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INDIRECT EFFECTS OF CLIMATE CHANGES: ROLE OF OCEAN WARMING AND ACIDIFICATION ON THE SUSCEPTIBILITY TO ENVIRONMENTAL CONTAMINATION IN MARINE ORGANISMS

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CONTENTS

ABBREVIATIONS

CAT – catalase

cat – catalase gene

Cu/Zn-sod – cytosolic superoxide dismutase gene

DNA – deoxyribonucleic acid

EEI – Earth energy imbalance

G/I – granulocytes versus hyalinocytes type cells ratio

GR – glutathione reductase

GSH – reduced glutathione

GSSG – oxidized glutathione

GST – glutathione S-transferase

gstpi – GST isoform pi gene

HO• - hydroxyl radical

HOCl – hypocloric acid

HONOO – peroxynitrite

Hsp70 – heatshock protein 70

hsp70 – heatshock protein 70 gene

HQ – hazard quotient

LMS – lysosomal membrane stability

MDA – malondialdehyde

MN – micronuclei

mRNA – messenger ribonucleic acid

MT – metallothioneins

mt-20 – metallothioneins isoform 20 gene

OHC – ocean heat content

PCR – polymerase chain reaction

RO• - alkoxyl radical

ROO• - peroxyl radical

ROS – reactive oxygen species

Se-dep. GPx – selenium dependent glutathione peroxidase

Se-dep. gpx – Se-dep. GPx gene

SOD – superoxide dismutase

TGSH – total glutathione

TOSC - total oxyradical scavenging capacity

Total GPx – selenium dependent and independent glutathione peroxidase

WOE – weight of evidence

1. INTRODUCTION

1.1. The CO_2 problem: ocean as a reservoir of heat and carbon

Atmospheric concentration of carbon dioxide (CO_2) has been growing since the beginning of the industrial revolution as a consequence of anthropogenic activities, and is now 40% higher than the pre-industrial times (IPCC, 2013). This rapid increase of anthropogenic emissions of carbon dioxide, and more in general of greenhouse gases, is the main cause of Earth Energy Imbalance (EEI) (von Schuckmann *et al.*, 2016).

Greenhouse gases are both natural and anthropogenic constituents of the atmosphere that effectively trap thermal infrared radiation within the surface-troposphere system (IPCC, 2007). As atmospheric concentration of carbon dioxide has increased, this has led to additional heat stored in the Earth system. The excess heat is causing a generalized warming of Earth system (IPCC, 2007 and IPCC, 2013). Since 70's, global surface temperature (combined land and sea surface temperature) has increased steadily and global warming has now reached ~1°C since the 19th century (Hansen *et al.*, 2016). Along with global warming, climate changes are strictly related to melting of snow, sea-ice and glaciers but also to intensification of extreme weather events and frequency of droughts and floods (IPCC, 2013; von Schuckmann *et al.*, 2016). Accurate calculations of EEI addressed the 93% of the excess heat to ocean uptake, with lower contribution of sea and land ice (~4%), atmosphere (~1%) and land (~2%) (von Schuckmann *et al.*, 2016). Ocean heat content (OHC) has effectively grown, and sea surface temperature (SST) has been increasing by ~0.13°C per decade since the 20th century, with ocean warming now unequivocal (Pörtner *et al.*, 2014; Reid, 2016). Side effects of this process can be detected in sea-level rise due to thermosteric expansion plus contribution from melting ice sheets, sea-ice and glaciers (IPCC, 2013; von Schuckmann *et al.*, 2016).

Anthropogenic CO_2 has three main fates: 45% remains in the atmosphere, 29% is adsorbed by terrestrial biosphere while the remaining 26% is adsorbed by the ocean (Le

Quéré *et al.*, 2009). Once in seawater, CO_2 influences changes in the inorganic carbon chemistry equilibria, known as "ocean acidification", explained by the following formulas:

$$CO_{2(aq)} + H_2O \longrightarrow H^+_{(aq)} + HCO_3^-{}_{(aq)}$$

$$HCO_3^-{}_{(aq)} \longrightarrow H^+_{(aq)} + CO_3^{2-}{}_{(aq)}$$

Carbon dioxide in seawater increases the concentration of bicarbonate ions ($[HCO_3^-]$) and dissolved inorganic carbon ($[C_T]$), and reduces seawater pH and the concentration of carbonate ions ($[CO_3^{2-}]$) which further decreases the saturation state of different forms of calcium carbonates, calcite, magnesium-calcite and aragonite (Orr, 2011; Pörtner *et al.*, 2014). It has been estimated that seawater pH has decreased from 8.17 in the pre-industrial era to 8.1 in the 1990s (Pörtner, 2008; Gattuso and Lavigne, 2009) and it could further decrease to 7.8 by the end of the century (Caldeira and Wickett, 2005; Feely *et al.*, 2009). However, the magnitude of these changes will not be homogeneous over the world oceans, but influenced by local features that characterize each ecosystem (IPCC, 2013; Hoegh-Guldberg *et al.*, 2014; Pörtner *et al.*, 2014; Popova *et. al*, 2016). In addition to this, seawater warming and acidification will be more pronounced in coastal areas due to upwelling of CO_2-enriched seawater, nutrients inputs, organic matter degradation, riverine waters inflow, eutrophication, deposition of atmospheric nitrogen and sulfur, water circulation that will alter both coastal waters temperature and pH fluctuations (Wang *et al.*, 2014).

1.2. Biochemistry behind the effects of ocean warming and acidification

There are now many evidences that ocean CO_2-related changes will affect marine ecosystems and organisms' health status, causing changes in species distribution, abundance, diversity, interactions, communities composition and therefore ecosystem functioning (Pörtner *et al.*, 2014; Magnan and Gattuso, 2016).

Each species is adapted to a limited range of temperature which determines physiological performance, and biological consequences of ocean warming will strictly depend on the wideness of this range (Pörtner, 2002; Bijima *et al.*, 2013; Pörtner *et al.*, 2014). Mechanisms underlying organisms' thermal tolerance have been explained by the concept of "oxygen and capacity limited thermal tolerance" (OCLTT, Pörtner, 2002; 2010): organism performance is function of oxygen supply, which becomes limiting at the extremes of the temperature range (Fig.1.1). Once temperature change exceeds the optimum range, organisms experience loss of performance; exacerbation of temperature change leads firstly to shift to anaerobic metabolism and secondly to cells damage. These processes determine whether thermal stress elicits biochemical to physiological effects in organisms. Temperature elevation has been demonstrated to affect mitochondrial functioning and antioxidants enzymes activity, leading to onset of oxidative stress and energy unbalance (Abele *et al.*, 1998, 2001, 2002; Sokolova and Pörtner, 2001; Sartoris *et al.*, 2003; Sokolova *et al.*, 2006) which can be followed by trade-offs of energy allocated for growth, fecundity, reproduction and activity (Munday *et al.*, 2008; Donelson *et al.*, 2010; Rosa *et al.*, 2012). Nevertheless, there are evidences highlighting that temperature elevation has effects on immune system efficiency increasing the susceptibility to diseases (Cheng *et al.*, 2004; Malagoli *et al.*, 2007; Monari

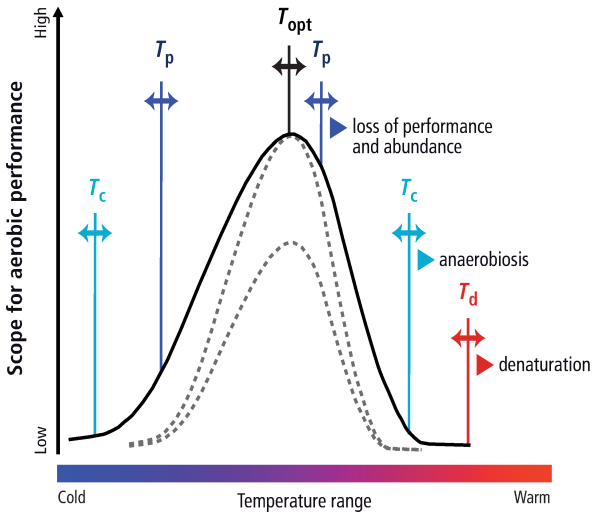


Figure 1.1 Performance curve and thermal tolerance.
T_{opt}= optimum; T_p= pejus; T_c= critical; T_d= denaturation. From Pörtner

et al., 2007; Matozzo *et al.*, 2009; Matozzo and Marin, 2011). It is worth noting that the width of thermal window is highly influenced by life stages, which is narrower in eggs, embryos, early larvae but also spawners than in juveniles and growing adults (Rosa *et al.*, 2012; Pörtner *et al.*, 2014).

On the other hand, also CO2-driven reduction of pH and of the saturation state of $CaCO_3$ have implications with organisms biochemical and physiological processes. Increase in ambient pCO_2 its reflected by changes in organisms' fluids pCO_2 and concentration of carbonate, bicarbonate and hydrogen ions (pH) (Figure 1.2). Higher ambient pCO_2 affects calcification rates by affecting HCO_3^- transportation in inner shell/skeleton-forming tissues. Cellular hypercapnia, increase in cellular CO_2, may also reflect in a decrease of both extracellular and intracellular pH, which have implications with oxygen transport, ion-exchange rates, mitochondrial processes and gene expression (Pörtner, 2008; Pörtner *et al.*, 2014). Organisms that have limited capability to regulate extracellular acid-base balance, such as invertebrates, will be more susceptible to ocean acidification. Adverse biological effects of ocean acidification have been addressed in reduced calcification rates (Gazeau *et al.*, 2007; Jokiel *et al.*, 2008; Dupont *et al.*, 2010; Cerrano *et al.*, 2013), acid-base balance disturbance (Miles *et al.*, 2007; Spicer *et al.*, 2007; Gutowska *et al.*, 2010), impaired metabolism (Lannig *et al.*, 2010; Stumpp *et al.*, 2012; Pan *et al.*, 2015), altered immune response (Bibby *et al.*, 2008; Hernroth *et al.*, 2011, 2012, 2016), incorrect larval development (Kurihara *et al.*, 2007; Dupont *et al.*, 2008; Ellis *et al.*, 2009; Stumpp *et al.*, 2011) and onset of oxidative stress (Tomanek *et al.*, 2011; Rokkita *et al.*, 2012; Soriano-Santiago *et al.*, 2012; Pimentel *et al.*, 2015; Freitas *et al.*, 2016).

