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

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

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Abstract

Ocean warming and acidification could represent additional threat to marine organisms already coping with other anthropogenic impacts, such as chemical contamination in coastal areas. In this study, interactions between such multiple stressors and their synergistic effects in terms of accumulation, detoxification and biological effects of metals were investigated in the Mediterranean mussel *Mytilus galloprovincialis*. Organisms sampled during the winter period were exposed for 28 days to different combinations of two temperatures (10°C and 15°C), two pH/pCO_2 (8.20/~400μatm and 7.4/~3000μatm) and two doses of cadmium (0 and 20 μg/L). Cadmium concentrations increased in digestive glands and gills of metal-exposed mussels, and were further enhanced by co-exposure at higher temperature. Interactive effects of temperature and/or pH were observed on Cd-mediated metallothioneins induction, responsiveness of antioxidant system and onset of oxidative damages to lipids, with tissue-specific effects. Immunological effects showed a generalized sensitivity of lysosomal membrane stability toward the investigated stressors with major effects in co-exposed organisms. Cadmium and temperature affected phagocytosis efficiency and haemocytes population composition probably influencing the micronuclei frequency through varied mitotic rate. Several differences were highlighted between these results and those previously obtained from mussels exposed in summer, supporting the importance of season when addressing the tolerance of temperate organisms to variations of environmental factors. The elaboration of the whole biomarkers results through weighted criteria allowed to summarize specific hazard indices, highlighting tissue-specific sensitivity toward multiple stressors and the need of improving the knowledge on multiple stressors 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 CO_2-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 CO_2 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/pCO_2. 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 (Sediqualsoft):
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

2.Materials and Methods

2.1 Animal collection and experimental design

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

After acclimation, mussels were randomly assigned and exposed to one of the following treatments, each containing 36 organisms in 20 L tanks: 1) control condition (CTRL), at environmental temperature of 10°C, normocapnia with pH=8.20/ pCO_2=~400 μatm; 2) Cd exposure (Cd), 10°C, pH=8.20/ pCO_2=~400 μatm and 20 μg/L Cd; 3) acidification (A), 10°C, hypercapnia with pH=7.40/ pCO_2=~3000 μatm; 4) warming (W), 15°C and pH=8.20/ pCO_2=~400 μatm; 5) acidification + Cd (A-Cd), 10°C, pH=7.40/ pCO_2=~3000 μatm and 20 μg/L Cd ; 6) warming + Cd (W-Cd), 15°C, pH=8.20/ pCO_2=~400 μatm and 20 μg/L Cd ; 7) acidification + warming (A-W), 15°C and pH=7.40/ pCO_2=~3000 μatm; 8) acidification + warming + Cd (A-W-Cd), 15°C, pH=7.40/ pCO_2=~3000 μatm and 20 μg/L Cd. Despite the effects related to climate changes are expected to occur over the course of decades, organisms were exposed to the tested environmental conditions of Cd, pH and temperature without gradual acclimation, thus simulating sudden changes as those more easily occurring in coastal or estuarine areas. The same experimental design had been previously applied on mussels sampled during the summer with the exception of temperature values, respectively at 20 and 25 °C for the control and warming treatments (Nardi *et al*., 2017). Cd dose is representative for a polluted but environmentally realistic condition in coastal waters, typically ranging from less than 1 up to hundreds μg/L in highly polluted areas (Neff, 2002; Bakary et al., 2015). Lowered pH was adapted from scenario RCP 8.5 and IPCC WGII AR5 (IPCC, 2014; Wong *et al*., 2014) reporting a mean pH value of 7.7 for open oceans, but predicting more pronounced reductions in coastal areas. The experimental pH was reached by adding to each treatment ASW (pH=8.2) small and defined amounts of CO_2-saturated ASW, obtained by bubbling pure CO_2 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 (pCO_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 μmol/kg and 0.05 μmol/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 μg/g dry weight (mean value ± standard
170 deviation, n = 5), limits of quantification and detection were respectively 0.002 and 0.001 μg/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 peroxyl radicals (TOSC
176 ROO•) and hydroxyl radicals (TOSC HO•), 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 (Sediqualsoft, 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

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

3. Results

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

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

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

4. Discussion

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

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

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

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

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

Different and more frequent oxidative variations were observed in gills. Cd, higher temperature 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

