



UNIVERSITÀ POLITECNICA DELLE MARCHE
Repository ISTITUZIONALE

Effects of ocean warming and acidification on accumulation and cellular responsiveness to cadmium in mussels *Mytilus galloprovincialis*: Importance of the seasonal status

This is the peer reviewed version of the following article:

Original

Effects of ocean warming and acidification on accumulation and cellular responsiveness to cadmium in mussels *Mytilus galloprovincialis*: Importance of the seasonal status / Nardi, A.; Benedetti, M.; D'Errico, G.; Fattorini, D.; Regoli, F.. - In: AQUATIC TOXICOLOGY. - ISSN 0166-445X. - STAMPA. - 204:(2018), pp. 171-179. [10.1016/j.aquatox.2018.09.009]

Availability:

This version is available at: 11566/264923 since: 2022-06-01T13:01:40Z

Publisher:

Published

DOI:10.1016/j.aquatox.2018.09.009

Terms of use:

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. The use of copyrighted works requires the consent of the rights' holder (author or publisher). Works made available under a Creative Commons license or a Publisher's custom-made license can be used according to the terms and conditions contained therein. See editor's website for further information and terms and conditions.

This item was downloaded from IRIS Università Politecnica delle Marche (<https://iris.univpm.it>). When citing, please refer to the published version.

(Article begins on next page)

1 **Effects of ocean warming and acidification on accumulation and cellular responsiveness to**
2 **cadmium in mussels *Mytilus galloprovincialis*: importance of the seasonal status.**

3
4
5
6 Alessandro Nardi^a, Maura Benedetti^{a,b}, Giuseppe d'Errico^a, Daniele Fattorini^a, and Francesco
7 Regoli^{a,b*}

8
9
10 ^aDipartimento di Scienze della Vita e dell'Ambiente (DiSVA), Università Politecnica delle Marche,
11 Ancona, Italy

12 ^bCoNISMa, Consorzio Interuniversitario per le Scienze del Mare, Roma, Italy

13
14
15
16
17 *Corresponding author: Prof. Francesco Regoli

18 Dipartimento di Scienze della Vita e dell'Ambiente (DiSVA),

19 Università Politecnica delle Marche,

20 Via Breccie Bianche 60131, Ancona, Italy

21 e-mail: f.regoli@univpm.it

22
23 **Keywords:** *Mytilus galloprovincialis*; ocean acidification; global warming; metal contamination;
24 bioaccumulation; cellular biomarkers

25

26 **Abstract**

27 Ocean warming and acidification could represent additional threat to marine organisms already
28 coping with other anthropogenic impacts, such as chemical contamination in coastal areas. In this
29 study, interactions between such multiple stressors and their synergistic effects in terms of
30 accumulation, detoxification and biological effects of metals were investigated in the Mediterranean
31 mussel *Mytilus galloprovincialis*. Organisms sampled during the winter period were exposed for 28
32 days to different combinations of two temperatures (10°C and 15°C), two pH/pCO₂ (8.20/~400μatm
33 and 7.4/~3000μatm) and two doses of cadmium (0 and 20 μg/L). Cadmium concentrations increased
34 in digestive glands and gills of metal-exposed mussels, and were further enhanced by co-exposure at
35 higher temperature. Interactive effects of temperature and/or pH were observed on Cd-mediated
36 metallothioneins induction, responsiveness of antioxidant system and onset of oxidative damages to
37 lipids, with tissue-specific effects. Immunological effects showed a generalized sensitivity of
38 lysosomal membrane stability toward the investigated stressors with major effects in co-exposed
39 organisms. Cadmium and temperature affected phagocytosis efficiency and haemocytes population
40 composition probably influencing the micronuclei frequency through varied mitotic rate. Several
41 differences were highlighted between these results and those previously obtained from mussels
42 exposed in summer, supporting the importance of season when addressing the tolerance of temperate
43 organisms to variations of environmental factors. The elaboration of the whole biomarkers results
44 through weighted criteria allowed to summarize specific hazard indices, highlighting tissue-specific
45 sensitivity toward multiple stressors and the need of improving the knowledge on multiple stressors
46 interaction.

47 **1.Introduction**

48 Ocean warming and acidification are posing serious threats to marine ecosystems. Since the pre-
49 industrial values, ocean pH has dropped by 0.1 units (Gattuso and Lavigne, 2009) and global mean
50 temperature has risen by almost 1°C (Hansen *et al.*, 2016). According to projections of future
51 scenarios, pH will further decrease down to 7.8 and temperature will rise of 2°C by 2100 (IPCC,
52 2013).

53 These changes can induce direct effects on health status and physiological performance of marine
54 organisms, but synergistic effects between multiple stressors may also occur with less documented
55 consequences (Crain *et al.*, 2008; Burton and Johnston, 2010; Kroeker *et al.*, 2013). Metal
56 contamination is a typical anthropogenic footprint in coastal areas (Doney, 2010; Bijima *et al.*, 2013;
57 Gilaranz *et al.*, 2016) and both ocean warming and acidification, through different mechanisms, can
58 individually influence distribution and fate of trace elements in sediments and seawater, as well as
59 their bioaccumulation in marine organisms. Temperature may increase bioaccumulation of metals
60 enhancing their solubility (Sokolova and Lannig, 2008), and the ventilation and feeding activity of
61 marine organisms due to higher energy demand (Viarengo *et al.*, 1988; Nichols and Playle, 2004;
62 Baines *et al.*, 2006; Cherkasov *et al.*, 2007; Mubiana and Blust, 2007; Guinot *et al.*, 2012; Negri *et*
63 *al.*, 2013; Coppola *et al.*, 2018). On the other side, also CO₂-enrichment in seawater can alter the
64 speciation and solubility of metals forming strong complexes with carbonate ions (Millero *et al.*,
65 2009; Hoffmann *et al.*, 2012; Stockdale *et al.*, 2016) or with organic compounds (Gledhill *et al.*,
66 2015). Increased fluxes of metals from sediments to seawater have been shown to occur at reduced
67 pH values or simulating CO₂ leakages from sub-seabed (Riba *et al.* 2003; Ardelan *et al.*, 2009; de
68 Orte *et al.*, 2014a, 2014b; Basallote *et al.*, 2015), and a higher uptake due to acidification has been
69 described in several marine invertebrates including bivalves (López *et al.*, 2010; Ivanina *et al.*, 2014;
70 Götze *et al.*, 2014; Rodríguez-Romero *et al.*, 2014a; Shi *et al.*, 2016; Velez *et al.*, 2016), polychaetes
71 (Rodríguez-Romero *et al.*, 2014b), and cephalopods (Lacoue-Labarthe *et al.*, 2009, 2011).

72 Various cellular mechanisms have been suggested to influence the vulnerability of marine
73 organisms to metals toxicity when also exposed to variations of temperature and pH/pCO₂. The
74 interaction between temperature and metals has been demonstrated to cause impairment of
75 mitochondrial function, pro-oxidative mechanisms, accumulation of lipid peroxidation products and
76 damages to lysosomes and DNA (Sokolova, 2004; Kefaloyianni *et al.*, 2005; Cherkasov *et al.*, 2007;
77 Mubiana and Blust, 2007; Sokolova and Lannig, 2008; Guinot *et al.*, 2012; Negri *et al.*, 2013; Attig
78 *et al.*, 2014; Banni *et al.*, 2014; Gomiero and Viarengo, 2014; Izagirre *et al.*, 2014; Múgica *et al.*,
79 2015). Similarly, interactive effects between metals and acidification are addressed in impaired larval
80 development, onset of DNA damage, pro-oxidative mechanisms and lower antioxidant efficiency,
81 alterations of the immune function (Lewis *et al.*, 2013, 2016; Roberts *et al.*, 2013; Campbell *et al.*,
82 2014; Ivanina *et al.*, 2015, 2016; Siddiqui and Bielmyer-Fraser, 2015).

83 Recently, cadmium (Cd) accumulation was shown to be unaffected by variations of temperature
84 and pH in the digestive gland and gills of the Mediterranean mussel *M. galloprovincialis* and of the
85 Antarctic scallop *A. colbecki*. At the same time, however, the effects of Cd on metal-binding proteins,
86 immune system, antioxidant responses and oxidative stress biomarkers were strongly modulated by
87 these co-factors, with different effects and magnitude depending on species and tissue (Benedetti *et*
88 *al.*, 2016; Nardi *et al.*, 2017). Beside species- and tissue-mediated specificity, seasonality of
89 environmental factors (notably temperature) and biological processes (i.e. reproductive cycle) could
90 be responsible for the different capability of organisms to tolerate variations of temperature and pH
91 related to climate change and their interactions with pollutants. In this respect, several cellular
92 responses typically involved in defence mechanisms and resistance to stressors exhibit marked
93 seasonal fluctuations in marine invertebrates and the ecophysiological performance of organisms is
94 also influenced by their seasonal metabolic status and energy available for processes like reproduction
95 and growth (Ringwood *et al.*, 2002; Bocchetti and Regoli, 2006; Farcy *et al.*, 2007; Bocchetti *et al.*,
96 2008; Pereira *et al.*, 2012).

97 Considering the complexity of interactions between environmental and biological factors, this
98 study aimed to elucidate whether seasonality can influence the responsiveness of *M. galloprovincialis*
99 to various combinations of temperature, pH and Cd: experiments were performed in winter and results
100 compared with those obtained in a similar study carried out in summer season (Nardi *et al.*, 2017).
101 Beside Cd accumulation, a wide battery of biomarkers was chosen to reflect the network of cellular
102 mechanisms underlying metal detoxification, oxyradical metabolism and onset of different forms of
103 cellular toxicity (Regoli and Giuliani, 2014): these biomarkers included induction of
104 metallothioneins, variations of single antioxidant defenses, total oxyradical scavenging capacity,
105 accumulation of lipid peroxidation products in digestive gland and gills, while lysosomal membrane
106 stability, phagocytosis, granulocytes/hyalinocytes ratio, DNA strand breaks and micronuclei
107 frequency were measured in haemocytes. To better summarize the biological significance of obtained
108 results and to facilitate the seasonal comparison of mussels susceptibility, the overall biomarkers
109 results have been elaborated through a widely validated quantitative hazard model (SediquaSoft):
110 this model applies weighted criteria based on the toxicological relevance of each analyzed biomarker
111 and on the magnitude of observed variations to summarize a synthetic cellular hazard index (Piva *et*
112 *al.*, 2011; Benedetti *et al.*, 2012).

