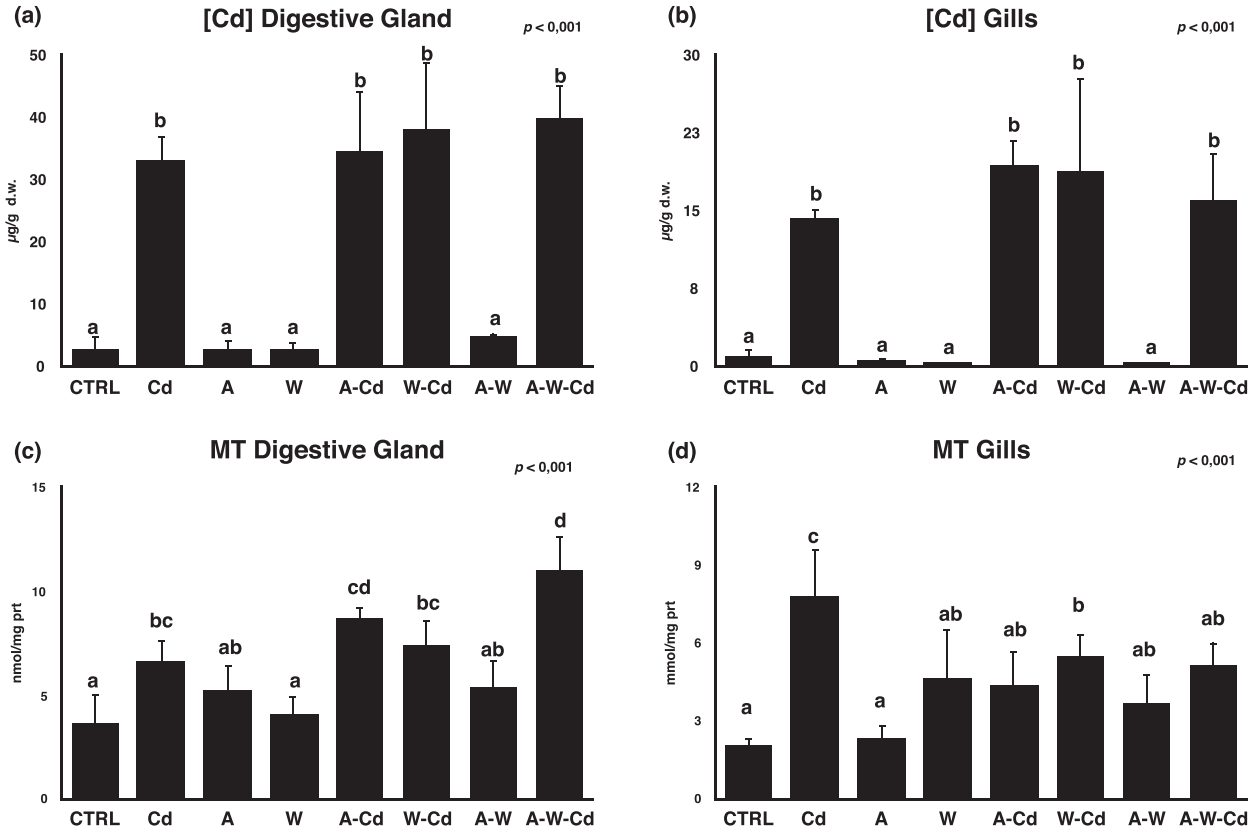

Fig. 1. Cadmium concentrations and level of metallothioneins in digestive gland (a and c) and gills (b and d) of mussels exposed to various treatments. Data are given as mean values ± standard deviations (n = 5). Different letters indicate significant differences between group of means (ANOVA and SNK *post-hoc*). CTRL= Control; Cd= Cadmium; A = acidification; W = warming; A-Cd = acidification + Cd; W-Cd = warming + Cd; A-W = acidification + warming; A-W-Cd = acidification + warming + Cd.

co-exposures to multiple stressors (Fig. 1d).