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

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

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

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

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

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

Treatment	measured parameters				calculated parameters		
	S	T (°C)	pH_{NBS}	A_T (µmol/kg)	*p*CO2 (µatm)	Ωc	Ωa
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

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

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

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

Figure 4 - Lysosomal membrane stability (a), phagocytosis rate (b), granulocytes/hyalinocytes ratio (c), DNA damage (d) and frequency of micronuclei (e) in haemocytes of mussels exposed to various treatments. Data are given as mean values ± standard 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

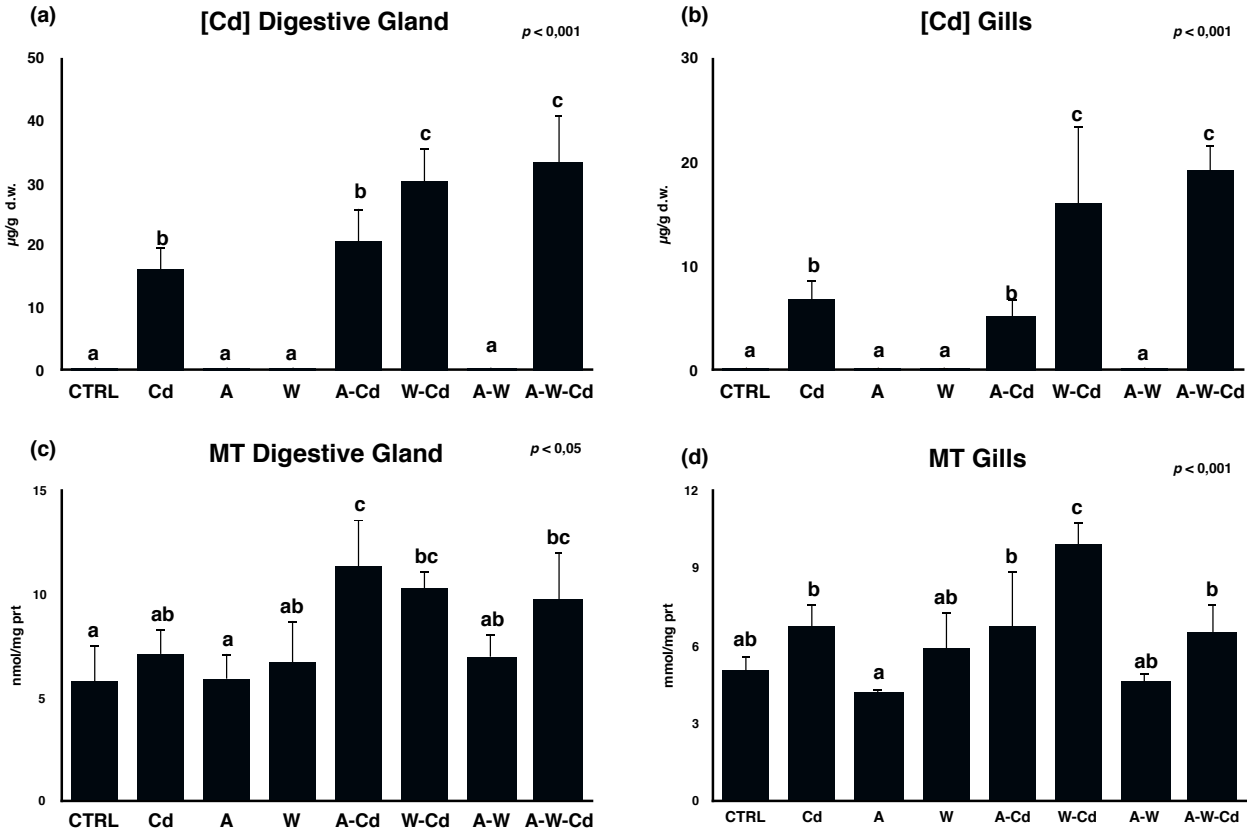
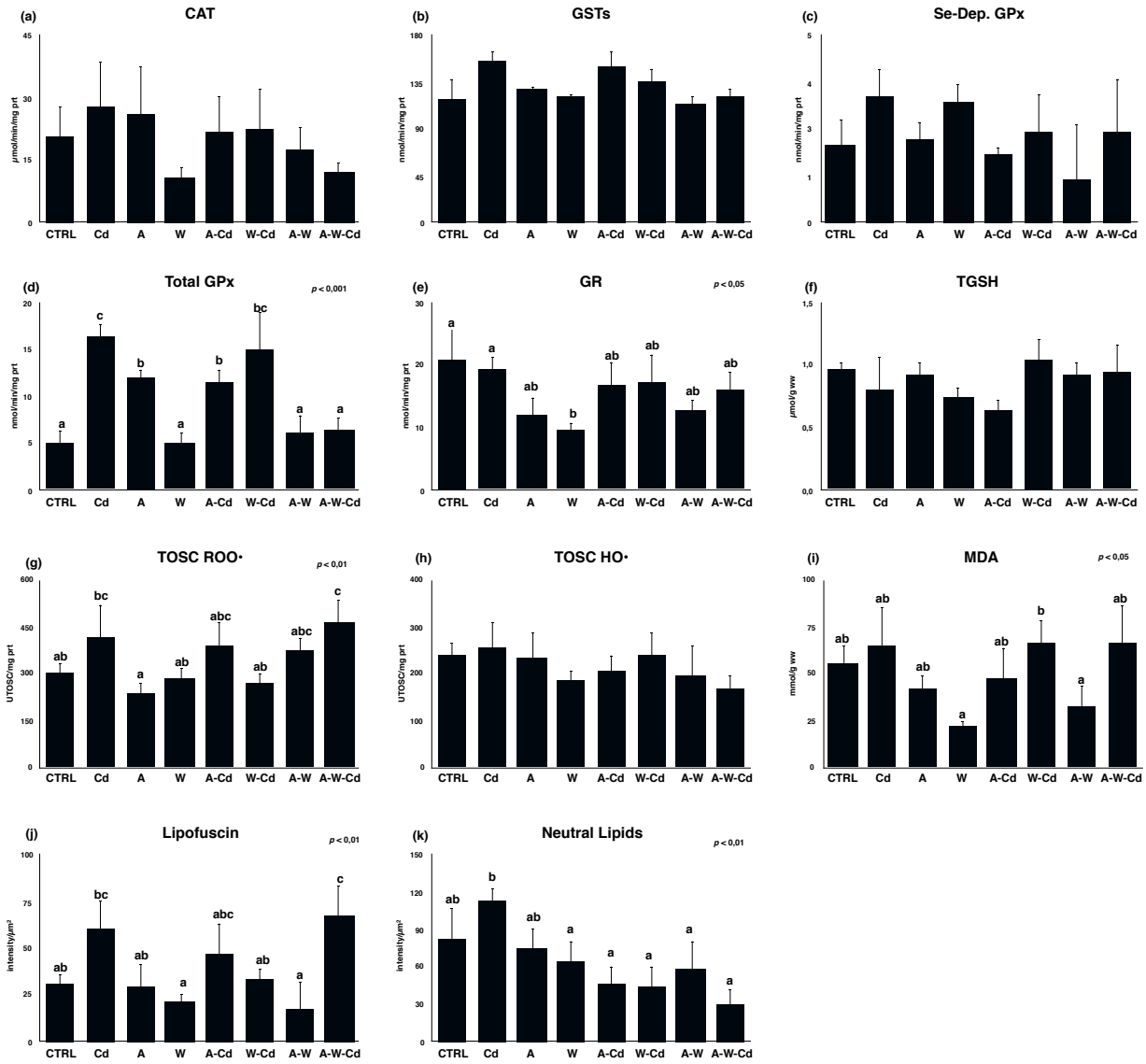