113 The obtained results are intended to expand our knowledge on the impacts of synergistic effects
114 of multiple stressors on cellular and tissue health status, and to relate them with the physiology of
115 mussels as a response to seasonal change.

116

117 **2. Materials and Methods**

118 *2.1 Animal collection and experimental design*

119 Mussels, *M. galloprovincialis* (6.0 ± 0.5 cm shell length), were obtained in January 2015 from
120 a shellfish farm in an unpolluted area of Central Adriatic Sea (Regoli *et al.*, 2014) and maintained for
121 7 days in aquaria with aerated artificial seawater (ASW; Instant Ocean®) at local environmental
122 conditions of salinity (37 practical salinity units), temperature (10°C) and pH (8.20).

123 After acclimation, mussels were randomly assigned and exposed to one of the following
124 treatments, each containing 36 organisms in 20 L tanks: 1) control condition (CTRL), at
125 environmental temperature of 10°C, normocapnia with pH=8.20/ $p\text{CO}_2 \sim 400 \mu\text{atm}$; 2) Cd exposure
126 (Cd), 10°C, pH=8.20/ $p\text{CO}_2 \sim 400 \mu\text{atm}$ and 20 $\mu\text{g/L}$ Cd; 3) acidification (A), 10°C, hypercapnia
127 with pH=7.40/ $p\text{CO}_2 \sim 3000 \mu\text{atm}$; 4) warming (W), 15°C and pH=8.20/ $p\text{CO}_2 \sim 400 \mu\text{atm}$; 5)
128 acidification + Cd (A-Cd), 10°C, pH=7.40/ $p\text{CO}_2 \sim 3000 \mu\text{atm}$ and 20 $\mu\text{g/L}$ Cd ; 6) warming + Cd
129 (W-Cd), 15°C, pH=8.20/ $p\text{CO}_2 \sim 400 \mu\text{atm}$ and 20 $\mu\text{g/L}$ Cd ; 7) acidification + warming (A-W), 15°C
130 and pH=7.40/ $p\text{CO}_2 \sim 3000 \mu\text{atm}$; 8) acidification + warming + Cd (A-W-Cd), 15°C, pH=7.40/
131 $p\text{CO}_2 \sim 3000 \mu\text{atm}$ and 20 $\mu\text{g/L}$ Cd. Despite the effects related to climate changes are expected to
132 occur over the course of decades, organisms were exposed to the tested environmental conditions of
133 Cd, pH and temperature without gradual acclimation, thus simulating sudden changes as those more
134 easily occurring in coastal or estuarine areas. The same experimental design had been previously
135 applied on mussels sampled during the summer with the exception of temperature values, respectively
136 at 20 and 25 °C for the control and warming treatments (Nardi *et al.*, 2017). Cd dose is representative
137 for a polluted but environmentally realistic condition in coastal waters, typically ranging from less
138 than 1 up to hundreds $\mu\text{g/L}$ in highly polluted areas (Neff, 2002; Bakary *et al.*, 2015). Lowered pH
139 was adapted from scenario RCP 8.5 and IPCC WGII AR5 (IPCC, 2014; Wong *et al.*, 2014) reporting
140 a mean pH value of 7.7 for open oceans, but predicting more pronounced reductions in coastal areas.
141 The experimental pH was reached by adding to each treatment ASW (pH=8.2) small and defined
142 amounts of CO₂-saturated ASW, obtained by bubbling pure CO₂ in ASW for at least 24h, until

143 reaching the target pH within a couple of hours (Nardi *et al.*, 2017). Salinity and pH were measured
144 daily, while total alkalinity (A_T) was measured twice per week according to Dickson *et al.* (2007),
145 and used for calculating seawater carbonate parameters ($p\text{CO}_2$, and saturation state (Ω) for calcite
146 and aragonite) in CO2SYS (Pierrott *et al.*, 2006) using barometric pressure values (full seawater
147 chemistry is provided in Table 1). For calculations, we used NBS scale for seawater pH, carbonate
148 constants from Millero (2010), KSO_4^- constant from Dickson *et al.* (2007), and concentrations of
149 silicate and phosphate from Instant Ocean® seawater ($0.21 \mu\text{mol/kg}$ and $0.05 \mu\text{mol/kg}$, respectively).
150 Water in each treatment was changed every other day using water at the same pH and temperature to
151 avoid fluctuations of these parameters during the exposure period. Mussels fed 12 hours prior the
152 water change with a commercial mixture of zooplankton for filter-feeding organisms (Zooplanktos-
153 S 50-300 μm , Brightwell Aquatics, Elysburg, PA).

154 After four weeks, animals were sampled for chemical and biological analyses. Specifically gills
155 and digestive glands were excised from the 36 specimens for each treatment, pooled in 12 samples,
156 each constituted by the tissues of 3 individuals, rapidly frozen in liquid nitrogen and maintained at -
157 80°C : these 12 pooled samples were shared for analyses of Cd and biomarker responses, to guarantee
158 a n value = 5 for replicates analysed for each parameter in each treatment. Haemolymph was also
159 withdrawn from the adductor muscle of 5 specimens for each treatment and immediately used for
160 measurement of immunity parameters and genotoxic damage.

161 2.2 Cd determination

162 Concentration of Cd in digestive gland and gills of mussels was analyzed according to
163 previously described methods (Regoli *et al.*, 2005). For each treatment, tissues were dried at 60°C
164 overnight and digested in a microwave system (Mars V, CEM). After digestion samples were
165 analyzed by atomic absorption spectrophotometry with graphite furnace atomization and Zeeman
166 effect. Quality assurance and control were assessed by processing blank samples and reference
167 standard material (Mussel Tissue Standard Reference Material SRM NIST-2977, National Institute
168 of Standards and Technology Gaithersburg, MD, USA), which always resulted within the 95%

169 confidence interval of certified values. Data are expressed as $\mu\text{g/g}$ dry weight (mean value \pm standard
170 deviation, $n = 5$), limits of quantification and detection were respectively 0.002 and 0.001 $\mu\text{g/g}$.

171 2.3 Biomarkers responses

172 Standardized methods, described in detail in Supplementary Material 1 (SM1), were used for
173 biomarkers analyses. Digestive gland and gills were analyzed for metallothioneins (MTs), antioxidant
174 enzymes and scavenger (catalase, glutathione S-transferases, glutathione peroxidases, glutathione
175 reductase, total glutathione), total oxyradical scavenging capacity toward peroxy radicals (TOSC
176 $\text{ROO}\bullet$) and hydroxyl radicals (TOSC $\text{HO}\bullet$), malondialdehyde content (MDA). Lipofuscin and neutral
177 lipids were histochemically determined on 8 μm thick cryostat sections of digestive glands.
178 Haemocytes were immediately processed for immune-related alterations, such as lysosomal
179 membrane stability (neutral red retention time, NRRT), phagocytosis activity and granulocytes versus
180 hyalinocytes ratio. Also genotoxic effects were evaluated in haemocytes in terms of percentage of
181 DNA integrity (Comet assay) and micronuclei frequency (MN).

182 2.7 Statistical analyses

183 Cd accumulation and biological responses results were submitted to analysis of variance
184 (One-way ANOVA), after checking the normal distribution (Shapiro-Wilk test) and homogeneity of
185 variances (Levene's Test). The null hypothesis tested was that no significant difference existed
186 between different treatments, level of significance was set to $p < 0.05$, and *post-hoc* tests (Student-
187 Newman-Keuls, SNK), were used to compare group of means. All statistical analyses were
188 performed using RStudio (version 0.99.491).

189 The overall significance of biomarkers results was summarized in a cellular hazard index
190 elaborated through a previously developed quantitative model which applies weighted criteria to
191 discriminate different endpoints and the magnitude of effects (SediquaSoft, Piva *et al.*, 2011). Despite
192 whole calculations and assumptions have been fully given elsewhere (Piva *et al.*, 2011; Benedetti *et*
193 *al.*, 2012), the general rationale of the model is to assign a weight to various biomarkers depending
194 on their toxicological relevance and to compare variations of responses toward a threshold specific

195 for each biomarker, which considers the different responsiveness among various species and tissues,
196 and the possibility of biphasic responses. The calculated Hazard Quotient (HQ) does not include the
197 contribution of biomarkers with variations lower or equal to their threshold, and it averages or adds
198 the effects respectively for those biomarkers with variations up to 2-fold or more than 2-fold greater
199 than the specific threshold (Piva *et al.*, 2011; Benedetti *et al.*, 2012, 2014; Regoli *et al.*, 2014; Avio
200 *et al.*, 2015). The model finally assigns the elaborated HQ to one of five classes of hazard, from
201 Absent to Severe (Piva *et al.*, 2011).

202

203 **3. Results**

204 A significant increase of Cd concentrations was observed in digestive gland and gills of mussels
205 exposed to this metal (Fig.1a, b), and further enhanced in both the tissues by co-exposure at higher
206 temperature (Cd and A-Cd *versus* W-Cd and A-W-Cd) (Fig. 1a, b).