Antioxidant defenses showed a certain variability in their responsiveness depending on the analyzed parameter, tissue and stress factor. In digestive gland, interactions between cadmium and temperature caused more frequent and evident changes, with increased activities for GST (Fig. 2b) and similar, but not statistically significant trends for Se-dependent GPx and GR (Fig. 2c and e); total GPx were enhanced by temperature alone (Fig. 2d). Results on total oxyradical scavenging capacity in digestive gland revealed a lowered efficiency in neutralizing ROO• as synergistic effect of low pH and cadmium exposure, and enhanced values in mussels exposed to higher temperature alone (Fig. 2g). No variations were observed in this tissue for catalase, levels of total GSH and TOSC toward hydroxyl radical for any experimental treatment.

Quite limited variations were observed for MDA in digestive gland with decreased values in treatments combining lower pH and higher temperature (Fig. 2i); accumulation of lipofuscin was significantly increased by Cd, acidification and the interaction between these factors (Fig. 2j), while more variable effects occurred for neutral lipids with a statistically significant increase in mussels exposed to Cd alone and to the combination of higher temperature and acidification (Fig. 2k).

Slight and often not significant variations of antioxidants were observed in gills. Lowered values appeared for total GPx in response to A, W-Cd, A-W (Fig. 3d), and for TOSC HO• toward A, W, W-Cd, A-W (Fig. 3h); increased values were observed for Se-dependent GPx (Fig. 3c) and TOSC ROO• (Fig. 3g) as synergistic effects of temperature and Cd. No significant effects were measured in gills for catalase, GST, GR, levels of GSH and MDA.

Lysosomal membrane stability in haemocytes significantly decreased in all experimental groups with the only exception of mussels exposed to Cd alone (Fig. 4a). Phagocytosis rate (Fig. 4b) was lowered in all treatments with higher temperature (alone or in combination with other stressors), while cadmium (alone and with concomitant exposure to hypercapnia and higher temperature) increased the granulocytes versus hyalinocytes ratio (Fig. 4c). Exposure to cadmium in hypercapnic conditions (A-Cd, A-W-Cd) was responsible of higher micronuclei frequencies (Fig. 4e), while no clear significant variations were observed in terms of DNA strand breaks (Fig. 4d).

The biological significance of cellular responses observed in each experimental condition was summarized in a single hazard index through the application of weighted criteria (Fig. 5). The elaborated hazard quotient (HQ) was “Slight” for organisms exposed to individual stressors (Cd, A, W) or to lowered pH in combination with warmer temperature (A-W); the HQ raised to “Moderate” after all the co-exposures involving cadmium with other factors (A-Cd, W-Cd, A-W-Cd), further supporting synergistic effects of these factors on measured cellular responses.

4. Discussions

The present investigation provided clear evidence that interactions occur between global changes and exposure to toxic metals, and that significant effects on early cellular responses might be useful to understand responsiveness of marine organisms at physiological level.

