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Oxidative and interactive challenge of cadmium and ocean acidification on the smooth scallop *Flexopecten glaber*

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Abstract

Ocean acidification (OA) may affect sensitivity of marine organisms to metal pollution modulating chemical bioavailability, bioaccumulation and biological responsiveness of several cellular pathways. In this study, the smooth scallop *Flexopecten glaber* was exposed to various combinations of reduced pH (pH/pCO_2 7.4/~3000 µatm) and Cd (20 µg/L). The analyses on cadmium uptake were integrated with those of a wide battery of biomarkers including metallothioneins, single antioxidant defenses and total oxyradical scavenging capacity in digestive gland and gills, lysosomal membrane stability and onset of genotoxic damage in haemocytes. Reduced pH slightly enhanced concentration of Cd in scallops tissues, but no effects were measured in terms of metallothioneins. Induction of some antioxidants by Cd and/or low pH in the digestive gland was not reflected in variations of the total oxyradical scavenging capacity, while the investigated stressors caused a certain inhibition of antioxidants and reduction of the scavenging capacity toward peroxyl radical in the gills. Lysosomal membrane stability and onset of genotoxic damages showed high sensitivity with possible synergistic effects of the investigated factors. The overall results suggest that indirect effects of ocean acidification on metals accumulation and toxicity are tissue-specific and modulate oxidative balance through different mechanisms.

Keywords: Oxidative stress; ocean acidification; metal contamination; bioaccumulation; biomarkers; scallops

1. Introduction

World oceans have absorbed about the 30% of anthropogenic emissions of carbon dioxide (CO_2) in the atmosphere causing changes in the inorganic carbon system equilibrium (Le Quéré *et al.*, 2009). The consequent ocean acidification (OA) is responsible for the continuous reduction of ocean pH, dropped by 0.1 units since the beginning of industrial era (Gattuso and Lavigne, 2009), and expected to further decrease by 0.14 to 0.35 units depending on CO_2 emissions scenarios (Caldeira and Wickett, 2005). Scientific literature provides wide evidence that future projections of ocean pH/pCO_2 will affect health status of marine organisms by altering key physiological processes, like calcification rates (Cerrano *et al.*, 2013; Dupont *et al.*, 2010; Gazeau *et al.*, 2007; Jokiel *et al.*, 2008), acid-base balance and ionic homeostasis (Gutowska *et al.*, 2010; Miles *et al.*, 2007; Spicer *et al.*, 2007), metabolism (Lannig *et al.*, 2010; Pan *et al.*, 2015; Stumpp *et al.*, 2012), immune response (Bibby *et al.*, 2008; Hernroth *et al.*, 2011, 2012, 2016), larval development (Dupont *et al.*, 2008; Ellis *et al.*, 2009; Kurihara *et al.*, 2007; Stumpp *et al.*, 2011) and oxidative stress responses (Benedetti *et al.*, 2016; Freitas *et al.*, 2016; Nardi *et al.,* 2017; Pimentel *et al.*, 2015; Rokitta *et al.*, 2012; Soriano-Santiago *et al.*, 2012; Tomanek *et al.*, 2011).

Beside the direct effects, there is growing interest for the potential interaction of OA with other environmental stressors, such as the high levels of metal contamination in coastal environments (Ivanina and Sokolova, 2015). In this respect, OA is supposed to increase the ionic and bioavailable fraction of certain metals like copper (Cu^{2+}), which typically form strong complexes with carbonate (CO_3^{2-}) and hydroxide (OH^-) ions (Millero *et al.*, 2009). These model predictions have been confirmed by some experimental evidence revealing that high pCO_2/low pH regimes can increase release of metals from polluted sediments (Ardelan *et al.*, 2009; Ardelan and Steinnes, 2010; de Orte *et al.*, 2014a, 2014b) and enhance their bioaccumulation (Duckworth *et al.*, 2017; Götze *et al.*, 2014; Ivanina *et al.*, 2014; Lacoue-Labarthe *et al.*, 2009, 2011; López *et al.*, 2010; Rodríguez-Romero *et al.*, 2014). Synergistic effects of high pCO_2/low pH and metal exposure were recently reported on several cellular responses of marine invertebrates (Götze *et al.*, 2014; Ivanina *et al.*, 2013, 2015;

70 Lewis *et al.*, 2013), including the antioxidant status and the onset of oxidative stress (Benedetti *et al.*,
71 2016; Nardi *et al.*, 2017; Ricevuto *et al.*, 2015, 2016; Siddiqui *et al.*, 2015;), which is one of the most
72 relevant pathways by which trace elements exert their toxicity through a sophisticated array of
73 molecular and cellular effects (Regoli and Giuliani, 2014).