207 Metallothioneins in the digestive gland were not significantly induced in organisms exposed to
208 Cd alone, while increasing in those co-exposed to the metal at reduced pH and/or higher temperature
209 (A-Cd, W-Cd and A-W-Cd) (Fig. 1c). In gills, metallothioneins were significantly enhanced only in
210 organisms exposed to Cd at higher temperature and control pH (W-Cd) (Fig.1d).

211 Antioxidant defenses showed tissue-specific effects of the investigated factors. In the digestive
212 gland, no statistically significant variations were observed in various treatments for catalase, GSTs,
213 Se-dependent GPx and TGSH (Fig. 2a, b, c, f). On the other hand, Cd caused a marked induction of
214 total GPx, particularly evident at control pH (compare CTRL vs. Cd and W vs. W-Cd); at reduced
215 pH, Cd did not further modulate the increase of total GPx caused by acidification itself (A vs. A-Cd,
216 and A-W vs. A-W-Cd) (Fig. 2d). Higher temperature was the only treatment to significantly reduce
217 GR activity in digestive gland (Fig. 2e). Total oxyradical scavenging capacity toward peroxy radical
218 (TOSC ROO•), levels of malondialdehyde and lipofuscin were generally enhanced by treatments
219 involving Cd alone and/or in combination with other stressors, but the statistical significance was
220 obtained only for TOSC and lipofuscin in mussels co-exposed to Cd, acidification and higher

221 temperature (A-W-Cd) (Fig. 2g, i, j). Conversely, neutral lipids content was reduced in all Cd-
222 exposed organisms at reduced pH and/or higher temperature (A-Cd, W-Cd and A-W-Cd) compared
223 to organisms exposed to Cd alone (Fig. 2k).

224 In gills, significant variations of antioxidant enzymes were observed for glutathione S-
225 transferases, selenium-dependent and total glutathione peroxidases. GSTs were inhibited in mussels
226 exposed to Cd alone, Cd at higher temperature (W-Cd) and higher temperature at reduced pH (A-W)
227 (Fig. 3b). Opposite effects were observed on Se-dependent GPx which were increased by Cd and
228 temperature as individual stressors (Cd and W) and by the combination of these factors with
229 acidification (A-Cd, A-W, A-W-Cd) (Fig. 3c). Enhanced values were also observed for total GPx
230 activity, particularly for treatments involving higher temperature and/or acidification (Fig. 3d).
231 Limited variations were measured in the total oxyradical scavenging capacity toward hydroxyl radical
232 (TOSC HO•) with slightly higher values in organisms exposed to Cd or higher temperature (Fig. 3h).

233 Lysosomal membrane stability in haemocytes significantly decreased in all experimental
234 treatments, with major effects in organisms co-exposed to all multiple stressors (A-W-Cd) (Fig. 4a).
235 Phagocytosis rate (Fig. 4b) was reduced in mussels treated at higher temperature (alone or in
236 combination with Cd), and also granulocytes/hyalinocytes ratio (Fig. 4c) was significantly reduced
237 in organisms co-exposed to Cd at higher temperature (W-Cd and A-W-Cd). No significant variations
238 were observed in terms of DNA strand breaks (Fig. 4d) while micronuclei frequency was generally
239 enhanced in all organisms exposed to Cd alone or with higher temperature (Fig. 4e).

240 Biomarkers responses observed in each experimental condition were summarized in a single
241 hazard index through the application of weighted criteria (Fig. 5). The elaborated class of hazard
242 revealed that digestive gland was generally more sensitive to Cd dosed alone and in combination with
243 A or W, when the assigned class of hazard was “Moderate”, compared to “Slight” in all the other
244 treatments (Fig. 5). In gills, higher values of HQ were typically measured compared to digestive
245 gland, and variations of biomarkers were generally reflected by a “Moderate” class of hazard (Fig.
246 5).

247 **4. Discussion**

248 This study provided new evidence that climate change can affect accumulation and
249 responsiveness to metals in marine organisms.

250 The obtained results highlighted an increased Cd accumulation in mussels co-exposed to the
251 metal at higher temperature during the winter period, contrasting with our previous study in which
252 this effect was not observed during the summer (Nardi *et al.*, 2017). The winter environmental
253 temperature was 10°C and the increased value selected for this study (15°C) was still below the upper
254 thermal limit of *M. galloprovincialis* (Anestis *et al.*, 2007; Gazeau *et al.*, 2014). On the other hand,
255 the environmental and warmer values used for summer exposures were 20° and 25°C (Nardi *et al.*,
256 2017). In this respect, only during the colder period the increase of temperature would have enhanced
257 metabolism rate and consequent accumulation of metals, confirming that the effects of climate-related
258 stressors are closely related to thermal tolerance of organisms (Ioannou *et al.*, 2009; Pörtner, 2010),
259 as confirmed in mussels exposed to thermal stress (21°C). The effect of temperature on enhanced Cd
260 accumulation was noticed both in digestive gland and gills, suggesting a whole organism,
261 physiological response, rather than a tissue-specific pathway. The different seasonal effects of
262 temperature on Cd accumulation suggests that future warming scenarios should take into account this
263 variability in temperate marine organisms, as *M. galloprovincialis*, which experience wide natural
264 fluctuations of this environmental parameter. The metal uptake was not further modulated by
265 reduction of pH. Similar results were previously observed in mussels exposed during the summer and
266 in scallops from Antarctic region (*A. colbecki*) (Benedetti *et al.*, 2016; Nardi *et al.*, 2017), while a
267 slight influence of lowered pH on Cd accumulation occurred in the smooth Mediterranean scallop
268 *Flexopecten glaber* (Nardi *et al.*, 2018). Overall these results confirm the more limited influence of
269 acidification compared to temperature on the bioaccumulation of Cd, despite a certain influence of
270 species-specific characteristics.

271 Metallothioneins induction is typically associated to the increase of tissue metal concentrations,
272 but a similar parallelism was not always observed in our study. In both digestive gland and gills, MTs

273 were not induced in mussels exposed to Cd alone, suggesting that basal levels of these proteins were
274 able to compensate for the increased metal content. The lack of correlations between Cd
275 bioaccumulation and MTs induction observed during co-exposures supports the modulation on
276 protein synthesis by oxidative pressure, confirming the role of prooxidant mechanisms caused by
277 synergistic effects of multiple stressors, as also previously observed in mussels exposed during the
278 summer (Viarengo, 2000; Regoli and Giuliani, 2014; Nardi *et al.*, 2017).

279 The oxidative challenge was assessed through the analysis of an extensive array of biomarkers
280 which included antioxidant defenses, total oxyradical scavenging capacity and oxidative damages. In
281 digestive gland, total GPx appeared as responsive enzymes, particularly toward Cd and acidification.
282 However, the more relevant results in terms of varied susceptibility to oxidative stress were observed
283 in mussels co-exposed to Cd, acidification and higher temperature. In these treatments, the significant
284 increase of total oxyradical scavenging capacity toward peroxy radicals and of lipofuscin content,
285 confirm the importance of oxidative pathways in modulating the responsiveness of marine organisms
286 to multiple stressors, as also suggested for *Hediste diversicolor* co-exposed to Hg and pH reduction
287 (Freitas *et al.*, 2017). Neutral lipids content tended to increase in digestive gland after exposure to Cd
288 alone and to decrease during co-exposures to multiple stressors, suggesting a greater use of reserve
289 materials under increasing cellular stress, as already hypothesized for *M. galloprovincialis* challenged
290 by trace metals and organic pollutants (Regoli, 1992; Koukouzika and Dimitriadis, 2008). As for
291 MTs, also variations of oxidative parameters in digestive gland did not correlate with Cd content, and
292 thus confirmed the occurrence of synergistic cellular effects between multiple stressors. Compared
293 to actual results, mussels previously exposed during the summer showed a greater sensitivity of
294 antioxidants in digestive gland toward temperature rise (alone or in combination with Cd), further
295 indicating that pathways of response and susceptibility to changes of environmental stressors can
296 vary between seasons.

297 Different and more frequent oxidative variations were observed in gills. Cd, higher temperature
298 and acidification (alone or in combination) were generally responsible for a certain reduction of GSTs

299 and the induction of Se-dependent and Se-independent GPx, confirming the involvement of these
300 antioxidant pathways also reported for mussels exposed during the summer (Nardi *et al.*, 2017). The
301 results obtained in gills at higher temperature, conversely to digestive gland, highlight that metabolic
302 and physiological functions of tissues may be responsible for their different responsiveness and
303 sensitivity toward the investigated stressors. The increase of winter temperature might exert oxidative
304 challenge in the gills due to enhanced filtration rates, thus explaining the raise of antioxidant
305 protection (Fields *et al.*, 2012). The higher enzymatic activities caused by individual stressors were
306 not synergistically enhanced during co-exposures, and were not typically paralleled by considerable
307 variations of the total oxyradical scavenging capacity; the concomitant lack of changes in
308 malondialdehyde content indicates that reported variations of antioxidant system were efficient in
309 preventing an oxidative impairment in gills: once again, winter results are partly in contrast with those
310 obtained in summer experiments when the total oxyradical scavenging capacity was decreased toward
311 hydroxyl radicals (Nardi *et al.*, 2017). The generally higher efficiency of antioxidant protection
312 observed during the winter reinforce the evidence that responsiveness of mussels to climate changes
313 is influenced by seasonal-specific features.

314 Lysosomal membranes are well known targets of oxyradical toxicity and their destabilization is
315 a typical effect of chemical, environmental or biological factors. In our study, lysosomal impairment
316 was a reliable biomarker toward the investigated stressors, further evidencing the occurrence of
317 synergistic effects during co-exposures to multiple factors. At the same time, temperature, alone or
318 in combination with Cd lowered phagocytosis rate, probably due to a reduction of granulocytes,
319 which are the haemocytes responsible of this activity (Gorbi *et al.*, 2013). The generalized impairment
320 of lysosomal membranes stability and phagocytosis rate confirm the sensitivity of haemocytes in
321 marine invertebrates and their utility as early warning signal of environmental disturbance (Beesly *et*
322 *al.*, 2008; Matozzo *et al.*, 2012; Sureda *et al.*, 2013; Nardi *et al.*, 2017). Sensitivity of haemocytes to
323 Cd, both alone and in combination with temperature was supported also by the increase in micronuclei
324 frequency which, considering the absence of changes in DNA strand breaks, might be modulated by

325 changes in mitotic frequency, rather than a direct genotoxic mechanism, as previously hypothesized
326 in similar experiments (Nardi *et al.*, 2017; Benedetti *et al.*, 2016).