Although ocean acidification and warming have been suggested

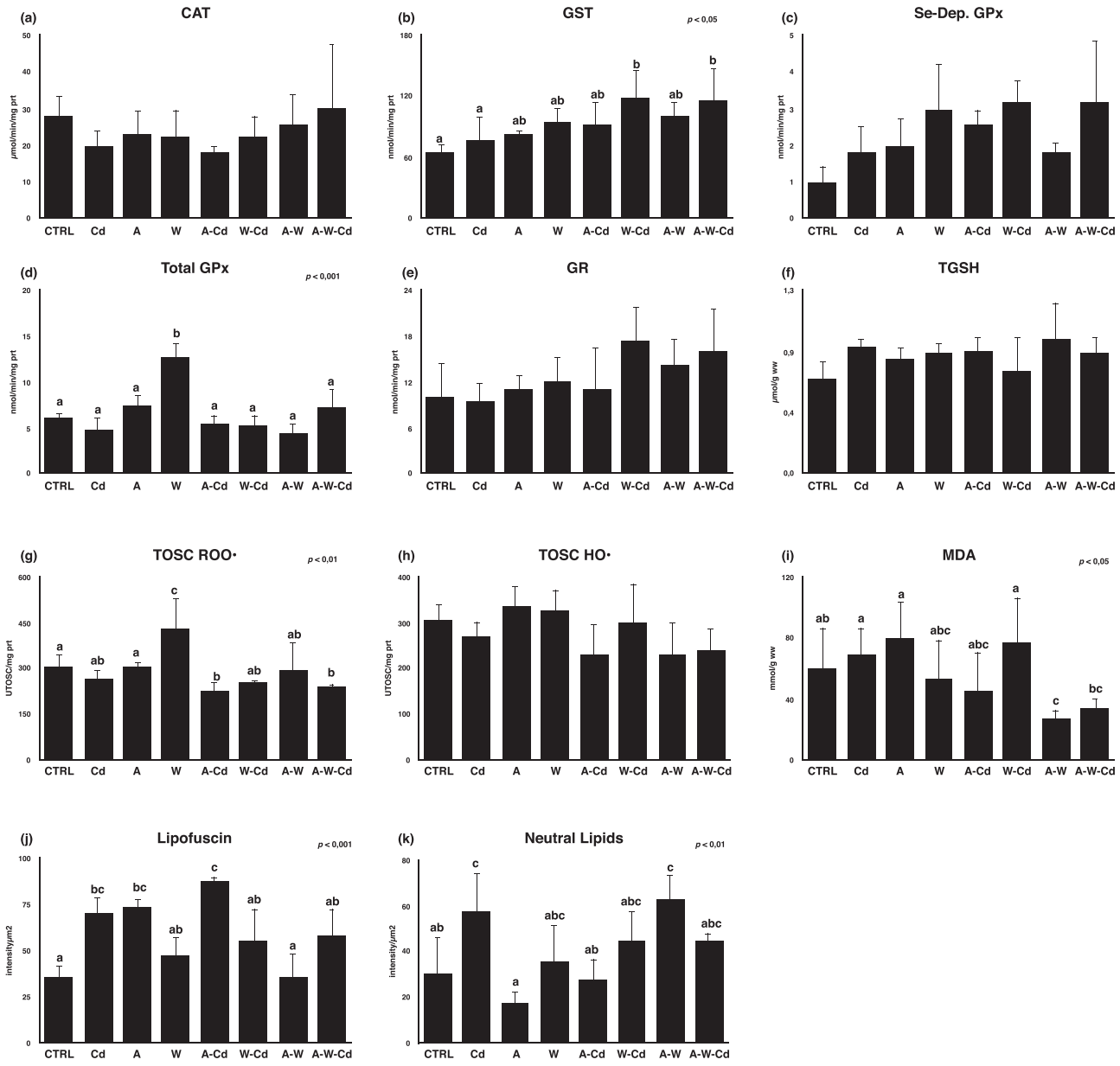

Fig. 2. Antioxidant defenses and oxidative stress biomarkers in digestive gland of mussels exposed to various treatments. CAT: catalase (a), GST: glutathione S-transferase (b), Se-Dep. GPx: Se-dependent glutathione peroxidases (c) total GPx: sum of Se-dependent and Se-independent glutathione peroxidases (d), GR: glutathione reductase (e), TGSH: total glutathione (f), TOSC ROO·: total oxyradical scavenging capacity toward peroxyl radical (g), TOSC HO·: total oxyradical scavenging capacity toward hydroxyl radical (h), MDA: levels of malondialdehyde (i), lipofuscin (j) and neutral lipids (k). Data are given as mean values ± standard deviations (n = 5). Different letters indicate significant differences between group of means (ANOVA and SNK *post-hoc*). CTRL= Control; Cd= Cadmium; A = acidification; W = warming; A-Cd = acidification + Cd; W-Cd = warming + Cd; A-W = acidification + warming; A-W-Cd = acidification + warming + Cd.