74 To provide new insights on the interactions between ocean acidification and metal
75 contamination, this study investigated whether high pCO_2/low pH may influence bioaccumulation
76 and sub-lethal effects of cadmium in the smooth scallop *Flexopecten glaber.* This species, widely
77 distributed throughout the Mediterranean Sea, has been recently addressed to be a key commercial
78 species, especially in Northwestern Adriatic Sea where it represents about 74% of shellfish fishery
79 (Marčeta *et al.*, 2016, Mazzoldi *et al.*, 2014; Pujolar *et al.*, 2010). Scallops are widely used in
80 ecotoxicological studies, they are typically characterized by high basal concentrations of cadmium in
81 the digestive gland (Bustamante *et al.*, 2002; Mauri *et al.*, 1990; Regoli *et al.*, 1998, 2000, 2002) and
82 they are recently addressed as highly sensitive to ocean acidification (Andersen *et al.*, 2013; Cooley
83 *et al.*, 2015; Schalkhausser *et al.*, 2013; White *et al.*, 2013). The effects of ocean acidification can be
84 exacerbated in shallow coastal and estuarine waters due to freshwaters inputs, which influence
85 carbonate chemistry, nutrients levels, organic matter degradation and pollutants concentrations
86 (Nikinmaa, 2013; Wallace *et al.*, 2014; Wong *et al.*, 2014). In this respect, scallops were exposed to
87 various combinations of Cd and high pCO_2/low pH, and a complex network of cellular responses was
88 investigated including levels of metallothioneins, variations of antioxidant defenses and total
89 antioxidant capacity in both digestive gland and gills, lysosomal alterations and onset of genotoxic
90 damages in haemocytes. The overall significance of biomarkers responses was synthetized in a
91 cellular hazard index through a quantitative hazard model (Sediqualsoft) which considers the number
92 and magnitude of observed variations, giving a different weight to each biomarker based on the
93 toxicological relevance of biological endpoints (Benedetti *et al.*, 2012; Piva *et al.*, 2011). Results
94 obtained in the present study were expected to contribute to the growing knowledge on the interactive
95 effects of ocean acidification and metals focusing on sensitivity of different tissues in a widely

96 distributed, potentially vulnerable but still poorly investigated species.

97

98 **2. Materials and Methods**

99 *2.1 Animal collection and experimental design*

100 Scallops, *Flexopecten glaber* (4.5 ± 0.5 cm shell length), were obtained in June 2015 from a
101 shellfish farm in an unpolluted area of Venice lagoon, Chioggia, Italy. Organisms were acclimatized
102 for 7 days in aquaria with aerated artificial seawater (ASW; Instant Ocean®) at local environmental
103 conditions of salinity (30 practical salinity units), temperature (20 °C) and pH_{NBS} (8.20).

104 Scallops were then randomly assigned to one of the following treatments, each containing 20
105 organisms in 20 L: 1) control condition (CTRL), at 20°C, pH=8.20/ pCO_2=~400 µatm; 2) cadmium
106 exposure (Cd), 20°C, pH=8.20/ pCO_2=~400 µatm and 20 µg/L cadmium; 3) acidification (A), 20°C,
107 pH=7.40/ pCO_2=~3000 µatm; 4) acidification + Cd (A - Cd), 20°C, pH=7.40/ pCO_2=~3000 µatm and
108 20 µg/L cadmium. Cadmium exposure was representative of a polluted but environmentally realistic
109 scenario (Neff, 2002), while selected target pH was based on scenario RCP 8.5 and the 2014 IPCC
110 WGII AR5 (IPCC, 2014) where future decrease in coastal waters is predicted to be higher than in
111 open ocean; target pH was reached by mixing ASW (pH=8.2) with small amounts of CO_2-saturated
112 ASW as described elsewhere (Nardi *et al.*, 2017). For each experimental condition temperature, pH
113 and salinity were measured daily, while total alkalinity (A_T) was measured every three days during
114 the experiment according to Dickson *et al.*, 2007. Seawater carbonate parameters (pCO_2, and
115 saturation state (Ω) for calcite and aragonite) were calculated in CO2SYS using barometric pressure
116 values (Pierrott *et al.*, 2006); A_T, pH, temperature, salinity values and full seawater chemistry are
117 provided in Table 1. For calculations, we used NBS scale for seawater pH, carbonate constants from
118 Millero (2010), KSO_4^- constant from Dickson *et al.* (2007) and concentration of silicate and phosphate
119 from Instant Ocean® composition (0.21 µmol/kg and 0.05 µmol/kg, respectively). Water was
120 changed every other day, and scallops fed 12 hours prior the water change with a commercial mixture
121 of zooplankton (50-300 µm) for filter-feeding organisms.

After ten days, animals were sampled from each tank and tissues collected for chemical and biological analyses. Gills and digestive glands were excised, pooled in 5 samples (each constituted by tissues of 4 individuals), rapidly frozen in liquid nitrogen and maintained at -80°C until analyzed for cadmium content or biomarker responses. Haemolymph was withdrawn from the adductor muscle of 5 specimens and immediately used for the measurement of lysosomal membranes stability and onset of genotoxic damages.

2.2 Cadmium determination

Cadmium (Cd) concentrations in scallops were analyzed according to previously described methods (Regoli *et al.*, 2005). For each treatment, digestive glands and gills were dried at 60°C overnight and digested in a microwave digestion system (Mars CEM, CEM Corporation, Matthews NC). Cd was analyzed by atomic absorption spectrophotometry (AAS) using graphite furnace atomization and Zeeman effect (SpectrAA 300 Zeeman, Varian, Mulgrave, VIC, Australia). 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). The concentrations obtained for the SRM were always within the 95% confidence interval of certified values. Data are expressed as μg/g dry weight (mean values ± standard deviation, n = 5).