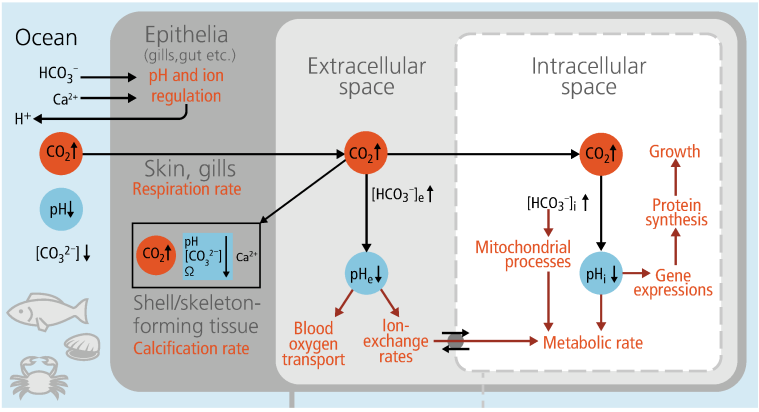


Figure 1.2 – Schematized responses to ocean acidification.
Effects are mediated via diffusive CO2 entry (black arrows) into body and cell compartments, resulting in a rise in pCO2 (highlighted in red), a drop in compartmental pH (highlighted in blue), and their effects (red arrows) on various processes (red text) in tissues and cellular compartments, as well as on calcium carbonate saturation state (Ω) at calcification sites. From Pörtner *et al.*, 2014.

A recent meta-analysis of 228 studies revealed significant adverse effects of ocean acidification on various physiological responses such as calcification, growth, development, abundance and survival, with marked variability depending on taxonomic group, life stage and biological interactions in multispecies exposures (Kroeker et al, 2013); the results further highlighted a trend of enhanced sensitivity toward acidification and co-occurring seawater warming. As explained by Pörtner *et al.*, 2014, the performance of organisms in relation to a temperature range could be narrowed under hypercapnia or hypoxia, which will occur with future scenarios of ocean acidification. Thus, beside the direct effects of ocean warming and acidification, it is important to consider also the interactions of these multiple stressors, which might be species-specific and related to the investigated pathways or physiological functions.

1.3. Multiple stressors in coastal ecosystems

In addition to ocean warming and acidification, marine ecosystems are threatened by a large number of stressors, as *i.e.* eutrophication, salinity variations, hypoxia and pollution (Heugens *et al.*, 2001; Crain *et al.*, 2008, 2009; Burton and Johnston, 2010; Hewitt *et al.*, 2016). Among these multiple stressors co-occuring in the marine environment, trace metal pollution is of particular interest in coastal and estuarine ecosystems, where anthropogenic footprint is higher than in open ocean (Doney, 2010; Bijima *et al.*, 2013; Gillaranz *et al.*, 2016). Trace metals inputs in the ocean can be both of natural or anthropogenic origin. Anthropogenic activities usually introduce trace metals in the environment through atmospheric and river inputs, dredging spoil, direct discharges, industrial dumping and sewage sludge. From a toxicological point of view, metals are distinguished between essentials and non-essentials depending whether they have a biological function for organisms. Among non-essential metals, cadmium is released in the environment as a consequence of combustion of coal and oil, the zinc industry, the recycling of scrap metal, plastics, cadmium paints and electroplating works (Theede, 1980; Neff, 2002). Cadmium concentration in seawater ranges from a few nanograms per liter up to a few tens

micrograms per liter, mainly present as cadmium chloride or bound to organic matter (Neff, 2002). Vertical distribution of cadmium in seawater is similar to that of nutrients, *i.e.* low at the surface, maximum at approximately 1000 m and then declining with depth. This is of particular interest in upwelling areas, which experience cadmium enrichment from deep waters (Neff, 2002; Regoli *et al.*, 2005). Cadmium is accumulated in marine organisms through respiratory epithelia, via active or passive transport, with the latter being more frequent; active uptake of cadmium involves calcium pump, suggesting competition between these elements (Neff, 2002). This is only one of the numerous mechanisms involving Cd toxicity, which may be exerted through competition on enzymes and membrane proteins active sites, damages to lysosomal membranes and onset of oxidative stress through enhancement of reactive oxygen species (ROS) or depletion-impairment of antioxidants with consequent damages to proteins, lipids and DNA (Theede, 1980; Rani *et al.*, 2014).

Both ocean warming and acidification have been described as potential factors affecting heavy metals distribution in coastal waters and their accumulation in marine organisms. Temperature may increase metals bioavailability by increasing their solubility, although this effect is negligible in an environmental realistic temperature range (Sokolova and Lannig, 2008). Higher temperature is a trigger for increased ventilation and/or feeding due to energy demand; higher metals uptake after temperature elevation has been widely described (Viarengo *et al.*, 1988; Hutchins *et al.*, 1996; Leung *et al.*, 2000; Nichols and Playle, 2004; Baines *et al.*, 2005, 2006; Cherkasov *et al.*, 2007; Mubiana and Blust, 2007; Guinot *et al.*, 2012; Negri *et al.*, 2013). On the other hand, the reduction of hydroxide and carbonate ions due to CO_2-enrichment (ocean acidification) may alter the speciation and solubility of metals that form strong complexes with these ligands (Millero *et al.*, 2009; Hoffmann *et al.*, 2012; Stockdale *et al.*, 2016), but ocean acidification will also affect metals that form complexes with organic ligands (Louis *et al.*, 2009; Gledhill *et al.*, 2015). Increased metals fluxes from sediments to water have been described under ocean acidification condition and simulation of CO_2 leakages (Ardelan *et al.*, 2009; de Orte *et al.*, 2014a,

2014b; Basallote *et al.*, 2015) and higher metals accumulation in organisms' tissues under ocean acidification condition was observed in bivalves *Ruditapes philippinarum*, *Crassostrea virginica*, *Mercenaria mercenaria, Mytilus edulis, Tegillarca granosa, Meretrix meretrix* (López *et al.*, 2010; Ivanina *et al.*, 2014a; Götze *et al.*, 2014; Rodriguez-Romero *et al.*, 2014a; Shi *et al.*, 2016), polychaetes *Hediste diversicolor* (Rodriguez-Romero *et al.*, 2014b), eggs and embryos of the squid *Loligo vulgaris* and of the cuttlefish *Sepia officinalis* (Lacoue-Labarthe *et al.*, 2009, 2011), .

Beside chemical speciation, bioavailability and bioaccumulation, temperature and pH/pCO_2 are also involved in biological effects of metals and their toxicity. The interaction between heat stress and metal exposure has been demonstrated to impair mitochondrial function, oxidative balance, accumulation of lipid peroxidation products, thus causing damages to lysosomal system and DNA (Sokolova *et al.*, 2004; Kefaloyianni *et al.*, 2005; Cherkasov *et al.*, 2007; Mubiana and Blust, 2007; Sokolova & Lannig, 2008; Guinot *et al.*, 2012; Negri *et al.*, 2013; Attig *et al.*, 2014; Banni *et al.*, 2014; Gomiero and Viarengo, 2014; Izagirre *et al.*, 2014; Múgica *et al.*, 2015). Nowadays, there is a growing body of knowledge on the biological adverse effects of interactions between metals and ocean acidification, which have been recognized as impaired larval development (Lewis *et al.*, 2013; Campbell *et al.*, 2014), DNA damage (Roberts *et al.*, 2013;Lewis *et al.*, 2016), onset of oxidative stress (Ivanina *et al.*, 2015; Siddiqui and Bielmyer-Fraser, 2015), alteration of the immune function (Ivanina *et al.*, 2016), mitochondrial functioning (Ivanina and Sokolova, 2013; Ivanina *et al.*, 2013), proteasome activity and energy metabolism (Götze *et al.*, 2014).

Despite the scientific literature provides increased evidence on the effects of ocean warming or acidification on accumulation and biological effects of metals, there's still a lack of knowledge on the effects of the co-occurrence of these three stressors which have been usually investigated two at a time.

1.4. Oxidative stress and antioxidant system

Several pathways of aerobic metabolism, including mitochondrial oxidative phosphorylation and electrons transport chains, oxido-reductase enzymes and immunological reactions (active phagocytosis), usually produce reactive oxygen species (ROS), like the singlet oxygen $^1O_2$, superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO•) (Regoli and Giuliani, 2014). These ROS can react with other molecules to form highly reactive radicals which can produce damages to proteins, lipids and DNA, as *i.e.* peroxynitrite (HONOO), hypochloric acid (HOCl), peroxyl radicals (ROO•) and alkoxyl radicals (RO•). To prevent damages, cells have a complex network of scavenging molecules and antioxidant enzymes which act both directly and indirectly on ROS. Low molecular scavengers, as reduced glutathione (GSH), directly bind radicals and oxidize themselves until being reconverted in reduced form by specific enzymes; antioxidant enzymes catalyze specific reactions toward specific substrates without being oxidized. Despite different specificity and mechanisms of actions, all the components of the antioxidant system are highly connected to form a sophisticated network of defense (Fig. 1.3).