(a)
[Cd] Digestive Gland
p < 0,001
µg/g d.w.
50
40
30
20
10
0
a
b
a
a
b
c
a
c
CTRL
Cd
A
W
A-Cd
W-Cd
A-W
A-W-Cd
(b)
[Cd] Gills
p < 0,001
µg/g d.w.
30
20
10
0
a
b
a
a
b
c
a
c
CTRL
Cd
A
W
A-Cd
W-Cd
A-W
A-W-Cd
(c)
MT Digestive Gland
p < 0,05
nmol/mg prt
15
10
5
0
a
ab
a
ab
c
bc
ab
bc
CTRL
Cd
A
W
A-Cd
W-Cd
A-W
A-W-Cd
(d)
MT Gills
p < 0,001
nmol/mg prt
12
9
6
3
0
ab
b
a
ab
b
c
ab
b
CTRL
Cd
A
W
A-Cd
W-Cd
A-W
A-W-Cd

Figure 1.



664

665 **Figure 2.**

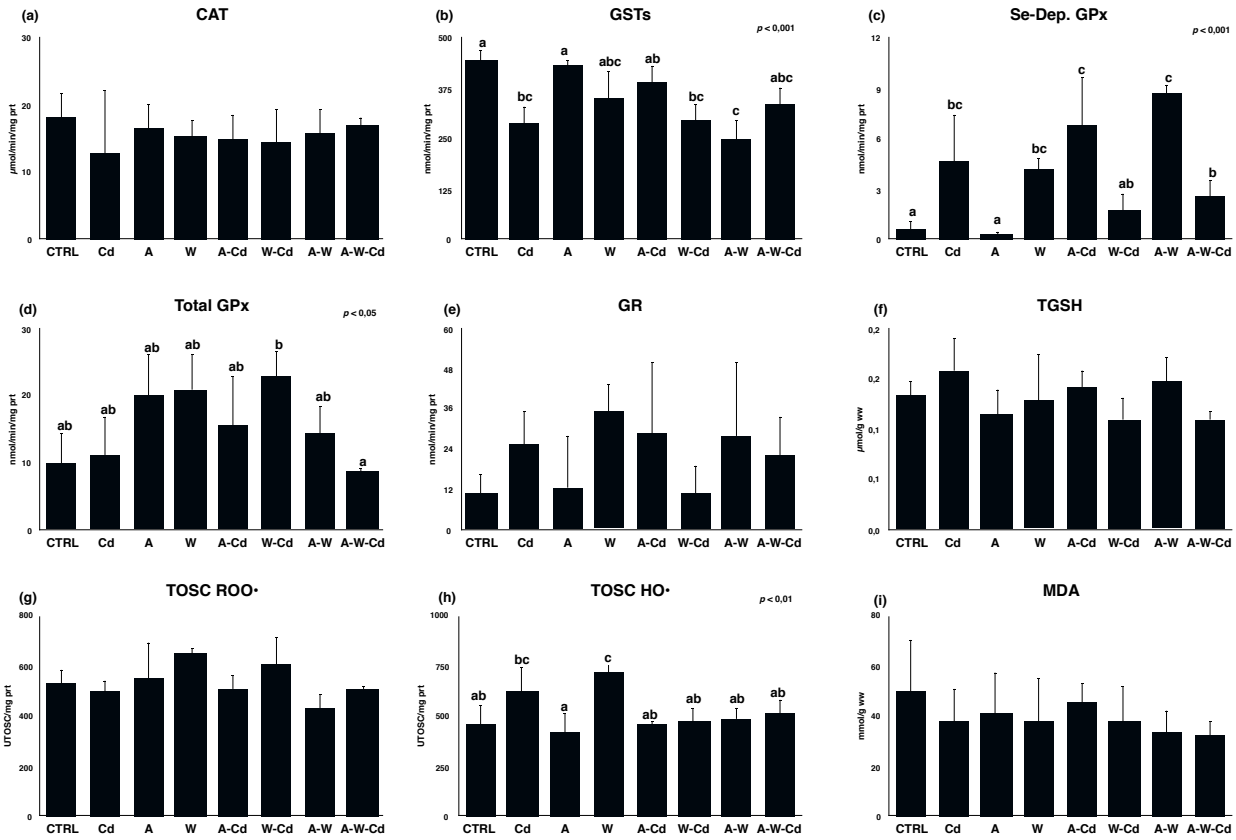
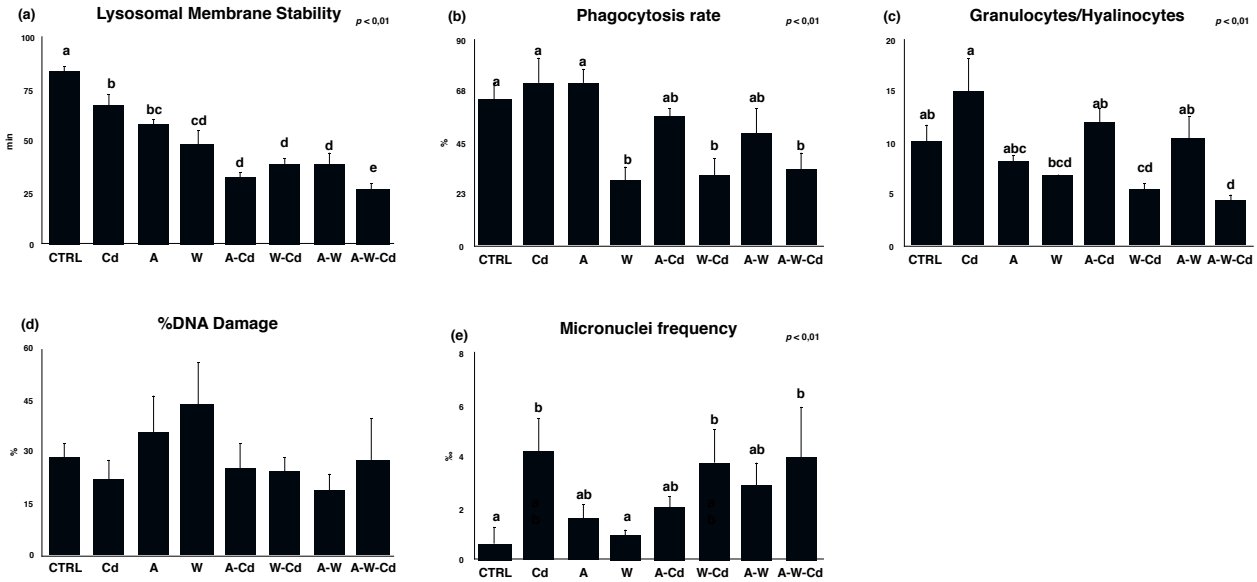
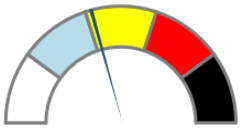
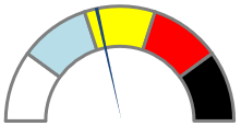
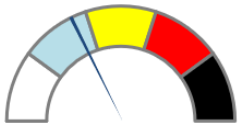
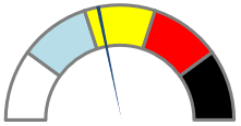
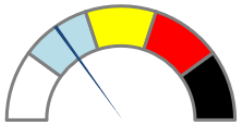