2.3 Biomarkers responses

Standardized protocols were used to analyze biomarkers and full methodological details are given in Supplementary Material 1 (SM1). Metallothioneins (MTs), single antioxidant defenses (catalase, glutathione S-transferases, glutathione peroxidases, glutathione reductase activities and total glutathione), total oxyradical scavenging capacity toward peroxyl radical (TOSC ROO•) and hydroxyl radical (TOSC HO•) were evaluated in digestive gland and gills. The analysis of the Total Oxyradical Scavenging Capacity (TOSC) is a reliable tool for quantitatively assess the biological

148 resistance to toxicity of different forms of ROS including peroxyl radicals, hydroxyl radicals and
149 peroxynitrite decomposition products (Regoli and Winston, 1998, 1999). The assay is based on the
150 capability of cellular antioxidants to reduce the oxidation of α-keto-ɣ-methiolbutyric acid (KMBA)
151 in the presence of artificially generated oxyradicals. Compared to individual antioxidants, variations
152 of TOSC have a greater biological relevance and prognostic value, being an impaired capability to
153 neutralize ROS associated to the onset of various forms of oxidative damages like lysosomal
154 dysfunctions, lipid peroxidation and genotoxic effects (Nigro et al., 2002; Camus et al., 2003; Gorbi
155 and Regoli, 2003; Moore et al., 2006). Lysosomal membrane stability (as Neutral Red Retention
156 Time, NRRT) and onset of genotoxic effects as DNA strand breaks (Comet assay) and micronuclei
157 frequency (MN) were analyzed in haemocytes.
158
159 *2.4 Statistical analyses*
160 Analysis of variance (One-way ANOVA) was used to evaluate the effects on all investigated
161 parameters, after ensuring that all data followed the normal distribution (Shapiro-Wilk test) and that
162 variances were homogeneous (Levene's Test). Level of significance was set to $p < 0.05$; *post-hoc*
163 Tukey HSD tests were used to compare group of means. Multivariate principal component analysis
164 (PCA) was applied to visualize the relationships among the different treatments and all statistical
165 analyses were performed using RStudio (version 1.0.143).
166 For each treatment, the whole dataset of biomarkers results was summarized in a hazard index
167 elaborated through weighted criteria which discriminate different endpoints and the magnitude of
168 effects (Sediqualsoft, Piva *et al.*, 2011). Within this quantitative model, each biomarker has a
169 "weight" based on its toxicological relevance and a "threshold" of biological significant changes
170 which consider the possibility of biphasic responses and the different responsiveness among various
171 species and tissues. Variations measured for various biomarkers are compared to their specific
172 thresholds and corrected for the weight and the statistical significance of the difference compared to
173 controls (for full details see Piva et al., 2011, Benedetti et al., 2012): the calculated Hazard Quotient

(HQ) does not include biomarkers with variations lower or equal to their threshold, while it 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 (Avio *et al.*, 2015; Benedetti *et al.*, 2012; Piva *et al.*, 2011; Regoli *et al.*, 2014). The model finally assigns the elaborated HQ in one of five classes of hazard, from Absent to Severe (Piva *et al.*, 2011). Whole calculations and assumptions have been fully given elsewhere (Benedetti *et al.*, 2012; Piva *et al.*, 2011).

3. Results

Exposures to Cd caused an increase of metal concentrations in both digestive gland and gills of scallops with a slightly greater, although not statistically significant accumulation in organisms exposed to the metal at lower pH (Fig.1a and 1b).

Levels of metallothioneins were not influenced neither by Cd-exposure nor by pH-reduction, in the digestive gland (Fig.2a) or gills (Fig.3a).

Sensitivity of antioxidant defenses showed tissue-specificities toward the investigated factors. In the digestive gland, catalase (Fig.2b) was enhanced in organisms exposed to lower pH, an effect which was not further modulated by concomitant presence of Cd; higher activities were observed also for GST activity and Se-dependent GPx in acidic conditions (Fig.2c and 2d), but for these enzymes an antagonistic effect occurred during co-exposure to Cd and high pCO_2/low pH; total glutathione (Fig.2g) was enhanced in all treatments involving Cd-exposure, pH reduction and their combination. Variations of individual antioxidants were not reflected by any significant difference among the experimental treatments of TOSC against both ROO• and HO• (Fig.2h and 2i).

Different interactive effects of Cd and acidification were observed on the antioxidant system of gills. The activity of GST was significantly lowered in organisms exposed to the combination of factors (Fig. 3c), while the inhibition of Se-dependent GPx caused by Cd alone was not observed when the metal was dosed at high pCO_2/low pH (fig. 3d). TOSC values toward ROO• were

199 significantly reduced by acidification with or without Cd (Fig. 3h), but no variations were observed
200 toward HO• (Fig.3i).

201 Lysosomal membrane stability (Fig.4a) was significantly lowered by the co-exposure to Cd and
202 acidification, no effects appeared in terms of DNA fragmentation (Fig.4b), while MN frequency (Fig.
203 4c) was strongly enhanced in all treatments with Cd and acidification, dosed alone or in combination.

204 The principal component analysis on the whole dataset of biomarkers provided a two-
205 dimensional pattern explaining almost 54.6% of the total variance (Fig.5). A clear separation was
206 observed between control and treated organisms, with a further discrimination between exposure to
207 Cd alone and those involving the acidification with or without the metal. The parameters determining
208 the separation along PC1 axis, i.e. between control pH vs. acidification-exposed organisms, were
209 antioxidants in digestive gland (CAT, GST, GPx and TOSC HO•), in gills (GST, GR and TOSC
210 ROO•), and micronuclei frequency in haemocytes; along the PC2 axis, the separation of Cd-exposure
211 was mostly determined by MTs and TGSH in both the tissues, GPxs in gills and TOSC ROO• in
212 digestive gland.

213 The elaboration of whole biomarkers data with the weighted criteria provided a synthetic hazard
214 quotient (HQ) for organisms of each experimental condition, with a level of hazard classified as
215 "Slight" for scallops exposed to Cd alone and "Moderate" for those exposed to the acidified
216 treatments; among these latter, a quantitatively higher value of HQ was summarized for scallops
217 exposed to acidification alone than in those exposed to cadmium and high pCO_2/low pH (Fig.6).