to influence chemical speciation and bioaccumulation of trace metals (Baines et al., 2005; Mubiana and Blust, 2007; Lacoue-Labarthe et al., 2009, 2011; Götze et al., 2014), our study did not reveal any variation of cadmium uptake in neither digestive gland nor in gills of mussels exposed to this element at higher temperature and/or lower pH. Similar results support the low influence of pH on chemical speciation of cadmium (Millero and DiTrolio, 2010), and the limited effect of temperature on accumulation of this element, as already been reported for *C. virginica, M. gallopro-vincialis* and the Antarctic scallops *A. colbeckii* (Cherkasov et al., 2006; Izagirre et al., 2014; Benedetti et al., 2016). These findings suggest that the effects of temperature and pH/pCO_2 on trace elements accumulation can not be generalized, depending on the species and the metal, thus being difficult to predict only from chemical models.

The present study confirmed that exposure to metals in mussels is usually associated with induction of metallothioneins. Despite cadmium accumulation was similar across various temperature-pH/pCO_2 combinations, levels of metallothioneins were differently affected by contemporary exposure to multiple stressors, with higher content of these proteins in digestive gland of organisms co-exposed to cadmium, hypercapnia and higher temperature. Since metallothioneins have a recognized scavenging capability toward oxyradicals (Regoli and Giuliani, 2014), their enhanced synthesis during co-exposures may reflect a greater prooxidant pressure due to synergistic interactions between the investigated stressors,