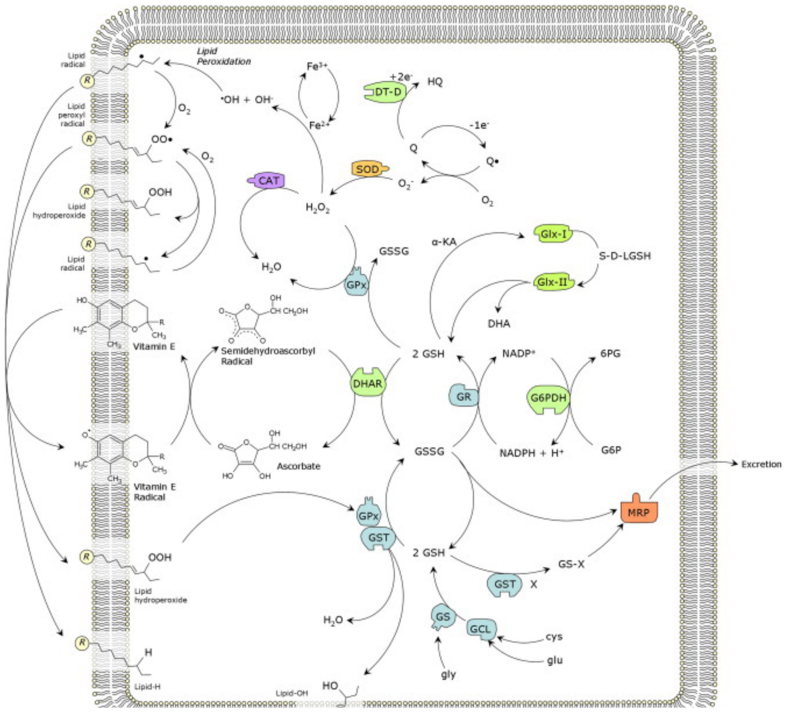


Figure 1.3 - Main cellular antioxidant defences and antioxidant pathways

6 PG: 6-phospogluconate; CAT: catalase; cys: cysteine; DHA: d-hydroxyacid; DHAR: dehydroascorbate reductase; DT-D: DT-diaphorase; G6P: glucose 6-phosphate; G6PDH: glucose 6-phosphate dehydrogenase; GCL: γ-glutamylcysteine synthetase; Glx-I: glyoxalase I; Glx-II: glyoxalase II; GPx: glutathione peroxidases; GR: glutathione reductase; gly: glycine; glu: glutamic acid; GS: glutathione synthetase; GSH: reduced glutathione; GSSG: oxidized glutathione; GST: glutathione S-transferases; GS-X: GSH conjugated xenobiotic; HQ: hydroquinone; αKA: α-keto aldehydes; MRP: multidrug resistance-related protein; Q: quinone; Q: semiquinone radica; S-D-LGSH: S-D-Lactoylglutathione; SOD: superoxide dismutase; X: xenobiotic From Regoli and Giuliani, 2014

Trace metals may disturb the balance between oxidative pressure and antioxidant system exhibiting different mechanisms of pro-oxidant challenge at molecular and cellular level; the most common mechanism for oxidative challenge by trace metals is represented by direct production of ROS through loss of electrons and catalyzation of Haber-Weiss and Fenton reactions that generate hydroxyl radical (HO•) (Halliwell and Gutteridge, 2007). Some trace metals, by reaction with cysteine, may generate thiol radicals (GS•) which can exert direct oxidative damage or react with other thiols and generate ROS (Shi and Dalal, 1988; Regoli, 2012), while others, with different oxidation states, undergo reduction by a pool of flavoenzymes; during this reduction process, O_2 is reduced to H_2O_2 which can react to generate HO• (Regoli, 2012). Indirect oxidative mechanisms of trace metals involve impairment of mitochondrial electrons transport chain and the depletion of antioxidant defenses: as an example, the high affinity of Cd for –SH groups of GSH leads to formation of mixed disulfides, and oxidized form of glutathione (GSSG) (Regoli, 2012; Regoli and Giuliani, 2014).

The study of the antioxidant defenses has been largely suggested as a useful tool in laboratory and field conditions to assess biological effects of pollutants (Livingstone 2001; Regoli and Principato, 1995; Bocchetti *et al.*, 2008; Gorbi *et al.*, 2008). Many environmental and physiological factors modulate antioxidant system efficiency and responsiveness of oxidative stress biomarkers: among these, seasonal variations of temperature, food availability, spawning, oxygen availability, phytoplankton blooms and even adaptation to specific environmental features (Regoli *et al.*, 2000; Gorbi *et al.*, 2005; Bocchetti and Regoli, 2006).

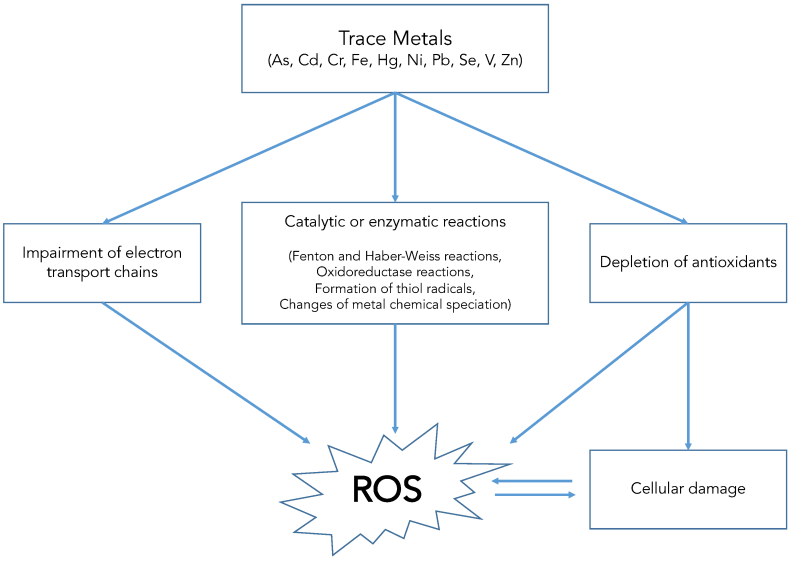


Figure 1.4 – Mechanisms of ROS generation by trace metals. (Regoli, 2012)

Among the antioxidant defenses, the main pathways commonly analyzed are the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione S-transferases (GSTs), glutathione peroxidases (GPx), glutathione reductase (GR) and the cytosolic scavenger glutathione (TGSH). SOD catalyzes the dismutation of $O_2^{\bullet-}$ into O_2 and H_2O_2., which is decomposed by CAT to H_2O and O_2; GSTs are a family of detoxification enzymes that act by conjugating GSH to lipid peroxides and xenobiotics; GPxs can be either selenium-dependent or Se-indipendent forms that protect the cell from oxidative damages by reducing respectively inorganic peroxides (as H_2O_2) to water and organic peroxides (as lipid hydroperoxides) to their respective alcohol; GR is the enzyme that reconverts oxidized glutathione (GSSG) to its reduced form (GSH), which acts both as a scavenger or as a cofactor for previously described enzymes (Regoli, 2012). Single antioxidants variations are useful in revealing early pro-oxidant challenges but the biological significance of this changes is difficult to summarize. For this reason, analysis of single antioxidant defenses is usually integrated with the analysis of the total oxyradical scavenging capacity (TOSC) toward different ROS, like the peroxyl (ROO•), hydroxyl (HO•) radicals and peroxynitrite (HOONO) (Regoli and Winston, 1999; Gorbi and Regoli, 2003; Regoli, 2012). Oxidative insult can lead to damages to lipids, with the formation of lipid hydroperoxides, further decomposed to aldehydes as malondialdehyde (MDA), and of lipofuscin, an end product of lipid oxidation; onset of DNA toxicity, with the formation of reversible damages, strand breaks, and less reversible effects, as chromosome breakage (Gorbi *et al.*, 2008). Further, transcriptional and catalytic responses can be non-synchronous, with the latter exhibiting delayed effects compared to the former; opposite trend of response can also be caused by oxidative insult directly affecting the enzyme but not the corresponding mRNA (Regoli and Giuliani, 2014).

2. AIMS OF THE RESEARCH ACTIVITY

The overall purpose of this thesis was to assess the interactive effects of temperature and pH/pCO2 variations on the health status of marine organisms exposed to pollutants. Focus of the investigation was if temperature elevation and pH reduction could modulate metals bioaccumulation and biological responses both directly and indirectly associated to metal exposure. Since the organisms' susceptibility to the investigated factors may be influenced by physiological characteristics and adaptation to features typical of each ecosystem, as tolerance to variations of environmental variables and pollutants accumulation, capability to activate detoxification mechanisms and protection toward oxidative insult, we compared the results of the investigated stressors effects at different level:

- Ecosystem (organisms from polar and temperate ecosystems)
- Species (species from the same ecosystem)
- Season (summer and winter)
- Tissue (digestive and respiratory tissue)
- Intracellular mechanism (transcriptional and catalytic responses)

Results of this research were expected to further elucidate interactive effects of multiple stressors on marine organisms in order to contribute to a deeper understanding of future impacts of climate changes.

3. METHODS

3.1. Model organism and overall experimental design

Three species of bivalve mollusks were used as model organisms in this thesis: the Antarctic scallop *Adamussium colbecki* (paper I), the Mediterranean mussel *Mytilus galloprovincialis* (paper II-III-IV), the smooth scallop *Flexopecten glaber* (paper V). Bivalve mollusks are widely used as bioindicator organisms in environmental toxicology studies due their ability to accumulate pollutants and their responsiveness toward chemical stress (Regoli and Orlando, 1993; Regoli *et al.*, 1998; Regoli, 2000; Camus *et al.*, 2003; Cajaraville *et al.*, 2000), but have also been addressed as highly sensitive to the negative effects of ocean warming and acidification (Ross *et al.*, 2011; Parker *et al.*, 2013). While *M. galloprovincialis* and *A. colbecki* are common species used for environmental monitoring and toxicological studies respectively in temperate and polar areas the use of *F. glaber* in this field is not documented to our knowledge. These species were selected as model organisms of specific ecosystems (*M. galloprovincialis* and *F. glaber* for the temperate area, *A. colbecki* for the Antarctic) and to allow the comparison between organisms from the same ecosystem (*M. galloprovincialis* and *F. glaber*) and between similar species from different ecosystems (*A. colbecki* and *F. glaber*).

Organisms, obtained from shellfish farms (paper II-III-IV) or by scuba diving sampling (paper I and V) were exposed in laboratory conditions in a multifactorial experimental design to all the possible combinations of two temperatures (control or warm condition), two pH/pCO2 (control/normocapnia or acid/hypercapnia) and two doses of cadmium (absent or present). The whole experimental details and duration of treatments are given in Table 3.1 along with temperature, pH and cadmium values, chosen according to environmental features typical of each biological model ecosystem. At the end of experimental phase, digestive glands and gills were excised, pooled in samples constituted by tissues of 3 individuals, rapidly frozen in liquid nitrogen and maintained at -80°C for analyses of cadmium or biomarkers. Haemolymph was withdrawn from the adductor muscle

of 5 specimens for each experimental treatment, and immediately used for measurement of immunity parameters and genotoxic damage.