218

219 **4. Discussions**

220 This study provided evidence that future projections of pH in coastal waters can modulate
221 bioaccumulation and biological effects of cadmium in the temperate scallop *F. glaber.*

222 The consequences of seawater acidification on metals bioavailability are variable: some authors
223 described increased uptake in marine organisms under reduced pH conditions (Götze *et al.*, 2014;
224 Ivanina *et al.*, 2014; Lacoue-Labarthe *et al.*, 2009, 2011; López *et al.*, 2010; Rodriguez-Romero *et*

225 *al.*, 2014; Shi *et al.*, 2016) while others did not report a similar effect (Benedetti *et al.*, 2016; Ivanina
226 *et al.*, 2016; Nardi *et al.*, 2017; Ricevuto *et al.*, 2016). In the present study, we observed a slight but
227 significant accumulation of cadmium in the digestive gland of scallops exposed to the metal, and a
228 more marked increase in the gills. The limited accumulation of cadmium in digestive gland can be
229 explained by the high basal levels of this metal in *F. glaber*, and more in general in digestive glands
230 of scallops which possess specific high molecular weight Cd-binding proteins (Bustamante *et al.*,
231 2002; Mauri *et al.*, 1990; Regoli *et al.*, 1998, 2000, 2002). When scallops were exposed under
232 hypercapnic condition, a further, slight increase of metal uptake was observed in both the tissues,
233 although this additional effect was not statistically significant. A limited influence of low pH on
234 cadmium accumulation has been reported in the Antarctic scallop *A. colbecki* and in the
235 Mediterranean mussel *M. galloprovincialis* supporting that effects of acidification on metals uptake
236 do not depend on the chemical speciation of the element, but rather reflect physiological effects of
237 CO_2 on an organism, which can not be generalized, being dependent on metal, tissue, species-specific
238 characteristics and duration of exposure (Benedetti *et al*., 2016; Nardi *et al*., 2017).
239 Despite cadmium is known to induce metallothioneins, levels of these proteins were not
240 modulated in *F. glaber* by metal-exposure in normocapnic or acidic conditions, neither in digestive
241 gland nor in the gills. Also this result might be related to the specific characteristics of scallops which,
242 beside the elevated tissue concentrations of cadmium and the presence of high molecular weight
243 binding proteins, also contain relatively high basal levels of MTs: these features might influence the
244 low sensitivity of metallothioneins induction pathway as previously observed in the Antarctic scallop
245 *A. colbecki* exposed to cadmium with and without acidification (Benedetti *et al.*, 2016).
246 The characterization of the antioxidant system, integrating the analyses of individual antioxidant
247 defenses with the total oxyradical scavenging capacity toward different ROS, revealed interactions
248 between cadmium and acidification. In the digestive gland, exposure to high pCO_2/low pH enhanced
249 the enzymatic activities of catalase, glutathione S-transferases, Se-dependent glutathione peroxidases
250 and levels of total glutathione. Catalase and Se-dependent GPx are responsible for the detoxification

of hydrogen peroxide, GST are also involved in metabolism of lipid hydroperoxides while GSH, beside acting as direct scavenger of ROS, is a fundamental co-factor for glutathione-dependent enzymes like GST and GPx (Regoli and Giuliani, 2014). These results suggest that pH reduction promotes oxidative insult through the generation of peroxides, as previously hypothesized for the oysters *Crassostrea virginica* and *Crassostrea gigas*, the polychaete *Sabella spallanzanii*, the scallop *A. colbecki* and the Mediterranean mussel *M. galloprovincialis* (Benedetti *et al.*, 2016; Moreira *et al.*, 2016; Nardi *et al.*, 2017; Ricevuto *et al.*, 2016; Tomanek *et al.*, 2011). With the exception of catalase, these inductive effects were typically less evident in organisms co-exposed to the metal and acidification, suggesting some antagonistic interaction and/or a certain Cd-mediated impairment of those antioxidant defenses (Regoli, 2012; Regoli and Giuliani, 2014). Despite the variation of these antioxidants, no effects were observed for the TOSC toward ROO• and HO•, indicating a good counteracting capability toward oxidative challenge in digestive gland of *F. glaber.* The observed inhibitions might be compensated by the involvement of other antioxidant defenses (Regoli and Giuliani, 2014), including low molecular weight scavengers or superoxide dismutase and peroxiredoxines, which were reported as important mechanisms affected by acidification in the mantle of *C. virginica* (Tomanek *et al.*, 2011).

Different effects were obtained in the gills with no induction of any antioxidant, but rather the inhibition of glutathione S-transferases by co-exposure to cadmium and acidification, and of Se-dependent GPx by Cd alone. Such results would indicate a lower capability to counteract oxidative stress in gills compared to digestive gland of *F. glaber*. In this respect, tissue-specific effects of multiple stressors were previously observed also in *A. colbecki* and *M. galloprovincialis* exposed to different combinations of cadmium, temperature and acidification (Benedetti *et al*., 2016; Nardi et al., 2017), confirming that within the complex network of oxidative interactions it is virtually impossible to predict the same responses toward multiple stressors when moving to different species, tissues, geographical latitudes or seasons (Camus *et al*., 2005; de Hoop *et al*., 2011). Despite such variability, changes of antioxidant defenses still remain useful and sensitive early indicators of a