327 The biological significance of the observed biomarker responses was better summarized by the
328 elaboration of specific hazard indices based on weighted criteria, which consider both the magnitude
329 of variations and the relative toxicological importance of investigated parameters (Piva *et al.*, 2011;
330 Benedetti *et al.*, 2012). Biomarkers have been widely used for early warning detection of
331 environmental disturbance, and various integrative methods and health indices have been recently
332 developed to improve their use in ecological risk assessment (Beliaeff and Burgeot, 2002; Piva *et al.*,
333 2011; Broeg *et al.*, 2005; Dagnino *et al.*, 2008; Benedetti *et al.*, 2012; Marigómez *et al.*, 2013). The
334 elaboration applied in the present study is part of a more articulated Weight Of Evidence model
335 (SediquaSoft) which integrates multiple typologies of data including sediment and seawater
336 chemistry, bioaccumulation, biomarker, ecotoxicological bioassays and benthic communities:
337 integrative rules and assumptions have been detailed in previous papers and already validated in
338 several risk assessment studies (Benedetti *et al.*, 2012, 2014; Regoli *et al.*, 2014; Avio *et al.*, 2015;
339 Bebianno *et al.*, 2015). The hazard indices derived from biomarker results highlighted that, toward
340 individual stressors, digestive gland is more sensitive to variations of Cd (HQ Moderate) compared
341 to acidification or warming alone (HQ Slight): despite different combinations of multiple stressors
342 were shown to highlight synergistic effects on various biological responses and mechanisms of action,
343 they did not determine an overall increase of the assigned class of cellular.

344 Gills typically exhibited a Moderate hazard index suggesting that the more direct contact with
345 seawater, and the lower basal metabolic activity could render these tissues more susceptible to
346 fluctuations of environmental factors. Once again, the variations observed for various biomarker
347 responses after contemporary exposure to multiple stressors did not change the elaborated class of
348 cellular hazard indices compared to individual factors:

349 Overall, the sensitivity of winter mussels is partly in contrast with results obtained in summer
350 season, when organisms exhibited more clear synergistic effects of Cd dosed in association to

351 warming and/or acidification, highlighting the importance of seasonal-specific sensitivity of
352 temperate organisms toward multiple stressors. Our data suggest also the need to still investigate
353 tissue-specific sensitivity, mechanisms of action related to physiological functions, and seasonal-
354 dependent effects to better understand tolerance to climate changes and consequences of multiple
355 stressors in marine organisms. Finally, the application of weighted criteria to elaborate hazard indices
356 was confirmed as a fundamental procedure to summarize the biological significance of large datasets
357 of complex biomarker data, thus facilitating prediction and comparisons between the effects of
358 individual and multiple stressors.

359 **5. References**

- 360 Anestis A, Lazou A, Pörtner HO, Michaelidis B (2007). Behavioral, metabolic, and molecular stress
361 responses of marine bivalve *Mytilus galloprovincialis* during long-term acclimation at increasing
362 ambient temperature. *American Journal of Physiology - Regulatory Integrative and Comparative*
363 *Physiology*, 293 (2), pp. R911-R921.
- 364 Ardelan MV, Steinnes E, Lierhagen S, Linde SO (2009) Effects of experimental CO₂ leakage on
365 solubility and transport of seven trace metals in seawater and sediment. *Science of the Total*
366 *Environment*, 407 (24), pp. 6255-6266.
- 367 Attig H, Kamel N, Sforzini S, Dagnino A, Jamel J, Boussetta H, Viarengo A, Banni M (2014). Effects
368 of thermal stress and nickel exposure on biomarkers responses in *Mytilus galloprovincialis* (Lam).
369 *Marine Environmental Research*, 94, pp. 65-71.
- 370 Avio CG, Gorbi S, Milan M, Benedetti M, Fattorini D, d'Errico G, Pauletto M, Bargelloni L, Regoli
371 F (2015). Pollutants bioavailability and toxicological risk from microplastics to marine mussels.
372 *Environmental Pollution*, 198, pp. 211-222.
- 373 Baines SB, Fisher NS, Kinney EL (2006). Effects of temperature on uptake of aqueous metals by
374 blue mussels *Mytilus edulis* from Arctic and temperate waters. *Marine Ecology Progress Series*, 308,
375 pp. 117-128.
- 376 Bakary I, Yao KM, Etchian OA, Soro MB, Trokourey A, Bokra Y (2015). Zinc, copper, cadmium,
377 and lead concentrations in water, sediment, and *Anadara senilis* in a tropical estuary. *Environmental*
378 *Monitoring and Assessment*, 187 (12), pp. 1-11.
- 379 Banni M, Attig H, Sforzini S, Oliveri C, Mignone F, Boussetta H, Viarengo A (2014). Transcriptomic
380 responses to heat stress and nickel in the mussel *Mytilus galloprovincialis*. *Aquatic Toxicology*, 148,
381 pp. 104-112.
- 382 Basallote MD, Rodríguez-Romero A, De Orte MR, Del Valls TÁ, Riba I (2015). Evaluation of the
383 threat of marine CO₂ leakage-associated acidification on the toxicity of sediment metals to juvenile
384 bivalves. *Aquatic Toxicology*, 166, pp. 63-71.
- 385 Bebianno MJ, Pereira CG, Rey F, Cravo A, Duarte D, d'Errico G, Regoli F (2015). Integrated
386 approach to assess ecosystem health in harbor areas. *Science of the Total Environment*, 514, pp. 92-
387 107.
- 388 Beesley A, Lowe DM, Pascoe CK, Widdicombe S (2008) Effects of CO₂-induced seawater
389 acidification on the health of *Mytilus edulis*. *Climate Research*, 37, pp. 215-225.
- 390 Beliaeff B, Burgeot T (2002) Integrated biomarker response: A useful tool for ecological risk
391 assessment. *Environmental Toxicology and Chemistry*, 21, pp. 1316-1322.
- 392 Benedetti M, Ciaprini F, Piva F, Onorati F, Fattorini D, Notti A, Ausili A, Regoli F (2012). A
393 multidisciplinary weight of evidence approach for classifying polluted sediments: Integrating
394 sediment chemistry, bioavailability, biomarkers responses and bioassays. *Environment International*,
395 38, pp. 17-28.
- 396 Benedetti M, Gorbi S, Fattorini D, d'Errico G, Piva F, Pacitti D, Regoli F (2014) Environmental
397 hazards from natural hydrocarbons seepage: Integrated classification of risk from sediment chemistry,
398 bioavailability and biomarkers responses in sentinel species. *Environmental Pollution*, 185, pp. 116-
399 126.

- 400 Benedetti M, Lanzoni I, Nardi A, d'Errico G, Di Carlo M, Fattorini D, Nigro M, Regoli F (2016).
401 Oxidative responsiveness to multiple stressors in the key Antarctic species, *Adamussium colbecki*:
402 Interactions between temperature, acidification and cadmium exposure. *Marine Environmental*
403 *Research*, 121, pp. 20-30.
- 404 Bijima J, Pörtner H-O, Yesson C, Rogers AD (2013). Climate change and the oceans – What does
405 the future hold? *Marine Pollution Bulletin*, 74, pp. 494-505.
- 406 Bocchetti R, Regoli F (2006). Seasonal variability of oxidative biomarkers, lysosomal parameters,
407 metallothioneins and peroxisomal enzymes in the Mediterranean mussel *Mytilus galloprovincialis*
408 from Adriatic Sea. *Chemosphere*, 65, pp. 913-921.
- 409 Bocchetti R, Lamberti CV, Pisanelli B, Razzetti EM, Maggi C, Catalano B, Sesta G, Martuccio G,
410 Gabellini M, Regoli F (2008). Seasonal variations of exposure biomarkers, oxidative stress responses
411 and cell damage in the clams, *Tapes philippinarum*, and mussels, *Mytilus galloprovincialis*, from
412 Adriatic sea. *Marine Environmental Research*, 66, pp. 24-26.
- 413 Broeg K, Westernhagen HV, Zander S, Körting W, Koehler A (2005) The Bioeffect Assessment
414 Index (BAI) a concept for the quantification of effects of marine pollution by an integrated biomarker
415 approach. *Marine Pollution Bulletin*, 50, pp. 495–503.
- 416 Burton GA, Johnston EL (2010). Assessing contaminated sediments in the context of multiple
417 stressors. *Environmental Toxicology and Chemistry*, 29, pp. 2625–2643.
- 418 Campbell AL, Mangan S, Ellis RP, Lewis C (2014). Ocean acidification increases copper toxicity to
419 the early life history stages of the polychaete *Arenicola marina* in artificial seawater. *Environmental*
420 *Science and Technology*, 48, pp. 9745-9753.
- 421 Cherkasov AS, Grewal S, Sokolova IM (2007). Combined effects of temperature and cadmium
422 exposure on haemocyte apoptosis and cadmium accumulation in the eastern oyster *Crassostrea*
423 *virginica* (Gmelin). *Journal of Thermal Biology*, 32, pp. 162-170.
- 424 Coppola F, Almeida Â, Henriques B, Soares AMVM, Figueira E, Pereira E, Freitas R (2018).
425 Biochemical responses and accumulation patterns of *Mytilus galloprovincialis* exposed to thermal
426 stress and Arsenic contamination. *Ecotoxicology and Environmental Safety*, 147, pp. 954-962.
- 427 Crain CM, Kroeker K, Halpern BS (2008). Interactive and cumulative effects of multiple human
428 stressors in marine systems. *Ecology Letters*, 11, pp. 1304-1315.
- 429 Dagnino A, Sforzini S, Dondero F, Fenoglio S, Bona E, Jensen J, Viarengo A (2008) A "Weight-of-
430 Evidence" approach for the integration of environmental "Triad" data to assess ecological risk and
431 biological vulnerability. *Integrated Environmental Assessment and Management*, 4, pp. 314-326.
- 432 de Orte MR, Sarmiento AM, Basallote MD, Rodríguez-Romero A, Riba I, delValls A (2014a). Effects
433 on the mobility of metals from acidification caused by possible CO₂ leakage from sub-seabed
434 geological formations. *Science of the Total Environment*, 470-471, pp. 356-363.
- 435 de Orte MR, Sarmiento AM, DelValls T, Riba I (2014b). Simulation of the potential effects of CO₂
436 leakage from carbon capture and storage activities on the mobilization and speciation of metals.
437 *Marine Pollution Bulletin*, 86, pp. 59-67.
- 438 Dickson AG, Sabine CL, Christian JR (2007). Guide to best practices for ocean CO₂ measurements.
439 PICES Special Publication, 3, 191.