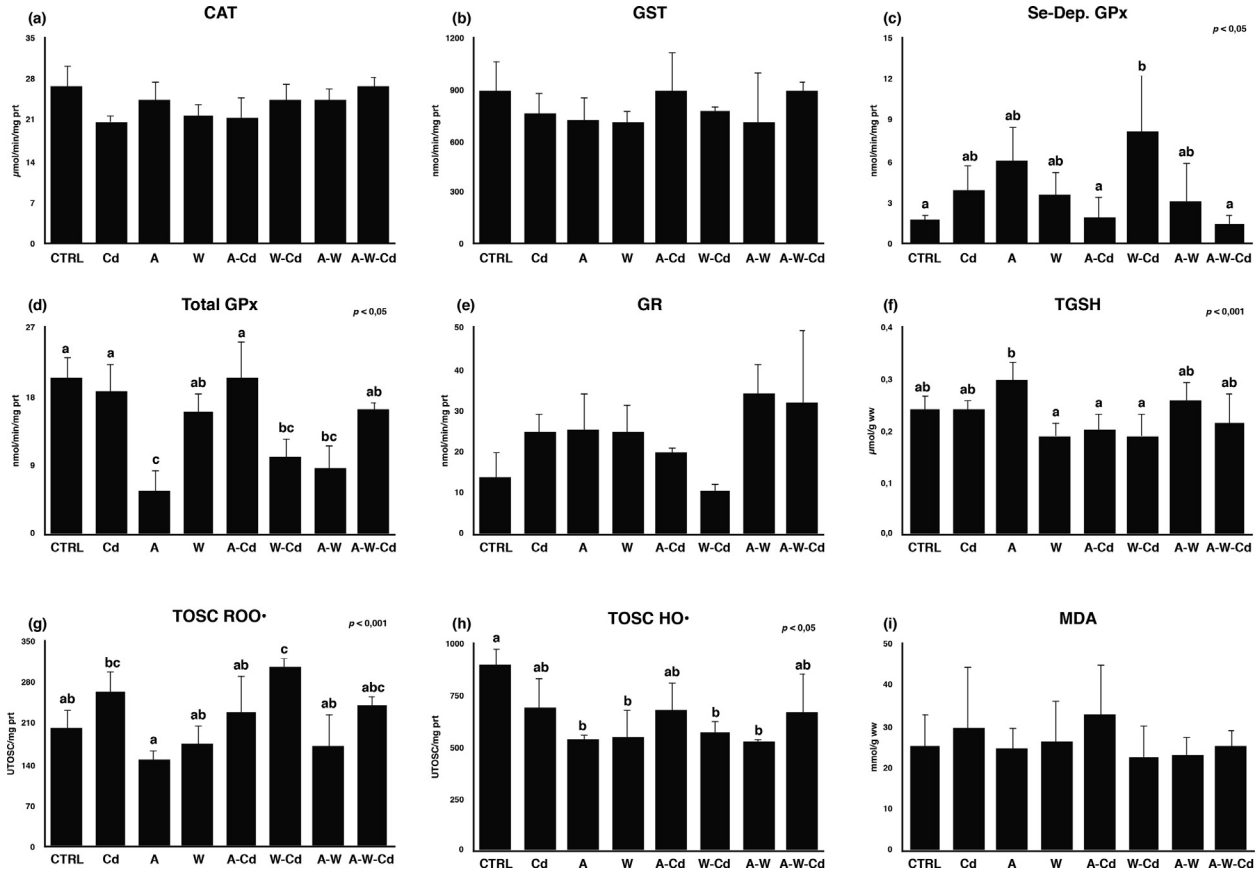

Fig. 3. Antioxidant defenses and oxidative stress biomarkers in gills of mussels exposed to various treatments. CAT: catalase (a), GST: glutathione S-transferase (b), Se-Dep. GPx: Se-dependent glutathione peroxidases (c) total GPx: sum of Se-dependent and Se-independent glutathione peroxidases (d), GR: glutathione reductase (e), TGSH: total glutathione (f), TOSC ROO·: total oxyradical scavenging capacity toward peroxyl radical (g), TOSC HO·: total oxyradical scavenging capacity toward hydroxyl radical (h), MDA: levels of malondialdehyde. Data are given as mean values ± standard deviations (n = 5). Different letters indicate significant differences between group of means (ANOVA and SNK *post-hoc*). CTRL= Control; Cd= Cadmium; A = acidification; W = warming; A-Cd = acidification + Cd; W-Cd = warming + Cd; A-W = acidification + warming; A-W-Cd = acidification + warming + Cd.