277 varied oxidative challenge, whose biological significance can be reflected by the total antioxidant
278 capacity. In our study, the marked decrease in the capability to neutralize peroxyl radicals confirmed
279 the sensitivity of gills in organisms exposed to reduced pH with or without cadmium: compared to
280 digestive gland, the direct contact with seawater and the filter feeding activity can probably explain
281 the higher vulnerability of these tissues toward acidified conditions. In addition, digestive gland is a
282 detoxification/storage tissue, with the possibility to sequester cadmium in soluble or insoluble
283 compounds in Pectinidae such as *Pecten maximus* and *Chlamys varia* (Metian et al., 2007).
284 Interactive, pro-oxidant effects of acidification and cadmium exposure reduced the stability of
285 lysosomal membranes more than the single stressors, in agreement with similar effects observed in
286 haemocytes of mussels *M. edulis* and *M. galloprovincialis* (Beeseley *et al.*, 2008; Nardi *et al.*, 2017).
287 Concerning the onset of genotoxic damages, DNA fragmentation did not vary in terms of strand
288 breaks, while micronuclei frequency was enhanced in all treatments with comparable effects in
289 scallops exposed to cadmium, acidification or their combination. Levels of MN increased also in the
290 Antarctic scallop *A. colbecki* after exposure to various combinations of Cd and reduced pH (Benedetti
291 *et al.*, 2016), and synergistic effects of cadmium and acidification were observed in haemocytes of
292 *M. galloprovincialis* (Nardi *et al.*, 2017). The increased genotoxicity caused by these stressors in
293 terms of MN frequency but not as DNA strand breaks, has been hypothesized to reflect an increased
294 mitotic rate on haemocytes of such bivalves, rather than a direct effect on DNA integrity (Benedetti
295 *et al*., 2016; Nardi *et al*., 2017).
296 Principal components analysis revealed clear separation between organisms exposed to Cd alone
297 and those exposed to acidification, with or without the metal suggesting that this scallop might be
298 more sensitive to low pH than metal exposure. This hypothesis seems to be quantitatively confirmed
299 by the elaboration of the overall results through weighted criteria based on number, magnitude of
300 variations and toxicological relevance of biomarkers. The summarized hazard index was “Slight”
301 after Cd exposure, “Moderate” in organisms treated at high pCO_2/low pH: among these, the
302 quantitative HQ value increased from Cd-acidified to acidified scallops. The relative high tolerance

of *F. glaber* to cadmium might reflect a certain cellular adaptation of this species to the elevated basal content of the metal, both in terms of binding proteins and efficiency of antioxidant defenses. A similar possibility has already been supposed for the Antarctic scallop *A. colbecki*, characterized by Cd levels up to 5-15 folds higher than in *F. glaber* due to the presence of upwelling phenomena and a natural Cd enrichment of the area: in this organism, however, the homeostatic equilibrium conferring resistance to various stressors appeared overwhelmed by an additional exposure and accumulation of this element, making *A. colbecki* highly sensitive to further changes of environmental stressors.

In conclusion, this study supported the role of acidification as an environmental disturbance acting both through direct mechanisms and indirect modulation of responsiveness to metal exposure: however, uptake and sub-lethal effects of elements like Cd can vary in a species and tissue-specific manner, highlighting the need of additional studies to elucidate the impact of multiple stressors, particularly in species with elevated ecological or commercial importance.

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586 Table 1 - Water chemistry parameters during experimental exposure. T (temperature), S (salinity),
587 pH_{NBS} (pH calibrated with National Bureau of Standard scale), A_T (total alkalinity), pCO_2 (partial
588 pressure of $CO_{2)}$, Ωc and Ωa (saturation state of respectively calcite and aragonite). Data are presented
589 as means ± standard deviations.
590

	measured parameters				calculated parameters		
treatment	T (°C)	S	pH (NBS scale)	A_T (µmol/kg)	*p*CO2 (µatm)	Ωc	Ωa
CTRL	20.02 ± 0.08	30 ± 0.5	8.21 ± 0.04	3464.9 ± 138.8	407.7 ± 20.1	8.6 ± 0.4	5.5 ± 0.2
Cd	20.00 ± 0.10	30 ± 0.5	8.19 ± 0.04	3482.5 ± 80.1	409.3 ± 22.5	8.6 ± 0.3	5.5 ± 0.3
A	19.98 ± 0.06	30 ± 0.5	7.41 ± 0.04	3431.2 ± 179.1	3144.6 ± 160.2	1.7 ± 0.1	1.1 ± 0.1
A-Cd	20.92 ± 0.08	30 ± 0.5	7.43 ± 0.04	3464.9 ± 138.8	3124.3 ± 162.3	1.7 ± 0.2	1.1 ± 0.1