- 440 Doney SC (2010). The growing human footprint on coastal and open-ocean biogeochemistry.
441 Science, 328, pp. 1512-1516.
- 442 Farcy E, Voiseux C, Lebel J-M, Fievet B (2007). Seasonal changes in mRNA encoding for cell stress
443 markers in the oyster *Crassostrea gigas* exposed to radioactive discharges in their natural
444 environment. Science of the Total Environment, 374, pp. 328-341.
- 445 Fields PA, Zuzow MJ, Tomanek L (2012). Proteomic responses of blue mussel (*Mytilus*) congeners
446 to temperature acclimation. Journal of Experimental Biology, 215 (7), pp. 1106-1116.
- 447 Freitas R, de Marchi L, Moreira A, Pestana JLT, Wrona FJ, Figueira E, Soares AMVM (2017).
448 Physiological and biochemical impacts induced by mercury pollution and seawater acidification in
449 *Hediste diversicolor* (2017). Science of the Total Environment, 595, pp. 691-701.
- 450 Gattuso J-P and Lavigne H (2009). Technical note: approaches and software tools to investigate the
451 impact of ocean acidification. Biogeosciences, 6, pp. 2121–2133.
- 452 Gazeau F, Alliouane S, Bock C, Bramanti L, Correa ML, Gentile M, Hirse T, Pörtner H-O, Ziveri P
453 (2014). Impact of ocean acidification and warming on the Mediterranean mussel (*Mytilus*
454 *galloprovincialis*). Frontiers in Marine Science, 1.
- 455 Gilarranz, L.J., Mora, C., Bascompte, J. (2016). Anthropogenic effects are associated with a lower
456 persistence of marine food webs. Nature Communications, 7.
- 457 Gledhill M, Achterberg EP, Li K, Mohamed KN, Rijkenberg MJA (2015). Influence of ocean
458 acidification on the complexation of iron and copper by organic ligands in estuarine waters. Marine
459 Chemistry, 177, pp. 421-433.
- 460 Gomiero A, Viarengo A (2014). Effects of elevated temperature on the toxicity of copper and
461 oxytetracycline in the marine model, *Euplotes crassus*: A climate change perspective. Environmental
462 Pollution, 194, pp. 262-271.
- 463 Gorbi S, Avio GC, Benedetti M, Totti C, Accoroni S, Pichierri S, Bacchiocchi S, Orletti R, Graziosi
464 T, Regoli F (2013). Effects of harmful dinoflagellate *Ostreopsis cf. ovata* exposure on immunological,
465 histological and oxidative responses of mussels *Mytilus galloprovincialis*. Fish and Shellfish
466 Immunology, 35, 941-950.
- 467 Götze S, Matoo OB, Beniash E, Saborowski R, Sokolova IM (2014). Interactive effects of CO₂ and
468 trace metals on the proteasome activity and cellular stress response of marine bivalves *Crassostrea*
469 *virginica* and *Mercenaria mercenaria*. Aquatic Toxicology, 149, 65-82.
- 470 Guinot D, Ureña R, Pastor A, Varó I, Ramo JD, Torreblanca A (2012). Long-term effect of
471 temperature on bioaccumulation of dietary metals and metallothionein induction in *Sparus aurata*.
472 Chemosphere, 87, pp. 1215-1221.
- 473 Hansen J, Sato M, Ruedy R, Schmidt GA, Lo K. 2016b. Global Temperature in 2015. In: NASA
474 News Release 16-008. New York City, NY, NOAA GISS.
- 475 Hoffmann LJ, Breitbarth E, Boyd PW, Hunter KA (2012). Influence of ocean warming and
476 acidification on trace metal biogeochemistry. Marine Ecology Progress Series, 470, pp. 191-205.
- 477 IPCC (2013). IPCC Climate Change 2013: The Physical Science Basis. Contribution of Working
478 Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change.
479 Cambridge University Press, Cambridge, UK and New York, NY, USA.

- 480 IPCC (2014). IPCC Climate Change 2014: Impacts, Adaptation, and Vulnerability. Contribution of
481 Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change.
482 Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.
- 483 Ioannou S, Anestis A, Pörtner HO, Michaelidis B (2009). Seasonal patterns of metabolism and the
484 heat shock response (HSR) in farmed mussels *Mytilus galloprovincialis*. Journal of Experimental
485 Marine Biology and Ecology, 381 (2), pp. 136-144.
- 486 Ivanina AV, Beniash E, Etzkorn M, Meyers TB, Ringwood AH, Sokolova IM (2014). Short-term
487 acute hypercapnia affects cellular responses to trace metals in the hard clams *Mercenaria mercenaria*.
488 Aquatic Toxicology, 140-141, pp. 123-133.
- 489 Ivanina AV, Hawkins C, Beniash E, Sokolova IM (2015). Effects of environmental hypercapnia and
490 metal (Cd and Cu) exposure on acid-base and metal homeostasis of marine bivalves. Comparative
491 Biochemistry and Physiology Part - C: Toxicology and Pharmacology, 174-175, pp. 1-12.
- 492 Ivanina AV, Hawkins C, Sokolova IM (2016). Interactive effects of copper exposure and
493 environmental hypercapnia on immune functions of marine bivalves *Crassostrea virginica* and
494 *Mercenaria mercenaria*. Fish and Shellfish Immunology, 49, pp. 54-65.
- 495 Izagirre U, Errasti A, Bilbao E, Múgica M, Marigómez I (2014) Combined effects of thermal stress
496 and Cd on lysosomal biomarkers and transcription of genes encoding lysosomal enzymes and HSP70
497 in mussels, *Mytilus galloprovincialis*. Aquatic Toxicology, 149, pp. 145-156.
- 498 Kefaloyianni E, Gourgou E, Ferle V, Kotsakis E, Gaitanaki C, Beis I (2005). Acute thermal stress
499 and various heavy metals induce tissue-specific pro- or anti-apoptotic events via the p38-MAPK
500 signal transduction pathway in *Mytilus galloprovincialis* (Lam.). Journal of Experimental Biology,
501 208, pp. 4427-4436.
- 502 Koukouzika N, Dimitriadis VK (2008). Aspects of the usefulness of five marine pollution biomarkers,
503 with emphasis on MN and lipid content. Marine Pollution Bulletin, 56, pp. 941-949.
- 504 Kroeker KJ, Kordas RL, Crim R, Hendriks IE, Ramajo L, Singh GS, Duarte CM, Gattuso J-P (2013).
505 Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with
506 warming. Global Change Biololgy, 19, pp. 1884-1896.
- 507 Lacoue-Labarthe T, Martin S, Oberhänsli F, Teyssié J-L, Markich S, Ross J, Bustamante P (2009).
508 Effects of increased pCO₂ and temperature on trace element (Ag, Cd and Zn) bioaccumulation in the
509 eggs of the common cuttlefish, *Sepia officinalis*. Biogeosciences, 6, pp. 2561-2573.
- 510 Lacoue-Labarthe T, Réveillac E, Oberhänsli F, Teyssié J-L, Jeffree R, Gattuso J-P (2011). Effects of
511 ocean acidification on trace element accumulation in the early-life stages of squid *Loligo vulgaris*.
512 Aquatic Toxicology, 105, pp. 166-176.
- 513 Lewis C, Clemow K, Holt WV (2013). Metal contamination increases the sensitivity of larvae but
514 not gametes to ocean acidification in the polychaete *Pomatoceros lamarckii* (Quatrefages). Marine
515 Biology, 160, pp. 2089-2101.
- 516 Lewis C, Ellis RP, Vernon E, Elliot K, Newbatt S, Wilson RW (2016). Ocean acidification increases
517 copper toxicity differentially in two key marine invertebrates with distinct acid-base responses.
518 Scientific Reports, 6.
- 519 López IR, Kalman J, Vale C, Blasco J (2010). Influence of sediment acidification on the
520 bioaccumulation of metals in *Ruditapes philippinarum*. Environmental Science and Pollution
521 Research, 17, pp. 1519-1528.