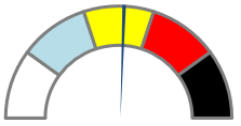
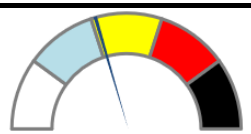
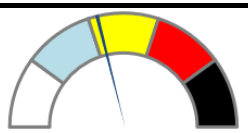
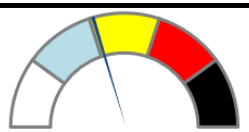
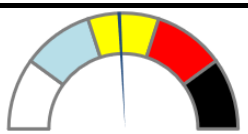
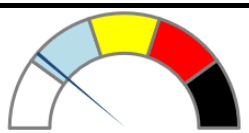
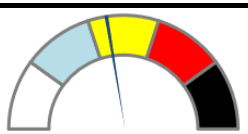
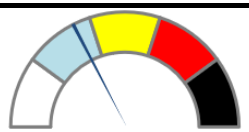
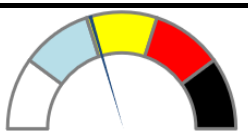

(a)
CAT
μmol/min/mg prt
(b)
GSTs
p < 0,001
nmol/min/mg prt
(c)
Se-Dep. GPx
p < 0,001
nmol/min/mg prt
(d)
Total GPx
p < 0,05
nmol/min/mg prt
(e)
GR
nmol/min/mg prt
(f)
TGSH
μmol/g ww
(g)
TOSC ROO·
UTOSC/mg prt
(h)
TOSC HO·
p < 0,01
UTOSC/mg prt
(i)
MDA
nmol/g ww
CTRL Cd A W A-Cd W-Cd A-W A-W-Cd

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.**

672