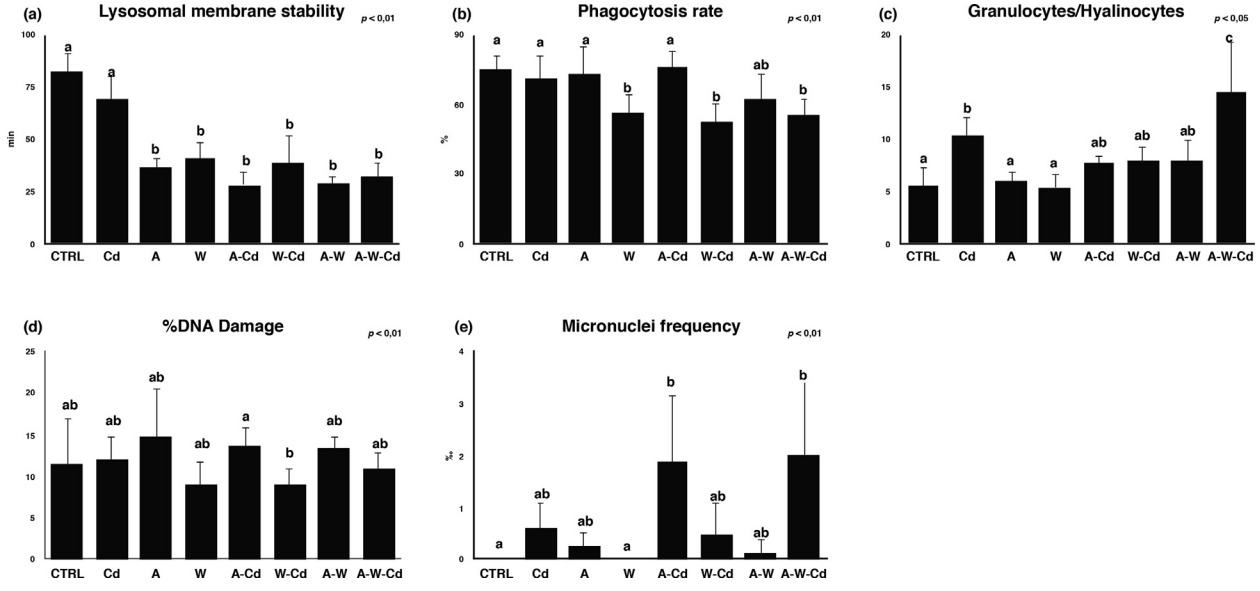

Fig. 4. Lysosomal membrane stability (a), phagocytosis rate (b), granulocytes/hyalinocytes ratio (c), DNA damage (d) and frequency of micronuclei (e) in haemocytes of mussels exposed to various treatments. Data are given as mean values ± standard deviations (n = 5). Different letters indicate significant differences between group of means (ANOVA and SNK *post-hoc*). CTRL= Control; Cd= Cadmium; A = acidification; W = warming; A-Cd = acidification + Cd; W-Cd = warming + Cd; A-W = acidification + warming; A-W-Cd = acidification + warming + Cd.

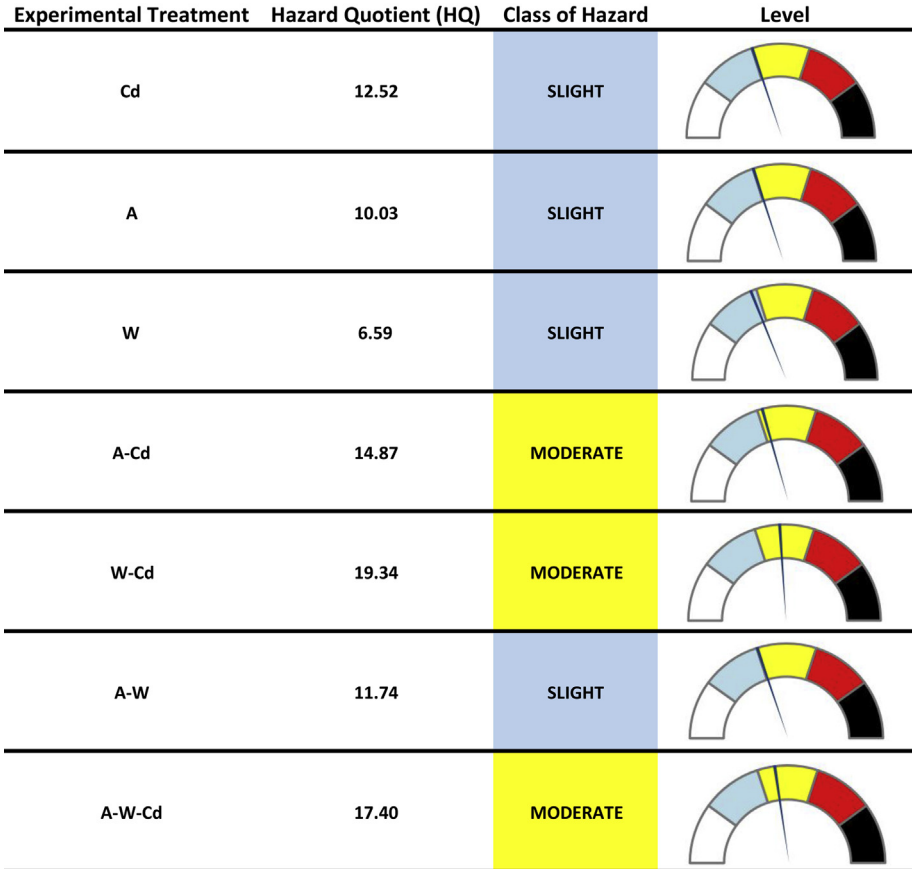

Fig. 5. Weight of Evidence (WOE) classification of biomarkers data for the whole dataset of analyzed parameters for each different laboratory condition. The quantitative hazard quotients (HQ) and the assigned class of hazard are given.