- 522 Marigómez I, Garmendia L, Soto M, Orbea A, Izagirre U, Cajaraville MP (2013) Marine ecosystem
523 health status assessment through integrative biomarker indices: a comparative study after the
524 Prestige-oil spill Mussel Watch. *Ecotoxicology*, 22, pp. 486–505.
- 525 Matozzo V, Chinellato A, Munari M, Finos L, Bressan M, Marin MG (2012). First evidence of
526 immunomodulation in bivalves under seawater acidification and increased temperature. *PLoS ONE*,
527 7, e33820.
- 528 Millero FJ, Woosley R, Ditrolio B, Waters J (2009). Effect of ocean acidification on the speciation
529 of metals in seawater. *Oceanography*, 22 (SPL.ISS. 4), pp. 72-85.
- 530 Millero FJ (2010). Carbonate constants for estuarine waters. *Marine Freshwater Research*, 61,
531 139e142.
- 532 Mubiana VK, Blust R (2007). Effects of temperature on scope for growth and accumulation of Cd,
533 Co, Cu and Pb by the marine bivalve *Mytilus edulis*. *Marine Environmental Research*, 63, pp. 219-
534 235.
- 535 Múgica M, Izagirre U, Marigómez I (2015). Lysosomal responses to heat-shock of seasonal
536 temperature extremes in Cd-exposed mussels. *Aquatic Toxicology*, 164, pp. 99-107.
- 537 Nardi A, Mincarelli LF, Benedetti M, Fattorini D, d'Errico G, Regoli F (2017). Indirect effects of
538 climate changes on cadmium bioavailability and biological effects in the Mediterranean mussel
539 *Mytilus galloprovincialis*. *Chemosphere*, 169, 493-502.
- 540 Nardi A, Benedetti M, Fattorini D, Regoli F (2018). Oxidative and interactive challenge of cadmium
541 and ocean acification on the smooth scallop *Flexopecten glaber*. *Aquatic Toxicology*, 196, pp. 53-60.
- 542 Neff JM (2002). *Bioaccumulation in Marine Organisms*. Elsevier Science, Oxford, UK.
- 543 Negri A, Oliveri C, Sforzini S, Mignione F, Viarengo A, Banni M (2013). Transcriptional response
544 of the mussel *Mytilus galloprovincialis* (Lam.) following exposure to heat stress and copper. *PLoS*
545 *ONE*, 8.
- 546 Nichols JW, Playle RC (2004). Influence of temperature on silver accumulation and depuration in
547 rainbow trout. *Journal of Fish Biology*, 64, pp. 1638–1654.
- 548 Pereira CDS, Martín-Díaz ML, Catharino MGM, Cesar A, Choueri RB, Taniguchi S, Abessa DMS,
549 Bicego MC, Vasconcellos MBA, Bairy ACD, Sousa ECPM, Delvalls TA (2012). Chronic
550 contamination assessment integrating biomarkers' responses in transplanted mussels-A seasonal
551 monitoring. *Environmental Toxicology*, 27 (5), pp. 257-267.
- 552 Pierrot D, Lewis E, Wallace DWR (2006). MS Excel Program Developed for CO₂ System
553 Calculations. ORNL/CDIAC-105a. Carbon Dioxide Information Analysis Center, Oak Ridge
554 National Laboratory, U.S. Department of Energy, Oak Ridge, Tennessee. doi:509
555 10.3334/CDIAC/otg.CO2SYS_XLS_CDIA105a
- 556 Piva F, Ciaprini F, Onorati F, Benedetti M, Fattorini D, Ausili A, Regoli F (2011). Assessing sediment
557 hazard through a weight of evidence approach with bioindicator organisms: A practical model to
558 elaborate data from sediment chemistry, bioavailability, biomarkers and ecotoxicological bioassays.
559 *Chemosphere*, 83, pp. 475-485.
- 560 Pörtner H-O (2010). Oxygen- and capacity-limitation of thermal tolerance: A matrix for integrating
561 climate-related stressor effects in marine ecosystems. *Journal of Experimental Biology*, 213, pp. 881-
562 893.

- 563 Regoli F (1992). Lysosomal responses as sensitive stress index in biomonitoring heavy metal
564 pollution. *Marine Ecology Progress Series*, 84, pp. 63-69.
- 565 Regoli F, Nigro M, Benedetti M, Gorbi S, Pretti C, Gervasi PG, Fattorini D (2005) Interactions
566 between metabolism of trace metals and xenobiotic agonists of the aryl hydrocarbon receptor in the
567 antarctic fish *Trematomus bernacchii*: Environmental perspectives. *Environmental Toxicology and*
568 *Chemistry*, 24, pp. 1475-1482.
- 569 Regoli F, Giuliani ME (2014). Oxidative pathways of chemical toxicity and biomarkers of oxidative
570 stress in marine organisms. *Marine Environmental Research*, 93, pp. 106-117.
- 571 Regoli F, Pellegrini D, Cicero AM, Nigro M, Benedetti M, Gorbi S, Fattorini D, d'Errico G, Di Carlo
572 M, Nardi A, Gaion A, Scuderi A, Giuliani S, Romanelli G, Berto D, Trabucco B, Guidi P,
573 Bernardeschi M, Scarcelli V, Frenzilli G (2014) A multidisciplinary weight of evidence approach for
574 environmental risk assessment at the Costa Concordia wreck: Integrative indices from Mussel Watch.
575 *Marine Environmental Research*, 96, pp. 92-104.
- 576 Riba I, García-Luquea RE, Blasco J, DelValls TA (2003). Bioavailability of heavy metals bound to
577 estuarine sediments as a function of pH and salinity values. *Chemical Speciation and Bioavailability*,
578 15 (4), pp. 101-114.
- 579 Ringwood AH, Hoguet J, Keppler CJ (2002). Seasonal variation in lysosomal destabilization in
580 oysters, *Crassostrea virginica*. *Marine Environmental Research*, 54, pp. 793-797.
- 581 Roberts DA, Birchenough SNR, Lewis C, Sanders MB, Bolam T, Sheahan D (2013). Ocean
582 acidification increases the toxicity of contaminated sediments. *Global Change Biology*, 19, pp. 340-
583 351.
- 584 Rodríguez-Romero A, Jiménez-Tenorio N, Basallote MD, Orte MRD, Blasco J, Riba I (2014a).
585 Predicting the impacts of CO₂ leakage from subseabed storage: Effects of metal accumulation and
586 toxicity on the model benthic organism *Ruditapes philippinarum*. *Environmental Science and*
587 *Technology*, 48, pp. 12292-12301.
- 588 Rodríguez-Romero A, Basallote MD, De Orte MR, DelValls TT, Riba I, Blasco J (2014b). Simulation
589 of CO₂ leakages during injection and storage in sub-seabed geological formations: Metal
590 mobilization and biota effects. *Environment International*, 68, pp. 105-117.
- 591 Shi W, Zhao X, Han Y, Che Z, Chai X, Liu G (2016). Ocean acidification increases cadmium
592 accumulation in marine bivalves: A potential threat to seafood safety. *Scientific Reports*, 6.
- 593 Siddiqui S, Bielmyer-Fraser GK (2015). Responses of the sea anemone, *Exaiptasia pallida*, to ocean
594 acidification conditions and copper exposure. *Aquatic Toxicology*, 167, pp. 228-239.
- 595 Sokolova IM (2004). Cadmium effects on mitochondrial function are enhanced by elevated
596 temperatures in a marine poikilotherm, *Crassostrea virginica* Gmelin (Bivalvia: Ostreidae). *Journal*
597 *of Experimental Biology*, 207, pp. 2639-2648.
- 598 Sokolova IM, Lannig G (2008). Interactive effects of metal pollution and temperature on metabolism
599 in aquatic ectotherms: Implications of global climate change. *Climate Research*, 37, pp. 181-20.
- 600 Stockdale A, Tipping E, Lofts S, Mortimer RJG (2016). Effect of Ocean Acidification on Organic
601 and Inorganic Speciation of Trace Metals. *Environmental Science and Technology*, 50, pp. 1906-
602 1913.

- 603 Sureda A, Natalotto A, Álvarez E, Deudero S (2013). Increased antioxidant response and capability
604 to produce ROS in hemocytes of *Pinna nobilis* L. exposed to anthropogenic activity. *Environmental*
605 *Pollution*, 181, pp. 321-324.
- 606 Velez C, Figueira E, Soares AMVM, Freitas R (2016). The impacts of As accumulation under
607 different pH levels: Comparing *Ruditapes decussatus* and *Ruditapes philippinarum* biochemical
608 performance. *Environmental Research*, 151, pp. 653-662.
- 609 Viarengo A, Mancinelli G, Orunesu M, Martino G, Faranda F, Mazzucotelli A (1988). Effects of
610 sublethal copper concentrations, temperature, salinity and oxygen levels on calcium content and on
611 cellular distribution of copper in the gills of *Mytilus galloprovincialis* lam.: A multifactorial
612 experiment. *Marine Environmental Research*, 24, pp. 227-231.
- 613 Viarengo A, Burlando B, Ceratto N, Panfoli I (2000). Antioxidant role of metallothioneins: a
614 comparative overview. *Cellular and Molecular Biology*, 46, pp. 407-417.
- 615 Wong PP, Losada IJ, Gattuso J-P, Hinkel J, Khattabi A, McInnes KL, Saito Y, Sallenger A (2014).
616 Coastal systems and low-lying areas. In: *Climate Change 2014: Impacts, Adaptation, and*
617 *Vulnerability. Part A: Global and Sectoral Aspects. Contribution of Working Group II to the Fifth*
618 *Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge University Press,
619 Cambridge, United Kingdom and New York, NY, USA, pp. 361-409.

620 **Table 1** - Summary of water chemistry parameters during experimental exposure. S (salinity), T (temperature),
 621 pH_{NBS} (pH calibrated with National Bureau of Standard scale), AT (total alkalinity), pCO₂ (partial pressure of
 622 CO₂), Ω_c and Ω_a (saturation state of respectively calcite and aragonite). Data are presented as means \pm standard
 623 deviations.