3.2. Cd bioaccumulation analyses and biomarker analyses (I, II, III and V)

Cadmium (Cd) concentration in digestive glands and gills of exposed organisms was analyzed by atomic absorption spectrophotometry with graphite furnace atomization and Zeeman effect, according to previously described methods (Regoli *et al.*, 2005).

Metal accumulation is usually associated to metallothioneins synthesis, sulfydryl-rich proteins involved in metal homeostasis and detoxification, and also induced by ROS (Viarengo *et al.*, 2000; Regoli and Giuliani, 2014). Since cadmium, thermal stress and hypercapnia were demonstrated to have pro-oxidant capacity (Theede, 1980; Tomanek *et al.*, 2011; Banni *et al.*, 2014; Vinagre *et al.*, 2014), antioxidant system efficiency was assessed analyzing individual antioxidant defenses in terms of activity of catalase, glutathione S-transferase, selenium-dependent and total glutathione peroxidases, glutathione reductase along with the evaluation of total glutathione (Gorbi *et al.*, 2008). In addition, total oxyradical scavenging capacity toward peroxyl (ROO•) and hydroxyl (HO•) radical was assessed, thus providing an integrated image of the whole tissue antioxidant status (Regoli and Winston, 1999). Oxidative insult was evaluated in terms of lipid peroxidation and lipid mobilization measuring malondialdehyde content, lipofuscin and neutral lipids. All these analyses, except of lipofuscin and neutral lipids, were conducted in both digestive gland and gills in order to evaluate possible differences in tissue sensitivity to the investigated multiple stressors. Further, immune system and genotoxic damages were evaluated in haemocytes in terms of phagocytosis rate, granulocytes versus hyalinocytes cells ratio, lysosomal membrane stability, DNA fragmentation and frequency of micronuclei (Avio *et al.*, 2015). Detailed protocols of analyzed biomarkers are given in Supplementary Material 1 (SM1).

3.3. Transcriptional responses (IV)

A battery of selected genes was analyzed through Real Time PCR in digestive gland and gills of exposed Mediterranean mussels. Genes included expression of the Cd-inducible isoform of metallothioneins (*mt-20*), the heat shock protein 70 (*hsp70*), along with antioxidants such as cytosolic superoxide dismutase, catalase, Se-dependent glutathione peroxidase and pi isoform of glutathione S-transferase (*Cu/Zn-sod*, *cat*, *Se-gpx*, *gst-pi*). Since variations of proteins activity are not always synchronous with variations of corresponding mRNA levels due to time delayed effects and factors controlling gene transcription (Regoli and Giuliani, 2014), this approach was aimed to compare transcriptional and functional responses at cellular level in exposed organisms, exploring the possibility of different modulatory effects on transcription, translation or catalytic activity. Detailed procedures are described in Supplementary Material 2 (SM2).

3.4. Data analysis

Analysis of variance (One-way ANOVA) was applied in each experiment to evaluate the effects of the treatments for each evaluated biomarker, after check of normal distribution and homogeneity of variances. Level of significance was set to $p < 0.05$; *post-hoc* tests, Student – Newman – Keuls (SNK), were used to compare group of means. Non-parametric ANOVA (Kruskal-Wallis, $p < 0.05$) was applied for transcriptional responses, followed by Dunn's *post-hoc* test. The overall significance of biomarkers results was summarized in a cellular hazard index elaborated through a previously developed quantitative model which applies weighted criteria to discriminate different endpoints and the magnitude of effects (Sediqualsoft, Piva *et al.*, 2011). The general rationale of the model is to assign to each biomarker a weight (toxicological importance of the measured endpoint) and to compare variations of each biomarker to a specific threshold, which consider the possibility of biphasic responses (induction and inhibition) and the different responsiveness among various species and tissues. The results are then integrated to calculate a Hazard Quotient

(HQ) that is assigned in one of five classes of hazard: Absent, Slight, Moderate, Major or Severe. Details and conceptual bases of the model are given elsewhere (Piva *et al.*, 2011).

Table 3.1 Experimental designs: temperature, pH, cadmium dose combinations in each experimental treatment for each biological model. CTRL= control; Cd= cadmium; A= acidification; W= warming; A-Cd= acidification + Cd: W= warming + Cd; A-W= acidification + warming; A-W-Cd= acidification + warming + Cd.

	A. colbecki [paper I]			*M. galloprovincialis* (summer) [paper II and IV]			*M. galloprovincialis* (winter) [paper III and IV]			*F. glaber* [paper V]		
Treatment	T	pH	Cd	T	pH	Cd	T	pH	Cd	T	pH	Cd
CTRL	-1°C	8.1	0 µg/L	20°C	8.2	0 µg/L	10°C	8.2	0 µg/L	20°C	8.2	0 µg/L
Cd	-1°C	8.1	40 µg/L	20°C	8.2	20 µg/L	10°C	8.2	20 µg/L	20°C	8.2	20 µg/L
A	-1°C	7.6	0 µg/L	20°C	7.4	0 µg/L	10°C	7.4	0 µg/L	20°C	7.4	0 µg/L
W	+1°C	8.1	0 µg/L	25°C	8.2	0 µg/L	15°C	8.2	0 µg/L	25°C	8.2	0 µg/L
A-Cd	-1°C	7.6	40 µg/L	20°C	7.4	20 µg/L	10°C	7.4	20 µg/L	20°C	7.4	20 µg/L
W-Cd	+1°C	8.1	40 µg/L	25°C	8.2	20 µg/L	15°C	8.2	20 µg/L	25°C	8.2	20 µg/L
A-W	+1°C	7.6	0 µg/L	25°C	7.4	0 µg/L	15°C	7.4	0 µg/L	25°C	7.4	0 µg/L
A-W-Cd	+1°C	7.6	40 µg/L	25°C	7.4	20 µg/L	15°C	7.4	20 µg/L	25°C	7.4	20 µg/L
duration	14 days			28 days			28 days			10 days		

Table 3.2 Analyzed parameters in each tissue and each species. [Cd]: cadmium concentration; MT: metallothioneins; CAT: catalase; GST: glutathione S-transferase; GPx: glutathione peroxidase; TGSH: total glutathione; TOSC: total oxyradical scavenging capacity assay (ROO•: peroxyl radical, HO• hydroxyl radical); MDA: malondialdehyde; Granuloc/Hyalinoc: granulocytes vs. hyalinocytes type cells ratio; LMS: lysosomal membranes stability; *mt-20*: Cd-inducible isoform of MT mRNA; *hsp70*: heatshock protein70 mRNA; *Cu/Zn-sod*: cytosolic superoxide dismutase mRNA; *cat*: catalase mRNA; *Se-dep. gpx*: selenium-dependent glutathione peroxidase mRNA; *gstpi*: pi isoform of glutathione S-transferase.

PARAMETERS		tissue			species		
		DIG. GLAND	GILLS	HAEMOCYTES	*A. colbecki*	*M. galloprovincialis*	*F. glaber*
BIOACCUMULATION	[Cd]	✓	✓	✗	✓	✓	✓
METAL-EXPOSURE	MT	✓	✓	✗	✓	✓	✓
SINGLE ANTIOXIDANT DEFENSES	CAT activity	✓	✓	✗	✓	✓	✓
	GST activity	✓	✓	✗	✓	✓	✓
	Se-dep. GPx activity	✓	✓	✗	✓	✓	✓
	Total GPx activity	✓	✓	✗	✓	✓	✓
	TGSH	✓	✓	✗	✓	✓	✓
TOTAL ANTIOXIDANT CAPACITY	TOSC ROO•	✓	✓	✗	✓	✓	✓
	TOSC HO•	✓	✓	✗	✓	✓	✓
LIPID PEROXIDATION	Malondialdehyde	✓	✓	✗	✓	✓	✗
	Lipofuscin	✓	✗	✗	✓	✓	✗
IMMUNE SYSTEM	Phagocytosis rate	✗	✗	✓	✗	✓	✗
	Granuloc/Hyalinoc	✗	✗	✓	✗	✓	✗
	LMS	✗	✗	✓	✗	✓	✓
GENOTOXICITY	DNA fragmentation	✗	✗	✗	✗	✓	✓
	Micronuclei	✗	✗	✓	✓	✓	✓
STRESS, DETOXIFICATION AND ANTIOXIDANT GENES	*mt-20*	✓	✓	✗	✗	✓	✗
	hsp70	✓	✓	✗	✗	✓	✗
	Cu/Zn-sod	✓	✓	✗	✗	✓	✗
	cat	✓	✓	✗	✗	✓	✗
	Se-dep. gpx	✓	✓	✗	✗	✓	✗
	gstpi	✓	✓	✗	✗	✓	✗

4. MAIN RESULTS AND DISCUSSION

4.1. Antarctic scallop *Adamussium colbecki* (Paper I)

Scallops *Adamiussium colbecki* were exposed for 14 days to Cd, acidification and warming, dosed alone and in various combinations. Bioaccumulation analyses showed 3 to 5-fold increase of Cd concentration in metal-exposed organisms, but at the same time did not reveal any additive effect of temperature or pH variations neither in the digestive gland nor in the gills of the scallops (Table 4.1). Accumulation of cadmium was not reflected by enhancement of metallothioneins levels in digestive gland while this effect was observed in the gills, again without any additional effect of temperature and/or pH variations. The low responsiveness of MTs in the digestive gland is probably due the high basal levels of these proteins in this tissue, that reflect adaptation to natural occurring exposure to cadmium in Antarctic waters (Regoli *et al.*, 1998, 2005).