591

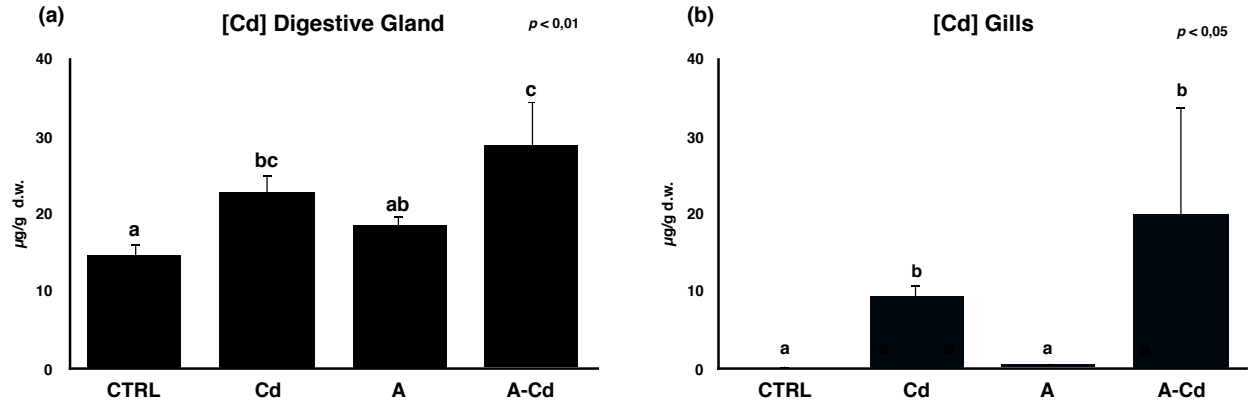


Figure 1. Cadmium concentrations in digestive gland (a) and gills (b) of exposed scallops. Data are expressed as mean values ± standard deviations (n=5). Different letters indicate significant differences between group of means (ANOVA and Tukey HSD *post-hoc*). CTRL= Control; Cd= Cadmium; A= acidification; A-Cd= acidification + Cd.

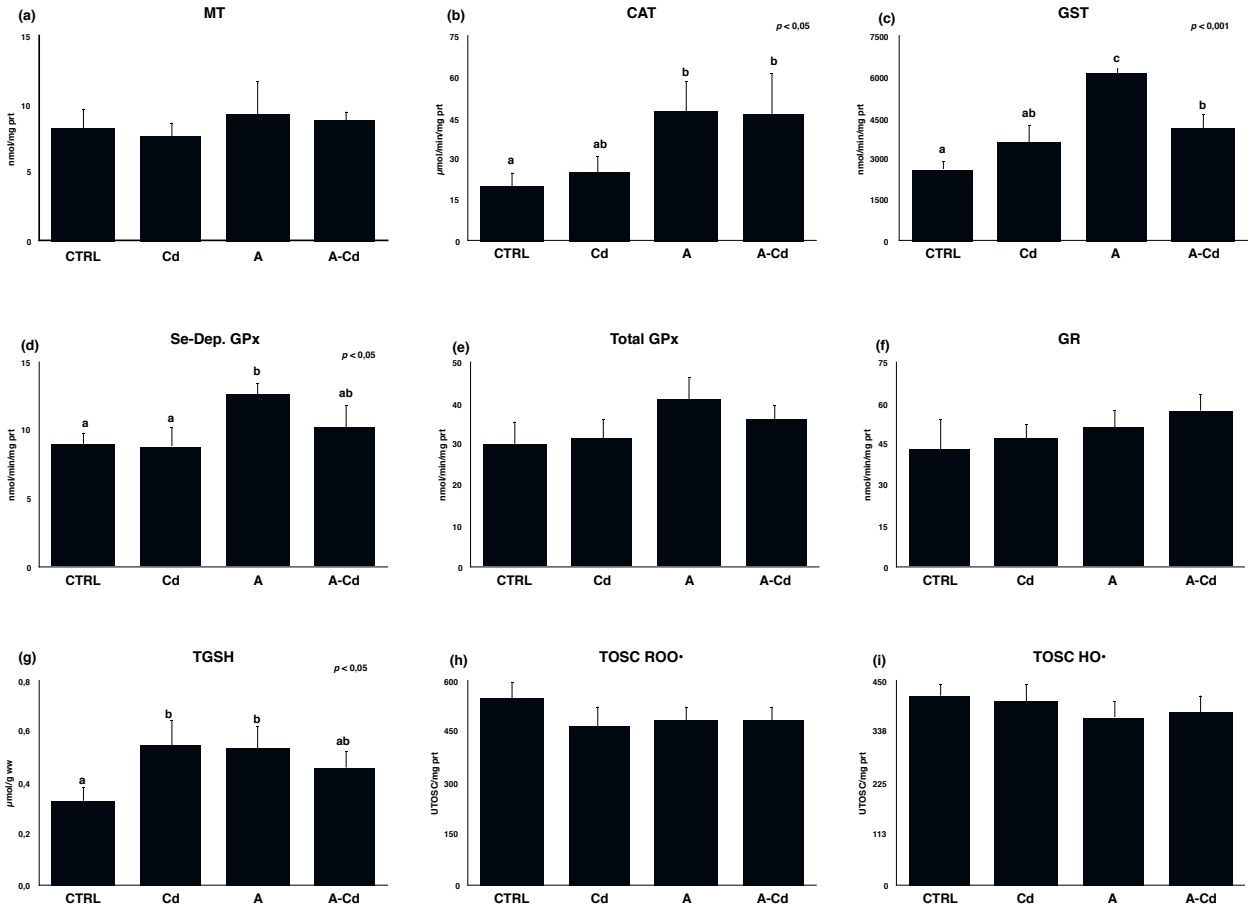
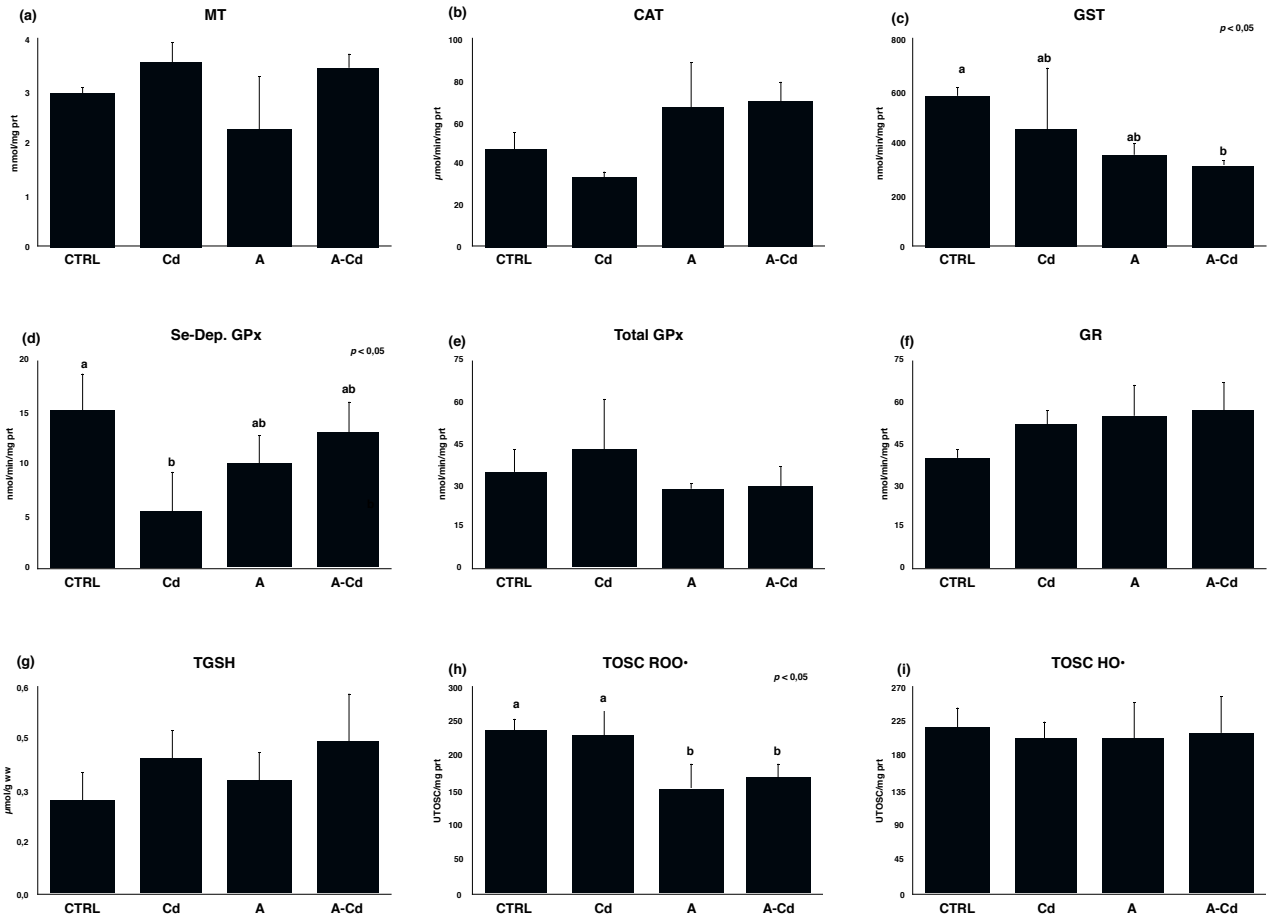