Treatment	measured parameters				calculated parameters		
	S	T (°C)	pH _{NBS}	A _T (μmol/kg)	pCO ₂ (μatm)	Ω_c	Ω_a
CTRL	37 ± 0.5	9.95 ± 0.11	8.18 ± 0.03	3283.2 ± 88.8	386.9 ± 26.2	6.2 ± 0.5	3.9 ± 0.3
Cd	37 ± 0.5	9.97 ± 0.06	8.16 ± 0.03	3334.2 ± 102.6	411.8 ± 35.8	6.1 ± 0.3	3.9 ± 0.2
A	37 ± 0.5	10.54 ± 0.08	7.40 ± 0.05	3364.1 ± 112.9	2882.2 ± 363.8	1.3 ± 0.2	0.8 ± 0.1
W	37 ± 0.5	14.95 ± 0.12	8.17 ± 0.03	3378.4 ± 121.2	416.8 ± 34.5	7.2 ± 0.5	4.7 ± 0.4
A-Cd	37 ± 0.5	10.04 ± 0.15	7.39 ± 0.02	3360.6 ± 36.8	2860.9 ± 207.2	1.2 ± 0.1	0.8 ± 0.1
W-Cd	37 ± 0.5	15.02 ± 0.11	8.17 ± 0.03	3350.5 ± 164.9	403.5 ± 53.1	7.3 ± 0.5	4.7 ± 0.3
A-W	37 ± 0.5	14.98 ± 0.05	7.39 ± 0.04	3354.5 ± 80.1	2916.3 ± 288.7	1.5 ± 0.2	1.0 ± 0.1
A-W-Cd	37 ± 0.5	14.92 ± 0.06	7.39 ± 0.02	3326.5 ± 67.1	2886.4 ± 174.1	1.5 ± 0.1	1.0 ± 0.1

624

625 **Figure 1** – Cd concentrations ($\mu\text{g/g}$ dry weight) and level of metallothioneins (nmol/mg of proteins) in digestive
626 gland (a and c) and gills (b and d) of mussels exposed to various treatments. Data are given as mean
627 values \pm standard deviations ($n=5$). Different letters indicate significant differences between group of means
628 (ANOVA and SNK *post-hoc*). CTRL= Control; Cd= Cadmium; A= acidification; W= warming; A-Cd=
629 acidification + Cd; W-Cd= warming + Cd; A-W= acidification + warming; A-W-Cd= acidification + warming +
630 Cd.

631 **Figure 2** – Antioxidant defenses and oxidative stress biomarkers in digestive gland of mussels exposed to various
632 treatments. CAT: catalase ($\mu\text{mol/mg}$ proteins) (a), GSTs: glutathione S-transferases (nmol/mg proteins) (b), Se-Dep. GPx:
633 Se-dependent glutathione peroxidases (nmol/mg proteins) (c) total GPx: sum of Se-dependent and Se-independent
634 glutathione peroxidases (nmol/mg proteins) (d), GR: glutathione reductase (nmol/mg proteins) (e), TGS: total
635 glutathione ($\mu\text{mol/g}$ wet weight) (f), TOSC ROO \cdot : total oxyradical scavenging capacity toward peroxy radical (TOSC
636 units/mg proteins) (g), TOSC HO \cdot : total oxyradical scavenging capacity toward hydroxyl radical (TOSC units/mg
637 proteins) (h), MDA: levels of malondialdehyde (mmol/g wet weight) (i), lipofuscin (intensity/ μm^2) (j) and neutral lipids
638 (intensity/ μm^2) (k). Data are given as mean values \pm standard deviations ($n=5$). Different letters indicate significant
639 differences between group of means (ANOVA and SNK *post-hoc*). CTRL= Control; Cd= Cadmium; A= acidification;
640 W= warming; A-Cd= acidification + Cd; W-Cd= warming + Cd; A-W= acidification + warming; A-W-Cd= acidification
641 + warming + Cd.

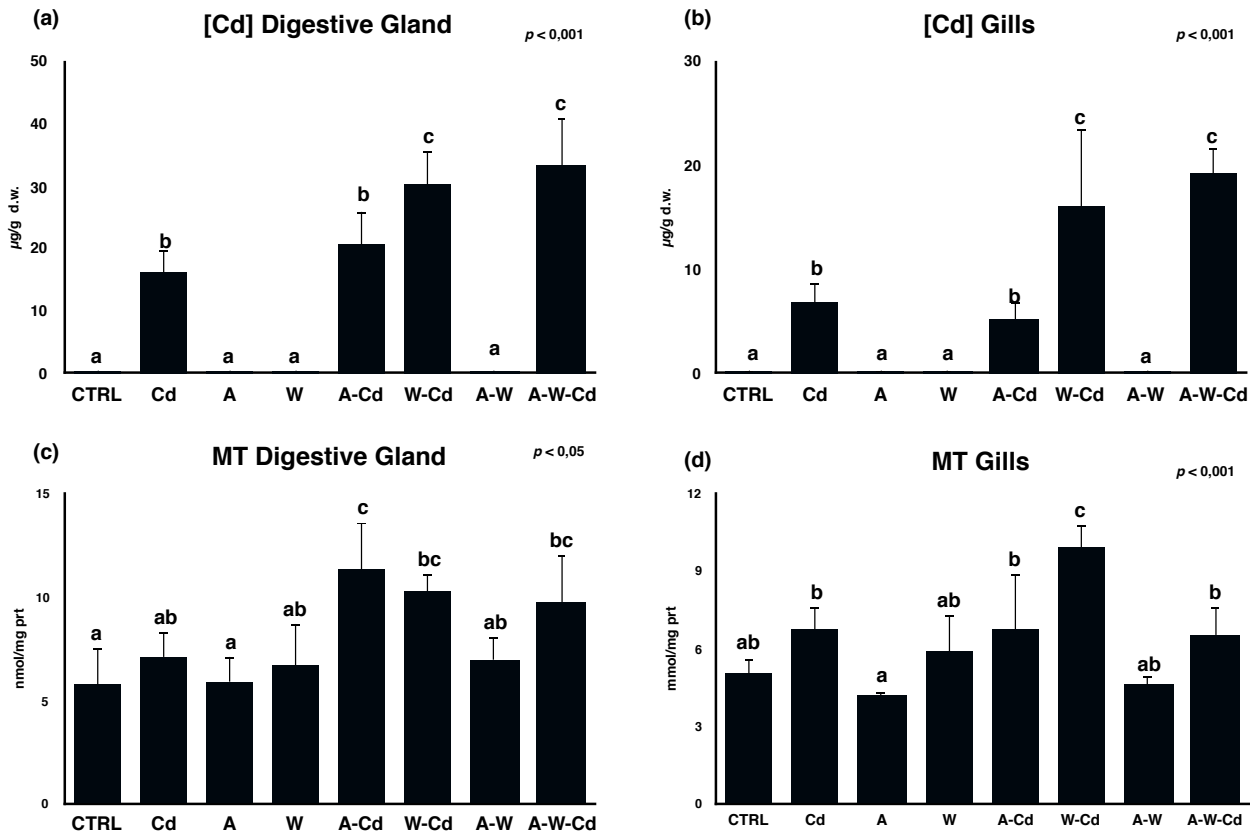
642 **Figure 3** - Antioxidant defenses and oxidative stress biomarkers in gills of mussels exposed to various treatments. CAT:
643 catalase ($\mu\text{mol/mg}$ proteins) (a), GSTs: glutathione S-transferases (nmol/mg proteins) (b), Se-Dep. GPx: Se-dependent
644 glutathione peroxidases (nmol/mg proteins) (c) total GPx: sum of Se-dependent and Se-independent glutathione
645 peroxidases (nmol/mg proteins) (d), GR: glutathione reductase (nmol/mg proteins) (e), TGS: total glutathione ($\mu\text{mol/g}$
646 wet weight) (f), TOSC ROO \cdot : total oxyradical scavenging capacity toward peroxy radical (TOSC units/mg proteins) (g),
647 TOSC HO \cdot : total oxyradical scavenging capacity toward hydroxyl radical (TOSC units/mg proteins) (h), MDA: levels
648 of malondialdehyde (mmol/g wet weight) (i). Data are given as mean values \pm standard deviations ($n=5$). Different letters
649 indicate significant differences between group of means (ANOVA and SNK *post-hoc*). CTRL= Control; Cd= Cadmium;
650 A= acidification; W= warming; A-Cd= acidification + Cd; W-Cd= warming + Cd; A-W= acidification + warming; A-W-
651 Cd= acidification + warming + Cd.

652 **Figure 4** - Lysosomal membrane stability (a), phagocytosis rate (b), granulocytes/hyalinocytes ratio (c), DNA damage
653 (d) and frequency of micronuclei (e) in haemocytes of mussels exposed to various treatments. Data are given as mean
654 values \pm standard deviation ($n=5$). Different letters indicate significant differences between group of means (ANOVA

655 and SNK *post-hoc*). CTRL= Control; Cd= Cadmium; A= acidification; W= warming; A-Cd= acidification + Cd; W-Cd=
656 warming + Cd; A-W= acidification + warming; A-W-Cd= acidification + warming + Cd.

657 **Figure 5** - Weight of Evidence (WOE) classification of biomarkers data for the whole dataset of analyzed
658 parameters in digestive gland and gills for each exposure condition. The quantitative hazard quotients (HQ) and the
659 assigned class of hazard are given.