Concerning the antioxidant system, the analysis of single antioxidants in the digestive gland revealed clear inhibitory effects of temperature, both alone and in combination with other stressors: catalase and Se-dep. glutathione peroxidase activities were inhibited by Cd-exposure and warming condition, total glutathione peroxidase activity was lowered when the metal was dosed in association with higher temperature and reduced pH, while glutathione S-transferases activity was lowered in organisms exposed to higher temperature alone; since these enzymes are involved in the metabolism of inorganic and organic hydroperoxides (Regoli and Giuliani 2014), observed variations suggest that temperature stress in association to Cd-exposure may impair peroxides metabolism in digestive gland. These changes were not paralleled by changes in total oxyradical scavenging capacity (TOSC) toward ROO• and HO•, and a slight increase of malondialdehyde (MDA) was observed in all the organisms exposed to at least two of the three investigated stressors; an increase of lipofuscin content was also observed in organisms exposed to cadmium and acidification, both alone or in combination, confirming involvement of pro-oxidative mechanisms.

On the other hand, the analysis of the antioxidant system in the gills revealed sensitivity toward multiple stressors but without clear trend: interactive effects of Cd with reduced pH or higher temperature inhibited the activity of catalase, while inducing glutathione S-transferases activity; on the other hand, Cd and higher temperature, both at control or reduced pH, increased total glutathione levels, while Se-dep. GPx were inhibited by individual and multiple stressors, showing a complex pattern of oxidative metabolism. An increased capacity to neutralize radicals (TOSC ROO• and HO•) was measured in organisms exposed to higher temperature with or without cadmium, without any increase of malondialdehyde content.

Lastly, the micronuclei frequency in scallops haemocytes showed high sensitivity to all the investigated factors, with marked effects after exposure to Cd at low pH, confirming pro-oxidative mechanisms of metal exposure at acidified conditions as suggested also by MDA and lipofuscin in the digestive gland and hypothesized by other authors previously (Tomanek *et al.*, 2011; Rokkita *et al.*, 2012; Soriano-Santiago *et al.*, 2012; Harms *et al.*, 2014; Pimentel *et al.*, 2015; Freitas *et al.*, 2016).

The elaboration for each tissue of the biomarkers dataset (Fig. 4.1) showed a higher responsiveness of digestive gland toward cadmium exposure, both alone or in combination; for the gills the major stressor was the higher temperature indicating a differential sensitivity to the multiple stressors in different tissues, probably reflecting their physiological and metabolic function.

Table 4.1 Cd bioaccumulation in A. colbecki tissues. Data are expressed as µg/g d.w. of tissue and given as mean ± standard deviation (n=5). Boxes that do not share the same lower case letters are significantly different (p <0,05).

Tissue	CTRL	Cd	A	W	A-Cd	W-Cd	AW	AW-Cd
Digestive gland	55.56 ± 15.90 (a)	151.26 ± 36.39 (b)	55.56 ± 15.90 (a)	55.56 ± 15.90 (a)	86.40 ± 26.50 (ab)	139.05 ± 65.69 (b)	55.56 ± 15.90 (a)	134.02 ± 66.54 (b)
Gills	4.95 ± 1.27 (a)	21.00 ± 6.59 (b)	4.95 ± 1.27 (a)	4.95 ± 1.27 (a)	18.21 ± 3.10 (b)	29.25 ± 7.62 (b)	4.95 ± 1.27 (a)	24.12 ± 16.18 (b)

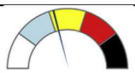
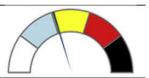
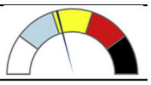
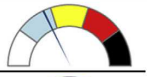
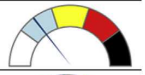
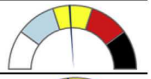
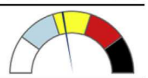
Tissues	Experimental groups	Hazard Quotients (HQ)	Class of Hazard	Level
Digestive glands	Cd	8.9	Moderate	
Digestive glands	Ac	7.99	Slight	
Digestive glands	W	5.92	Slight	
Digestive glands	Ac+Cd	8.62	Moderate	
Digestive glands	W+Cd	6.13	Moderate	
Digestive glands	W+Ac	1.9	Slight	
Digestive glands	W+Ac+Cd	5.5	Moderate	
Gills	Cd	3.53	Slight	
Gills	Ac	5.75	Slight	
Gills	w	2.35	Slight	
Gills	Ac+Cd	1.61	Slight	
Gills	W+Cd	10.58	Moderate	
Gills	W+Ac	9.03	Moderate	
Gills	W+Ac+Cd	13.15	Moderate	

Figure 4.1 - Weight of Evidence (WOE) classification of biomarkers data, in scallops exposed to different laboratory conditions. The quantitative Hazard Quotients (HQ) and the assigned classes of hazard are given.

4.2. Mediterranean mussels *Mytilus galloprovincialis*, summer (Paper II)

Mussels *Mytilus galloprovincialis* were exposed for 28 days to all the possible combinations of two temperatures, two levels of pH and two doses of Cd during summer 2014. Bioaccumulation analysis revealed increased concentrations of the metal in all the experimental treatments with Cd, and no additional effects of temperature or pH variations were measured in digestive glands or gills of exposed mussels. Conversely, metallothioneins levels were significantly induced by Cd and further modulated by temperature and pH: in digestive gland reduced, pH significantly increased MTs levels in Cd exposed organisms, while in the gills warming and/or acidification blocked the inductive effect of Cd on the synthesis of these proteins. Results observed in the digestive gland may be related to a possible antioxidant role described for MTs (Regoli and Giuliani, 2014); different typologies of variation of MTs mRNA have been described in various tissues of oyster *Crassostrea virginica* exposed to Cd, Cu and hypercapnia (Ivanina *et al.*, 2015).

Analyses of the antioxidant system showed increased glutathione S-transferases activity in organisms exposed to Cd at higher temperature, and similar although not significant trends were observed also for Se-dependent glutathione peroxidase and glutathione reductase activity; since these enzymes are involved in detoxification of hydrogen and lipid hydroperoxides, it is possible to hypothesize that higher metabolic rates and cadmium enhance ROS production stimulating an adaptive mechanisms of protection toward oxidative insult (Regoli and Giuliani, 2014). Despite the inductions of these enzymatic activities, a reduction of the total oxyradical scavenging capacity toward peroxyl radical (TOSC ROO•) was measured in digestive gland of organisms exposed to combined effects of Cd and acidification, at both control and higher temperature. Interestingly a lower level of MDA was measured in organisms exposed to acidification and Cd at higher temperature, while higher lipofuscin content was observed in those exposed to Cd and acidification at control temperature: this allows to hypothesize different roles of warming and acidification on oxyradicals metabolism, with the former promoting glutathione-dependent enzymes

protection and the latter promoting oxidative insult. Similar hypothesis was formulated also by other authors for the larval development of sea urchin *P. lividus* (Garcia *et al.*, 2015).

In the gills the individual antioxidant defenses in showed a more complex pathway of variations: co-exposure to warming and Cd led to increased activity of Se-dep. GPx, while acidification, alone or in combination with warming, and co-exposure to warming and Cd significantly inhibited Total GPx activity. These variations led to increased antioxidant capacity toward the peroxyl radical (TOSC ROO•) in organisms exposed to Cd at higher temperature, and to a reduction of the TOSC HO• in organisms exposed to almost all experimental treatments. Despite this, no accumulation of MDA was measured; the oxidative status of the gills was as high responsiveness to multiple stressors and suggesting involvement of complex pathways of oxidative insult.

Immunological analyses on haemocytes revealed high sensitivity of the lysosomal membranes to acidification and warming, as previously suggested by other authors (Beesley *et al.*, 2008; Matozzo *et al.*, 2012). In addition, decreased phagocytosis rate in organisms exposed to higher temperature, was in agreement with results obtained in oysters and clams (Hégaret *et al.*, 2003; Monari *et al.*, 2007). Mussels haemocytes can be divided in granulocytes, with phagocytic function, and hyalinocytes, deputed to encapsulation and coagulation, with low ROS protection (Gorbi *et al.*, 2013). In this study, we found an increased ratio between granulocytes versus hyalinocytes in Cd-exposed organisms at reduced pH and higher temperature; this finding, combined with the phagocytosis results, suggest a disruption of hyalinocytes health rather than of granulocytes, probably due to oxidative mechanisms of multiple stressors. This is further confimed by the higher frequency of micronuclei occurred in haemocytes of mussels exposed to Cd and reduced pH: in this respect, the genotoxicity of Cd-exposure, may be further increased by pro-oxidative pressure exerted by reduced pH, as reported also in Lewis *et al.*, 2016 in haemocytes of *M. edulis* and in coelomocytes of *P. lividus* exposed to copper and acidification.

This complex dataset of results on biomarkers was elaborated within a quantitative model which resumed through weighted criteria the observed variations (Fig. 4.2). The hazard was classified as "Moderate" in all the Cd-exposed experimental treatments at lower pH and/or higher temperature, confirming the possible negative effects of multiple stressors interactions on the Mediterranean mussels' health.

Table 4.2 Cd bioaccumulation in *M.galloprovincialis* tissues. Data are expressed as µg/g d.w. of tissue and given as mean ± standard deviation (n=5). Boxes that do not share the same lower case letters are significantly different (p <0,05).

Tissue	CTRL	Cd	A	W	A-Cd	W-Cd	AW	AW-Cd
Digestive gland	2.69 ± 3.31 (a)	33.15 ± 6.42(b)	3.01 ± 1.86 (a)	2.83 ± 1.98 (a)	34.53 ± 16.41 (b)	38.03 ± 18.39 (b)	5.08 ± 0.21 (a)	39.8 ± 9.06 (b)
Gills	1.04 ± 0.62 (a)	14.22 ± 1.03 (b)	0.55 ± 0.26 (a)	0.38 ± 0.12 (a)	19.47 ± 2.41 (b)	18.78 ± 8.94 (b)	0.33 ± 0.12 (a)	15.99 ± 4.39 (b)

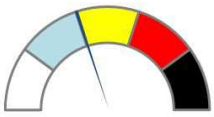
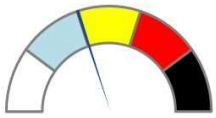
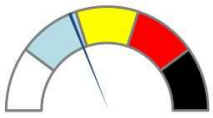
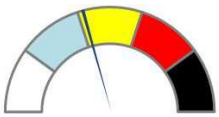
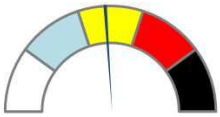
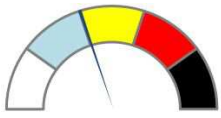
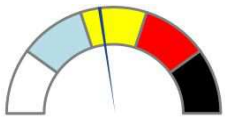
Experimental Treatment	Hazard Quotient (HQ)	Class of Hazard	Level
Cd	12.52	SLIGHT	
A	10.03	SLIGHT	
W	6.59	SLIGHT	
A-Cd	14.87	MODERATE	
W-Cd	19.34	MODERATE	
A-W	11.74	SLIGHT	
A-W-Cd	17.40	MODERATE	

Figure 4.2 - Weight of Evidence (WOE) classification of biomarkers data for the whole dataset of analyzed parameters for each different laboratory condition. The quantitative hazard quotients (HQs) and the assigned class of hazard are given.