Figure 2. Metallothioneins and antioxidant defenses in digestive gland of scallops exposed to various treatments. MT: metallothioneins (a), CAT: catalase (b), GST: glutathione S-transferase (c), Se-Dep. GPx: Se-dependent glutathione peroxidases (d) total GPx: sum of Se-dependent and Se-independent glutathione peroxidases (e), GR: glutathione reductase (f), TGSH: total glutathione (g), TOSC ROO•: total oxyradical scavenging capacity toward peroxyl radical (h), TOSC HO•: total oxyradical scavenging capacity toward hydroxyl radical (i). Data are given as mean values ± standard deviations (n=5). Different letters indicate significant differences between group of means (ANOVA and Tukey HSD *post-hoc*). CTRL= Control; Cd= Cadmium; A= acidification; A-Cd= acidification + Cd.



607

608 Figure 3. Metallothioneins and antioxidant defenses in gills of scallops exposed to various treatments.
609 MT: metallothioneins (a), CAT: catalase (b), GST: glutathione S-transferase (c), Se-Dep. GPx: Se-
610 dependent glutathione peroxidases (d) total GPx: sum of Se-dependent and Se-independent
611 glutathione peroxidases (e), GR: glutathione reductase (f), TGSH: total glutathione (g), TOSC ROO•:
612 total oxyradical scavenging capacity toward peroxyl radical (h), TOSC HO•: total oxyradical
613 scavenging capacity toward hydroxyl radical (i). Data are given as mean values ± standard deviations
614 (n=5). Different letters indicate significant differences between group of means (ANOVA and Tukey
615 HSD *post-hoc*). CTRL= Control; Cd= Cadmium; A= acidification; A-Cd= acidification + Cd.