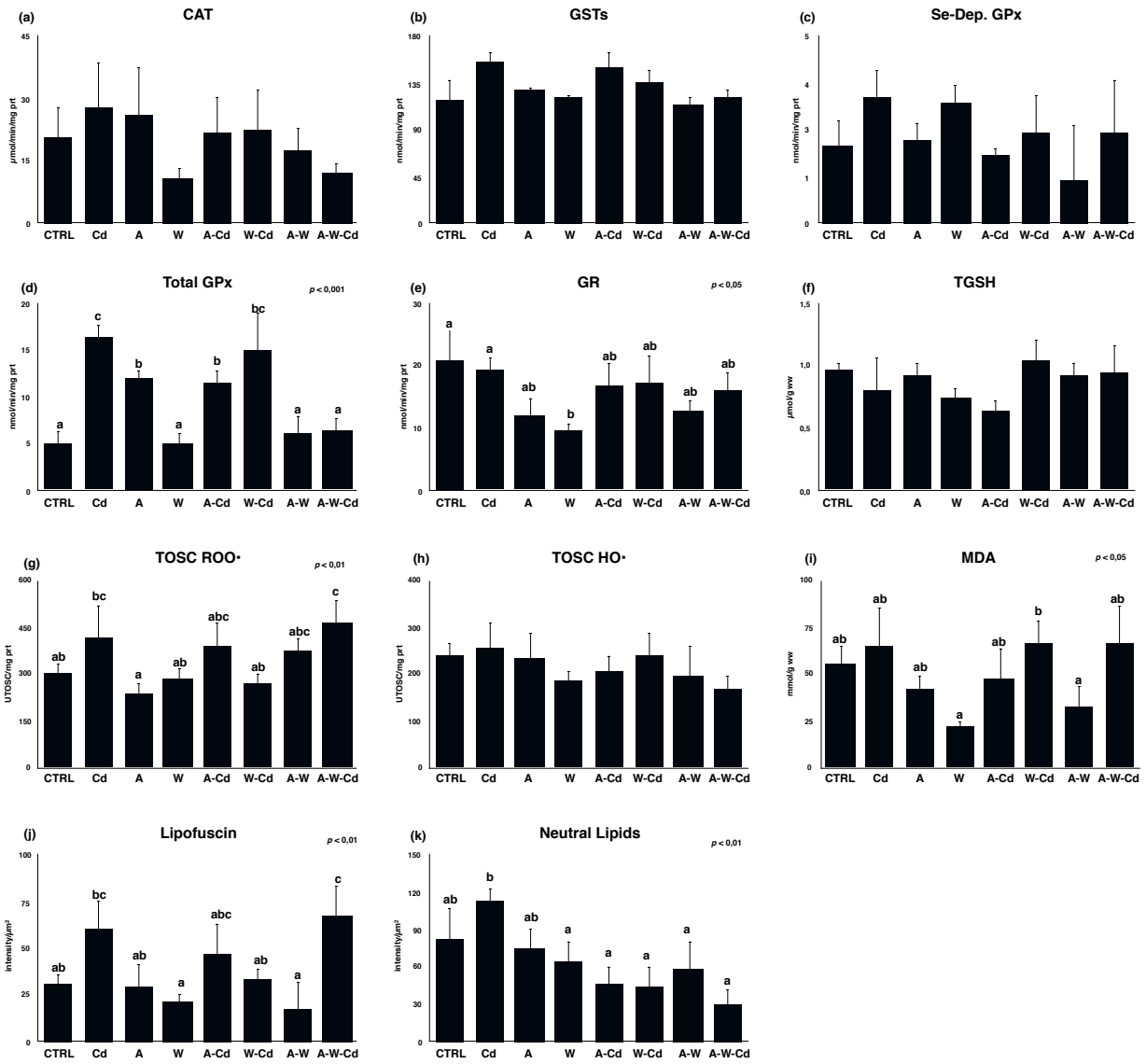
660



661

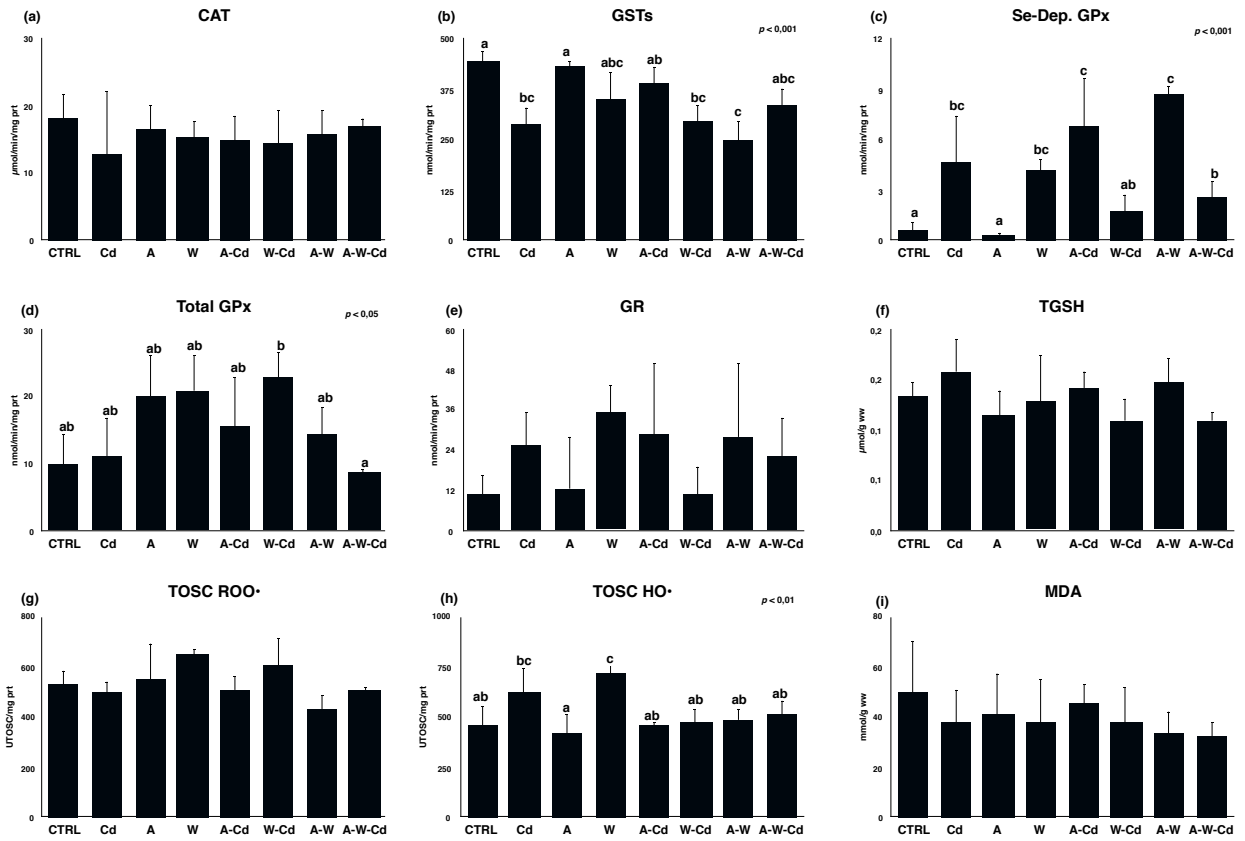
662 **Figure 1.**

663



664

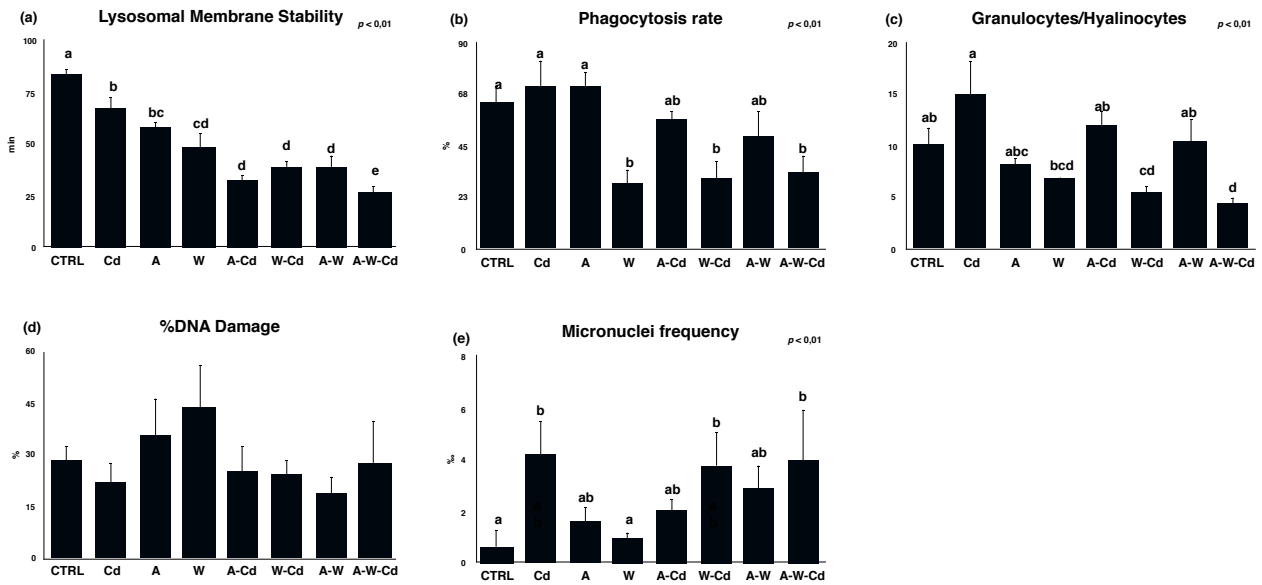
665 **Figure 2.**



666

667

Figure 3.



668

669

Figure 4.

Experimental Treatment	Digestive gland			Gills		
	Hazard Quotient (HQ)	Class of Hazard	Level	Hazard Quotient (HQ)	Class of Hazard	Level
Cd	9.87	MODERATE		13.82	MODERATE	
A	4.60	SLIGHT		7.48	MODERATE	
W	2.31	SLIGHT		31.16	MODERATE	
A-Cd	6.66	MODERATE		39.55	MODERATE	
W-Cd	8.57	MODERATE		9.66	MODERATE	
A-W	0	SLIGHT		50.84	MODERATE	
A-W-Cd	2.86	SLIGHT		12.47	MODERATE	

670
671

Figure 5.