4.3. Mediterranean mussels *Mytilus galloprovincialis*, winter (Paper III)

Mediterranean mussels were exposed to the same experimental design previously presented in section 4.2 for 28 days during winter 2015, to further investigate modulatory effects of temperature and pH variations on Cd accumulation and responsiveness, and to assess possible differences with the experiment performed during summer. In this experiment concentration of cadmium in the digestive gland and gills of mussels increased in the organisms exposed to the metal and was further enhanced in those treatments in which Cd was dosed in association to higher temperature (Table 4.3). This this may be due to temperature-controlled acceleration of metabolism: the temperature range used in this study is within the thermal optimum of mussels, and thus allows increase of metabolic increase at increasing temperature (Pörtner, 2010). Metallothioneins induction by cadmium was slight in this experiment: in the digestive gland MTs levels increased only when Cd-exposure was associated to acidification and/or warming, while in the gills a significant increase was observed only in organisms exposed to Cd at higher temperature and control pH. This confirms previously formulated hypothesis on MTs function: these proteins are not exclusively related to metal exposure but their induction can be further modulated by other environmental stressors as mechanism of antioxidant protection (Viarengo, 2000; Regoli and Giuliani, 2014).

The antioxidant system analyses aimed to investigate single antioxidants, total oxyradical scavenging capacity and onset of oxidative damages. In the digestive gland, significant differences were observed in total GPx activity: cadmium exposure led to induction of this enzyme activity in organisms exposed at control pH; on the contrary, this effect was masked in organisms exposed to Cd at reduced pH, with also temperature playing a major role in these experimental treatments. These variations however did not elicit effects on the total oxyradical scavenging capacity, except to an increased ability to neutralize ROO• in organisms exposed to Cd, warming and acidification. Despite this, the limited effects on MDA content were in contrast with the increase of lipofuscin content in these organisms which showed the higher content of this peroxidation marker. On the other

hand, a significant decrease of neutral lipids content in organisms exposed to Cd at higher temperature and/or reduced pH compared to those exposed to the metal at control conditions, suggested mobilization of energy reserves during stress condition.

In the gills, Se-dep. GPx activity was enhanced by Cd in organisms exposed at control temperature, but also by higher temperature; at higher temperature however Cd exposure inhibited Se-dep. GPx responsiveness. The same effect was shown to occur in total GPx activity due to acidification in organisms exposed to Cd and warming. As in the digestive gland these effects were paralleled by limited variations of the total oxyradical scavenging capacity toward HO•, which was increased in organisms exposed to warming, without any variation of MDA levels.

Haemocytes lysosomal membrane was highly sensitive to the investigated factors, but the strongest variations occurred in organisms co-exposed to Cd, acidification and warming, confirming previously reported observations for *M. galloprovincialis* (Beesley *et al.*, 2008; Matozzo *et al.*, 2012). High sensitivity of haemocytes to higher temperature was shown in terms of reduced phagocytosis rate, which occurred also in oysters and clams (Hégaret *et al.*, 2003; Monari *et al.*, 2007), while a significant reduction of the ratio between granulocytes and hyalinocytes was reported in organisms exposed to Cd at higher temperature; since similar results of phagocytosis were observed also in mussels exposed in the summer experiment (Paper II), it is possible to hypothesize an increase of hyalinocytes which were suggested as the most Cd- and temperature-sensitive hemolymph population. This is further corroborated by the higher frequency of micronuclei measured in organisms exposed to Cd alone and to Cd at higher temperature. The dataset of biomarkers responses was elaborated through a quantitative model that classified the effects of each treatment into one of five classes of hazard. The results (Fig 4.3) showed a generalized sensitivity, mainly due to Cd and warming, classifying as "Slight" only the hazard for organisms exposed to acidification without cadmium, while in all the other treatments the hazard was "Moderate", suggesting that during winter mussels are more responsive to metal and/or thermal stress.

Table 4.3 Cd bioaccumulation in *M.galloprovincialis* tissues. Data are expressed as µg/g d.w. of tissue and given as mean ± standard deviation (n=5). Boxes that do not share the same lower case letters are significantly different (p <0,05).

Tissue	CTRL	Cd	A	W	A-Cd	W-Cd	AW	AW-Cd
Digestive gland	0.52 ± 0.07 (a)	16.39 ± 3.32 (b)	0.44 ± 0.09 (a)	0.55 ± 0.11 (a)	20.67 ± 5.47 (b)	30.48 ± 5.31 (c)	0.49 ± 0.09 (a)	33.39 ± 7.57 (c)
Gills	0.22 ± 0.09 (a)	6.78 ± 1.93 (b)	0.19 ± 0.09 (a)	0.25 ± 0.12 (a)	5.21 ± 1.53 (b)	16.17 ± 7.44 (c)	0.31 ± 0.09 (a)	19.39 ± 2.18 (c)

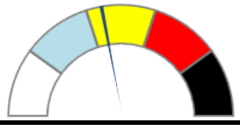
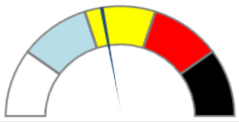
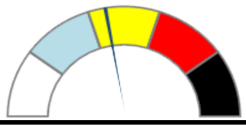
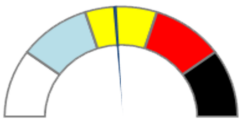
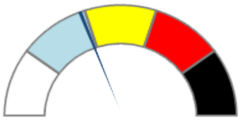
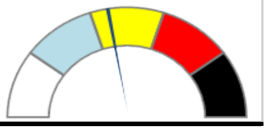
Experimental Treatment	Hazard Quotient (HQ)	Class of Hazard	Level
Cd	29.41	MODERATE	
A	14.5	SLIGHT	
W	37.39	MODERATE	
A-Cd	56.59	MODERATE	
W-Cd	22.62	MODERATE	
A-W	61.08	SLIGHT	
A-W-Cd	24.67	MODERATE	

Figura 4.3 - Weight of Evidence (WOE) classification of biomarkers data for the whole dataset of analyzed parameters for each different laboratory condition. The quantitative hazard quotients (HQs) and the assigned class of hazard are given.

4.4. Transcriptional responsiveness of *Mytilus galloprovincialis* (Paper IV)

The responsiveness to multiple stressors interactions was assessed also at the transcriptional level in *M. galloprovincialis* exposed to the previously presented experimental plans, both in summer and winter, both in digestive gland and gills. The selected genes battery included Cd-inducible isoform of metallothioneins (*mt-20*), the heat shock protein 70 (*hsp70*), along with antioxidants such as cytosolic superoxide dismutase, catalase, a Se-dependent glutathione peroxidase and pi isoform of glutathione S-transferase (*Cu/Zn-sod*, *cat*, *Se-gpx*, *gst-pi*).

Concerning *mt-20*, usually associated to metal exposure (Dondero *et al.*, 2005), Cd exposure led to higher mRNA levels in the digestive gland in both seasons, independently of temperature or pH variations. On the other hand, different effects were observed in gills in the two seasons: in summer, enhancement of temperature blocked the *mt-20* transcription in Cd exposed organisms, while in winter this inhibitory effect was observed only in organisms exposed to Cd and reduced pH; this may reflect differential responsiveness due to metabolic capacity and cellular mechanisms in tissues with different physiological function and thus biochemical characteristics (Regoli and Giuliani, 2014); nevertheless the opposite effects that investigated factors have in different seasons in the same tissue highlight a seasonal modulation on sensitivity to environmental variables.

Hsp70, usually associated to folding and degradation of damaged, unrepairable proteins (Mayer and Bukau, 2005), is often analyzed as marker of thermal or generic cellular stress (Wang *et al.*, 2013). In our study, *hsp70* gene expression showed to be mostly sensitive to acidification after 28 days of exposure, but without a clear pattern: in summer, acidification, alone or in combination with other stressors, significantly up-regulated *hsp70* gene transcription in digestive tissue, while in winter this effect was observed only in organisms exposed to acidification alone. On the other hand, a significant down-regulation of this transcript was observed in gills of organisms co-exposed to cadmium and acidification at control temperature in summer, while a slight upregulation by acidification without cadmium was observed in winter. Higher *hsp70* mRNA levels may be interpreted

as markers of cellular stress, but lower levels may suggest involvement of mRNA degradation (Lee *et al.*, 2015). Down-regulation of *hsp70* mRNA was observed in the haemocytes of clam *Mercenaria mercenaria* exposed to cadmium and hypercapnia (Ivanina *et al.*, 2014b) and in the haemocytes of oyster *Saccostrea glomerata* exposed to cadmium (Thompson *et al.*, 2012). These results highlight seasonal and tissue specific differences in stress response and further confirm a weak long-term activation of HSP70 in response to thermal stress. The sensitivity of this transcript to acidification may suggest mechanisms of protein damages or modulation of gene expression due to changes in the intracellular milieu (Lewis *et al.*, 2016; Pörtner *et al.*, 2014; Wang *et al.*, 2016).