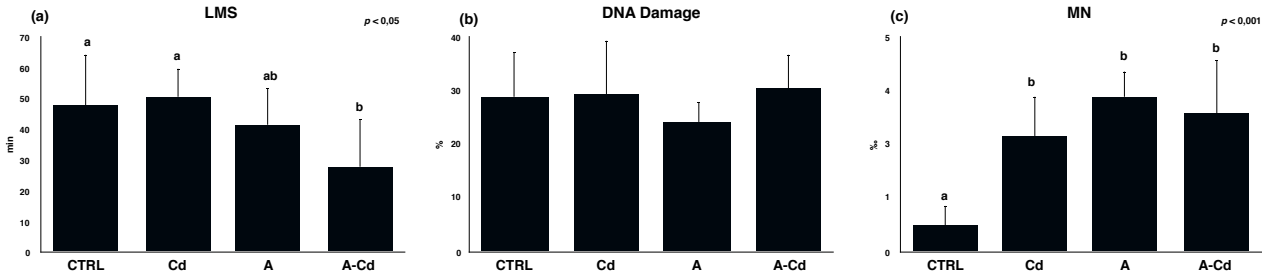
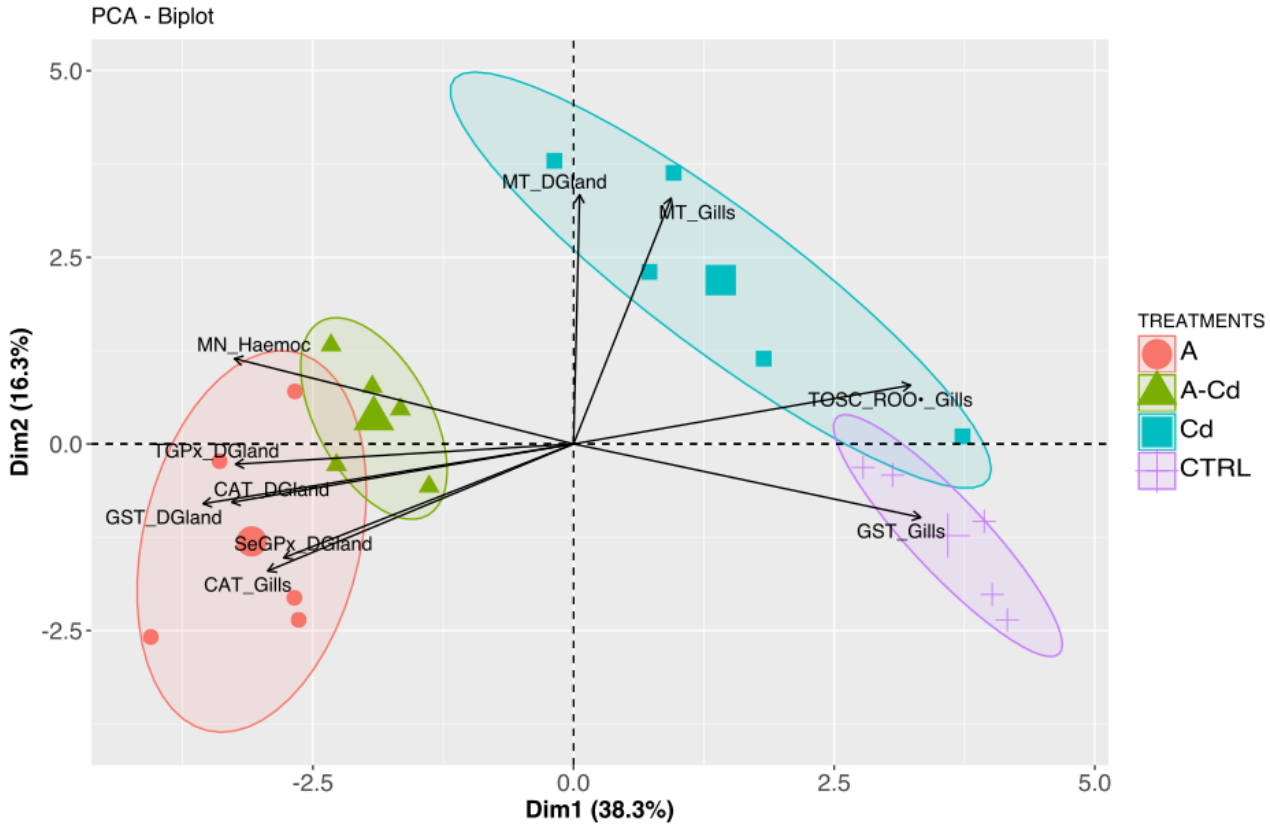


Figure 4. Lysosomal and genotoxic parameters in haemocytes of scallops exposed to various treatments. LMS: lysosomal membrane stability (a), DNA damage (b), MN: frequency of micronuclei (c). Data are given as mean values ± standard deviation (n=5). Different letters indicate significant differences between group of means (ANOVA and Tukey HSD *post-hoc*). CTRL= Control; Cd= Cadmium; A= acidification; A-Cd= acidification + Cd.



623

624 Figure 5. Graphical representation of principal components analysis conducted on biological
625 parameters analyzed in scallops tissues. CTRL= Control; Cd= Cadmium; A= acidification; A-Cd=
626 acidification + Cd. Arrows represent the ten major variables that contribute to the separation.

627

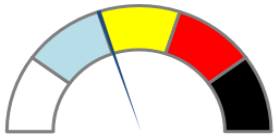
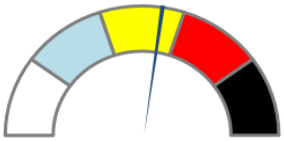
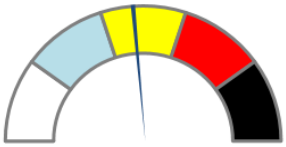
Experimental Treatment	Hazard Quotients (HQs)	Class of Hazard	Level
Cd	9.06	SLIGHT	
A	21.07	MODERATE	
A-Cd	13.13	MODERATE	

Figure 6. Weighted classification of biomarkers data for the whole dataset of analyzed parameters in each experimental condition. The quantitative hazard quotients (HQ) and the assigned class of hazard are given.