especially cadmium and pH/pCO_2. A similar interaction was observed in haemocytes of oysters exposed to cadmium and low pH (Ivanina et al., 2015), and further corroborated by the prooxidant effect of hypercapnia occurring in oysters, clams and polychaetes (Tomanek et al., 2011; Freitas et al., 2016a,b; Velez et al., 2016). A different modulatory effect of temperature and/or pH/pCO_2 was observed on metallothioneins levels in gills, where these factors appeared to lower the inductive capacity of cadmium. Interestingly different effects of hypercapnia and cadmium were shown in various tissues of oysters, showing often opposite variations of metallothioneins mRNA levels (Ivanina et al., 2015). Synergistic effects of multiple stressors might thus involve tissue-specific physiological processes and pathways, supporting the role of metallothioneins not only in metal detoxification but also in responsiveness to oxidative challenge (Regoli and Giuliani, 2014).

The antioxidant status of exposed mussels was assessed through a wide battery of biomarkers, including individual antioxidants, their integration with the total antioxidant capacity and the measurement of oxidative damages. In digestive gland, co-exposure to higher temperature and cadmium caused enhanced activities of glutathione-dependent enzymes, with significant variations for GST and similar trends for selenium-dependent GPx and GR; total GPx and TOSC toward ROO• were significantly increased in mussels exposed to warmer temperature alone. All these defenses are involved in reduction of lipid hydroperoxides and hydrogen peroxide, thus suggesting an adaptive mechanism to rapidly counteract the increased oxidative pressure from higher metabolic rates and metal-induced ROS production (Regoli and Giuliani, 2014). At the same time, co-exposure to reduced pH-higher CO_2 and cadmium lowered the capability to neutralize peroxyl radicals in digestive gland but without affecting the activities of glutathione-dependent enzymes: from these results, it might be hypothesized that CO_2-mediated prooxidant challenge interacts with other antioxidants or biochemical pathways, as those mediated by superoxide dismutase and peroxiredoxins, previously described as the main sensitive components of the proteasome in the mantle of *C. virginica* exposed to hypercapnia (Tomanek et al., 2011).

The synergistic effects of acidification and cadmium on lipofuscin content and the lower levels of malondialdehyde in organisms exposed to acidification and Cd at higher temperature, suggest that warming and acidification have contrasting effects on the oxyradical metabolism in digestive gland: the former enhancing the glutathione-dependent antioxidant protection while the latter acting as a pro-oxidant factor leading to accumulation of lipid peroxidation products. In this respect exposure to moderately higher temperature, within the already experienced environmental range, seems to protect the digestive gland from oxidative mechanism caused by hypercapnia, as already hypothesized for settlement of *P. lividus* (García et al., 2015).

Although gills did not reveal clear links between observed variations and different stressors, obtained results highlighted an elevated responsiveness of glutathione peroxidases in this tissue and subsequent changes in the total antioxidant capacity. The effects of cadmium were particularly marked in association to higher temperature at normocapnic condition, with enhanced activity of Se-dependent GPx and a concomitant reduction of the Se-independent forms. The higher efficiency of GPx toward inorganic

peroxides was supported by the greater capability to counteract peroxyl radical, while a reduced activity of Se-independent GPx was observed also in the gills of organisms exposed to hypercapnia, both alone or in combination with elevated temperature. The general decrease of the total antioxidant capacity toward hydroxyl radical in gills revealed an elevated responsiveness of this tissue to multiple stressors and a general stress condition after almost all the experimental treatments.