The analysis of the battery of antioxidant genes revealed a certain disturbance by the investigated stressors, although clear patterns of action were not identified due to seasonal and tissue specificity. In the digestive gland, acidification, alone or in combination with other stressors, was the main factor promoting up-regulation of *cat* and *gst-pi* mRNA both in summer and in winter with more evident variations in the former; similar finding would confirm a CO_2-mediated increase of oxidative challenge, as already hypothesized by other authors (Tomanek *et al.*, 2011; Benedetti *et al.*, 2016). On the other hand, *Se-dep. gpx* was down-regulated by Cd-exposure at control temperature, by warming without Cd in summer, and by higher temperature alone or in combination with other stressors in winter. Negative effects of cadmium on *Se-dependent gpx* expression have been already reported in *Danio rerio* and *Oncorhynchus kisutch* (Banni *et al.*, 2011; Wang *et al.*, 2012), while temperature was shown to up-regulate selenium-dependent glutathione peroxidases in the gastropod *Haliotis discus discus* (De Zoysa *et al.*, 2009). Lower sensitivity to the investigated factors was observed in the gills in both seasons. In summer, thermal stress was responsible for down-regulation of *Se-dep. gpx* at control pH as already observed in the digestive gland, while in winter acidification and warming, alone or combined with cadmium significantly and negatively affected *Se-dep. gpx* transcript levels. In addition to this, the down-regulation of *cat* mRNA due to the cadmium exposure and acidification, alone or in

combination, suggests lower protection toward oxidative insult deriving from these stressors in the gills.

The complex patterns of variations observed for antioxidants genes reflect the multiple pathways that regulates the transcription of each gene, which can be triggered by several transcription factors, binding different responsive elements on their promoter regions upon different stimuli (Miao *et al.*, 2005; Stoytcheva and Berry, 2009; Glorieux *et al.*, 2015).Such results, confirm that ocean acidification and warming can modulate transcriptional responsiveness toward cadmium-mediated cellular stress. Nevertheless, investigated tissues showed differential sensitivity to cadmium, acidification and warming. Digestive gland exhibited higher capability toward oxidative insult by enhanced mRNA levels of certain antioxidants in response mainly to cadmium and acidification; on the other hand gills, showed more complex patterns of sensitivity, but the investigated stressors led to punctual lowering of mRNA levels of the investigated responses, suggesting time-delayed effects or variable modulation of selected genes transcription.

4.5. Smooth scallop *Flexopecten glaber* (Paper V)

The smoot scallop *Flexopecten glaber* was exposed to the same experimental plans used for *M. galloprovincialis* in summer for 10 days. The first important result obtained in this species was the high mortality rates observed in organisms exposed at higher temperature (25°C), independently of Cd or pH. The results presented were obtained in organisms exposed at control temperature.

Bioaccumulation analyses showed increased concentration of Cd in digestive gland of metal exposed organisms, with further increase, although not significant, in organisms exposed under acidification condition; on the other hand, metal uptake in the gills significantly increased only in organism exposed to Cd at reduced pH (Tab. 4.5). Metallothioneins levels were not significantly affected by Cd exposure, probably due the high basal levels of Cd in the tissues of scallops, which makes these animals more adapted to high Cd concentration (Mauri *et al.*, 1990; Regoli *et al.*, 1998b, 2000b, 2002; Bustamante *et al.*, 2002). The antioxidant defenses analyzed in the digestive gland showed both single and interactive effects of Cd and acidification: catalase activity was inducted by acidification, as also glutathione S-transferases, Se-dep. GPx activities and concentration of total glutathione, suggesting a pro-oxidative mechanism of reduced pH exerted through the generation of hydroperoxides, as suggested for the oysters *Crassostrea virginica* and *Crassostrea gigas* and the polychaete *Sabella spallanzanii* (Tomanek *et al.*, 2011; Moreira *et al.*, 2016; Ricevuto *et al.*, 2016). These effects were however lower in organisms co-exposed to Cd and acidification, suggesting antagonist effects of Cd through depletion or impairment on antioxidant defenses (Regoli and Giuliani, 2014). The enhanced antioxidants responsiveness however guaranteed higher protection toward oxidative stress, as confirmed by the absence of any variation of the total oxyradical scavenging capacity.

In the gills, the complex interactions between acidification and cadmium were even more evident: synergistic effects of co-exposure to Cd and reduced pH inhibited the activity of GST, while Cd inhibited the Se-dep. GPx activity only at control pH. These

variations were followed by a generalized reduction of the total oxyradical scavenging capacity toward peroxyl radical, with no additive effects of investigated factors, confirming pro-oxidative mechanisms and lower efficiency of the gills compared to digestive gland to overwhelm these stressors.

Haemocytes showed synergistic effects of acidification and Cd on lysosomal membranes stability and a general increase of MN frequency, with no differences between experimental treatments involving Cd and acidification.

Finally, the elaboration of biomarkers data through the quantitative model gave "Moderate" hazard class for treatments at reduced pH, both with or without Cd, while the overall effects of Cd exposure at control pH were classified as "Slight" (Fig. 4.5). The naturally high basal levels of Cd in these organism may explain the lower effect of exposure to metal alone, while acidification pose a major threat; nevertheless, Cd effects become more evident under acidification conditions, suggesting that metal exposure can be modulated under stress conditions determined by reduced pH.

Table 4.4 Cd bioaccumulation in *F.glaber* tissues. Data are expressed as µg/g d.w. of tissue and given as mean ± standard deviation (n=5). Boxes that do not share the same lower case letters are significantly different (p <0,05).

Tissue	CTRL	Cd	A	A-Cd
Digestive gland	14.54 ± 1.40 (a)	22.87 ± 2.07 (bc)	18.50 ± 1.16 (ab)	28.68 ± 5.64 (c)
Gills	0.16 ± 0.04 (a)	9.35 ± 1.52 (ab)	0.56 ± 0.11 (a)	19.98 ± 13.65 (b)

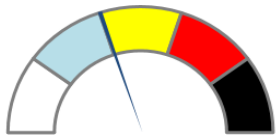
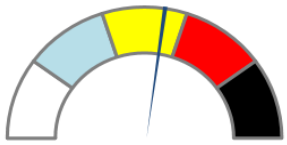
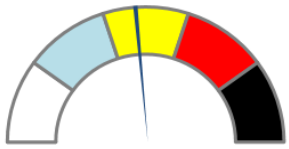
Experimental Treatment	Hazard Quotients (HQs)	Class of Hazard	Level
Cd	9.06	SLIGHT	
A	21.07	MODERATE	
A+Cd	13.13	MODERATE	

Figura 4.4 - Weight of Evidence (WOE) classification of biomarkers data for the whole dataset of analyzed parameters for each different laboratory condition. The quantitative hazard quotients (HQs) and the assigned class of hazard are given.

4.6. Comparison of scallops from different ecosystems (Paper I and V)

One of the main purposes of this research activity was to compare the sensitivity of organisms living in highly different ecosystems, as temperate scallop *F. glaber* and Antarctic scallop *A. colbecki*, both previously presented. Despite the cadmium dose and the magnitude of temperature and pH variation chosen in these experiments were different, they were selected to represent environmental realistic scenarios in temperate and Antarctic areas.

The first evident result is the higher sensitivity of temperate scallops to 5°C temperature elevation compared to +2°C which did not affect mortality of *A. colbecki*. Since the analyses on *F. glaber* were conducted only on organisms exposed at control temperature, the comparison between the two species will focus on metal uptake and on the cellular effects of cadmium and acidification. Concerning bioaccumulation, despite both species have high basal levels of Cd, cadmium exposure led to increased concentrations in digestive gland and gills of both the species. Nevertheless, acidification represented an additional threat only in temperate scallops, enhancing metal uptake in gills. Since Cd speciation has been predicted to be poorly affected by environmental pH changes (Millero *et al.*, 2009) this effect can be ascribed to increased gills permeability due to acidification effects on ions exchange on respiratory epithelia (Pörtner, 2010). In the digestive gland of both species we found low induction of MTs, while Cd-exposure led to an increase of these proteins in the gills of *A. colbecki* but not in *F. glaber*, probably due to higher naturally occurring Cd exposure in Antarctic upwelling zones (Mauri *et al.*, 1990; Regoli *et al.*, 1998b), which can explain the higher responsiveness. Lower sensitivity to the investigated stressors was found in *A. colbecki* antioxidant system in the digestive gland compared to *F. glaber*, in which acidification and cadmium increased single antioxidant protection without affecting total oxyradical scavenging capacity. On the contrary the same pathways were involved in the gills, as CAT, GST and Se-dep. GPx, but with different effects of acidification and cadmium in the two species: in *A. colbecki* co-exposure to Cd and acidification inhibited catalase and Se-dep. GPx while increased GST, in *F. glaber* inhibited GST activity. This is reflected by

the decreased total oxyradical scavenging capacity toward ROO• in *F. glaber*, which is unaffected in *A. colbecki*, and can be interpreted as higher capability of Antarctic scallop to counteract negative effects of acidification and cadmium. Despite these differences in antioxidant responsiveness, onset of genotoxic damage in terms of MN frequency showed a similar trend in the two species, with increased frequency of these nuclear abnormalities in all experimental treatments without differences between Cd and acidification effects.

These results suggest that *A. colbecki* is more capable to counteract oxidative insult deriving from the investigated stressors, probably due the higher environmental oxidative pressure in the Antarctic ecosystem (Regoli *et al.*, 2012).

4.7. Comparison of temperate species (Paper II and V)

Species living in the same ecosystems may have a different capability to counteract changes of environmental factors; one of our purposes was to elucidate whether scallops and mussels counteract variations of the same magnitude. In this respect this section aimed to compare the results obtained in the summer experiments with *M. galloprovincialis* and *F. glaber*.

In Mediterranean mussel, Cd uptake was not influenced by temperature or pH variations, while, as already shown, bioaccumulation of Cd was affected by reduced pH in *F. glaber*. These differences, anyhow, were not reflected by MTs levels, which were insensitive to metal exposure in *F. glaber*; on the contrary in *M. galloprovincialis* digestive gland Cd and acidification induced higher levels of MTs, while in the gills Cd inductivity was blocked by reduced pH. The single antioxidant defenses in *M. galloprovincialis* showed low sensitivity to acidification and Cd which was paralleled by decreased total antioxidant capacity in organisms co-exposed to Cd and acidification in the digestive gland; on the contrary in *F. galber* we observed high sensitivity of single antioxidants which guaranteed higher protection toward oxidative insult in digestive gland but not in the gills, where the total oxyradical scavenging capacity toward ROO• was lower in all experimental treatments compared to control. Haemocytes lysosomal membrane stability showed similar trends, with additive and negative effects of Cd and acidification; lastly the analysis of genotoxic damages, did not reveal effects in terms of DNA fragmentation in both species, while MN frequency showed strong differences: while in *F. glaber* these nuclear abnormalities increased in all organisms exposed to Cd and/or acidification, in *M. galloprovincialis* negative effects of Cd were amplified when this was associated to acidification, confirming pro-oxidative challenge of these stressors.