Immunological analyses provided clear evidence that haemocytes are a sensitive target for the effects of Cd, ocean acidification and temperature increase. Lysosomal membrane stability was compromised in all experimental treatments involving hypercapnia and higher temperature, confirming similar results reported in haemolymph of other invertebrate species (Beesley et al., 2008; Matozzo et al., 2012). Beside the general impairment of lysosomal stability, a slightly decreased phagocytosis rate was measured in all exposures involving higher temperature, in agreement with results obtained on haemocytes of *C. virginica* and *C. galina* (Hégaret et al., 2003; Monari et al., 2007); since the phagocytosis success was not affected in haemocytes of *M. galloprovincialis* when temperature was raised from 10 to 15 °C (Parry and Pipe, 2004), this function might be impaired only above a certain threshold of temperature. Mussels haemocytes contain two main populations of cells, i.e. the granulocytes with phagocytic function, and the hyalinocytes, more involved in coagulation and encapsulation processes (Gorbi et al., 2013). In this study, a higher ratio between granulocytes and hyalinocytes was observed after cadmium exposure, but the highest stimulation was evident in mussels exposed to cadmium at higher temperature and hypercapnic condition. Considering that phagocytosis was affected only by temperature, it is reasonable that variations in the ratio between these cellular populations are due to a reduction of hyalinocytes rather than an increase of granulocytes. Since hyalinocytes have lower phagocytic activity and a more limited protection against ROS, their loss might be ascribed to an oxidative mechanism exerted by cadmium, and amplified by co-exposure to warming and hypercapnia.

This hypothesis is corroborated by the results on genotoxic damage occurring as higher frequency of micronuclei in haemocytes of mussels exposed to cadmium in combination with low pH-high CO_2 condition. The lack of clear effects on DNA strand breaks would also indicate that variations of MN frequency might be, at least partly due to changes in cell division rate, rather than a direct damage on haemocytes DNA. Independently on the cellular mechanism, our data confirm an increased metal genotoxicity under moderate OA-conditions, as recently reported in haemocytes of *M. edulis* and in coelomocytes of *P. lividus* exposed to copper (Lewis et al., 2016). Future analyses on cadmium content in haemocytes could be useful to clarify whether pH/pCO_2 effects are modulated by enhanced accumulation of metals.

Synergistic effects of multiple stressors on responsiveness to cadmium in *M. galloprovincialis* were further highlighted when cellular responses were evaluated through the weighted criteria of the quantitative Sediqualsoft model, which elaborates a synthetic hazard index based on number of changed biomarkers, their toxicological relevance and magnitude of observed variations (Piva et al., 2011; Benedetti et al., 2012). Biomarkers have been widely recognized as early warning signals of environmental disturbance, and mechanisms of action have been deeply investigated for several anthropogenic and natural stressors. At the same time, the predictive utility of biomarkers has been debated for the complexity to summarize the toxicological relevance of variations occurring on multiple cellular pathways. Various integrative methods and health indices have been proposed in recent years to facilitate biomarkers data interpretation and, despite different mathematical calculations and assumptions, a recent comparison confirmed that such approaches are all useful to discriminate altered health conditions (Beliaeff and Burgeot, 2002; Piva et al., 2011; Broeg et al., 2005; Dagnino et al., 2008; Benedetti et al., 2012; Marigómez et al., 2013). The calculations applied in the present investigation are part of a more complex Weight of Evidence model which can elaborate multiple typologies of data, as previously validated in several risk assessment studies (Benedetti et al., 2012, 2014; Regoli et al., 2014; Avio et al., 2015; Bebianno et al., 2015): in this respect, the calculated HQ for biomarkers raised from “Slight” in organisms exposed to individual stressors to “Moderate” during exposures to cadmium with various combinations of temperature and/or acidification.

In conclusion, this study provided clear evidence that variations of temperature and pH/pCO_2 can modulate cellular effects of cadmium in marine organisms. Our data suggest that mechanisms of action can be highly tissue-dependent, probably interacting with specific metabolic functions, consequent biochemical specialization and responsiveness of various cell types. The analyses of cellular effects might be useful to better understand and predict physiological sensitivity of marine organisms and ecological effects of multiple stressors, including the potential for adaptation or resilience, the influence of environmental and biological factors, the role of seasonality and long term effects. Additional studies would be useful to test interactions between multiple stressors in different seasons, or when dosed at different levels of intensity, or with different frequencies of duration.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.11.093.

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