The overall conclusion on this comparison showed high capability of mussels to counteract the effects of single stressors conversely to scallops which exhibited generalized sensitivity to acidification with slight additive effects of Cd.

4.8. Seasonal differences of the responsiveness of Mediterranean mussels

Since the biological responsiveness of organisms can be modulated by biological and physiological characteristics (*e.g.* reproductive cycle) and even by seasonal fluctuations of abiotic factors (*e.g.* naturally occurring and pronounced temperature variations at temperate latitudes), we aimed to compare the results obtained in experiments performed with mussels in summer and in winter.

In this respect, Cd bioaccumulation was strongly different in the two experiments. In summer no additional effect of temperature and/or pH variations influenced the uptake of the metal, while in winter strong differences occurred in organisms exposed to Cd at higher and control temperature, with higher accumulation in the former compared to the latter. This may be explained by the different effect that a +5°C temperature elevation has on organisms in different seasons: in winter, organisms are well below their upper thermal limit, and temperature elevation leads to faster metabolic rates and higher ventilation with consequent increase in metal uptake; on the other hand, in summer, organisms are very close to their upper thermal limit and temperature elevation does not elicit metabolism acceleration, but rather a shift to loss of performance (Pörtner *et al.*, 2010). Also metallothioneins responsiveness showed high variability in the two experiments, with more pronounced effects in the gills. In summer Cd induction is blocked by variations of temperature and pH, while in winter this effect was not observed: this finding confirms the implications of thermal tolerance in the responsiveness to environmental pollution and the possible negative effects in metal binding efficiency under warming and acidification scenarios. Similarly, the antioxidant system in digestive gland showed responsiveness of antioxidant enzymes toward higher temperature in summer, while in winter the major effects where due to acidification that interfered with Cd-related responsiveness. These effects were partially translated into changes of the total oxyradical scavenging capacity, which in summer was reduced and in winter was enhanced by co-exposure to Cd and acidification. In the gills, the same antioxidant pathways were involved in the two seasons, namely the Se-dep. and total GPx, but with opposite trends: in summer Se-dep. GPx is

induced by Cd at control pH and inhibited at reduced pH, while in winter this trend was observed due to temperature elevation; on the other hand, total GPx activity was induced by acidification in Cd exposed organisms at higher temperature in winter, while in summer there was an inhibition. This effects where not reflected by evident variations of TOSC, which was only slightly reduced in summer in almost all experiment treatments. This confirms the high capability to adjust the antioxidant responsiveness, probably involving other antioxidants to counteract the oxidative pressure.

In both seasons, lipofuscin accumulation was increased by acidification and Cd, with slight differences due to higher temperature that in summer blocks this effect; similar trend was observed also for MDA, which decreased in summer in organisms co-exposed to higher temperature and low pH. On the contrary neutral lipids, which did not show differences in summer, decreased in winter in all organisms exposed to Cd and acidification and/or warming compared to those exposed to Cd at control temperature and pH. Since neutral lipids accumulation in lysosomes was observed due to exposure to environmental pollutants (Cajaraville *et al.*, 1992; Regoli *et al.*, 1992), this results may be interpreted as a mobilization of energy resources under moderate stress.

Both summer and winter mussels showed reduced lysosomal membrane stability in haemocytes, with more pronounced effects of single stressors in summer than in winter; similarly, phagocytosis rate was reduced at higher temperature in both seasons, but in winter the reduction was higher. Mussels haemocytes populations can be distinguished in granulocytes, with phagocytic functions, and hyalinocytes, deputed to encapsulation; in summer the ratio between these two populations was increased by Cd, particularly in organisms exposed to Cd, warming and acidification, while in winter this ratio decreased in organisms exposed to warming alone and in combination with cadmium. Since the phagocytosis rate followed similar trends in both seasons, it is possible to hypothesize that in summer the increase may be ascribed to hyalinocytes disruption due to Cd exposure, while in winter there is an increase of hyalinocytes respect to granulocytes. Lastly, the onset of genotoxic damages showed sensitivity to Cd and acidification in summer, while in winter

a more generalized sensitivity to all experimental stressors increased MN in all experimental treatments.

The overall conclusion on seasonal comparison is that seasonality effectively affects biochemical pathways of antioxidant protection; these pathways are differentially impacted by the investigated stressors in the two seasons, with summer organisms being sensitive to Cd only when associated to reduction of temperature and pH, while in winter Cd and warming led to major variations.

4.9. Comparison of transcriptional and catalytic responses in *M. galloprovincialis* (Paper II, III and IV)

Transcriptional and catalytic responses are not always synchronous. In our study, comparison of mRNA and protein activity of certain biological pathways confirmed the hypothesis of complex interactions and effects between multiple stressors in different seasons and tissues. These investigations were carried out on both experiments conducted with *M. galloprovincialis*.

In the digestive gland, metallothioneins concentration was affected by interactions of the investigated stressors, but this was not reflected by mRNA levels; since *mt-20* is the Cd-inducible isoform, it is possible to hypothesize that the discrepancies between mRNA and protein levels may be generated by the transcription of other MTs genes such as *mt-10*. In a similar way, this may have affected also the gills, where more evident interactive effects of the investigated stressors occurred at transcriptional level, suggesting time delayed responses effects (Regoli and Giuliani, 2014).

The other pathway compared at transcriptional and catalytic level is the antioxidant system, through the analysis of three antioxidant enzymes, catalase, Se-dep. glutathione peroxidases and glutathione S-transferase, and their respective genes, *cat*, *Se-dep. gpx* and *gst-pi*. In this respect, *cat* expression was up-regulated by acidification in the digestive gland of mussels, while catalase activity was not affected by investigated stressors; this difference between effects at transcriptional and catalytic level may be explained as a compensative mechanism resulting from increased oxidative challenge (Giuliani *et al.*, 2013). Non-synchronous effects were also observed for *Se-dep. gpx* expression and Se-dep. GPx activity, with the former being down-regulated by metal exposure and higher temperature in summer, while the enzyme activity was slightly enhanced by these stressors. On the contrary, the *gst-pi* expression was regulated by acidification while the activity of GST was induced by warming in summer and unaffected in winter; this may suggest both non-synchronous responses but also differential mechanisms triggering the responsiveness of genes transcription and the modulation of catalytic activity.

In the gills, catalase expression and catalytic activity were unaffected by the investigated stressors in summer, while in winter we observed down-regulation of mRNA by cadmium and acidification not paralleled by changes of the enzyme activity. Concerning selenium-dependent glutathione peroxidases, transcriptional expression was down-regulated by warming with or without cadmium but, on the contrary, catalytic activity was enhanced in these organisms, indicating once again non-synchronous effects. Also in winter the effects on transcript were not paralleled at catalytic level, which was enhanced in almost all treatments with slight differences between them. All these differences at transcriptional and catalytic levels may be explained by time-delayed effects of responsiveness of transcription and protein activity, but it is also possible the involvement of different mechanisms of action of the investigated stressors on the transcription and on the protein, including a greater damage on protein than on gene or a more limited cellular capability to synthesize proteins compared to mRNA.

5. CONCLUSIONS AND PERSPECTIVES

The general objective of this Thesis has been to contribute to the emerging concern on interactions between multiple stressors in the marine environment, such as ocean warming, acidification and anthropogenic pollutants. Laboratory exposures of different species of mollusks from polar and temperate ecosystems showed that these interactions can occur and cause alterations at lowest levels of biological organization (molecular and cellular), allowing to hypothesize implications for ecosystem functioning.

Bioaccumulation of Cd has been demonstrated to be influenced by temperature and pH variations, with species-, tissue- and seasonal-specific patterns that are hard to generalize. Metal-binding proteins and single antioxidants variations are affected by the investigated factors, and variations often reflect the physiological function of investigated tissue. Nevertheless, environmental features, such as high oxidative pressure or seasonal fluctuations of abiotic factors can further shape organisms' responsiveness to single and multiple stressors.

Cadmium accumulation, among the investigated species, was higher in mussels than scallops (both Antarctic or temperate), which typically contain higher basal levels of this metal; nevertheless, Cd bioaccumulation in mussels was strongly influenced by temperature elevation only in winter, probably related to seasonal physiological characteristics that involve metabolic rates acceleration; on the other hand, in temperate scallops cadmium uptake was slightly enhanced by reduced pH. The higher basal levels of cadmium may also explain the lower responsiveness of metallothioneins in scallops compared to mussels, in which the levels of these proteins were further modulated by temperature and/or pH/pCO2 variations, with tissue-specific and seasonal-dependent effects.

Concerning the oxidative status, temperate mussels and scallops resulted to be more responsive than the polar species to the investigated stressors, probably due to

physiological plasticity and capability of these organisms to counteract oxidative insult deriving from environmental variables; this high responsiveness acts as a protection mechanism toward oxidative insult at higher cellular level. On the other hand, the lower responsiveness measured in Antarctic scallops toward multiple stressors exposure can be explained by the higher basal level of antioxidant protection at both protein and enzymatic level, which represents a key adaptive mechanism to extreme environmental conditions.

Lastly, haemocytes confirmed to be particularly sensitive to multiple stressors: higher temperatures and Cd-exposure led to immune system disorders in mussels, while onset of genotoxic damage was strongly enhanced by all experimental factors, especially by cadmium and acidification in all investigated species. It is worth noting that the differential responsiveness observed between digestive gland and gills may be strongly influenced by metabolic function and physiological features typical of each tissue.

The overall conclusions of this research activity highlight the need of further experimentation, widening the number of investigated species, undergoing longer experimentation periods and testing different pollutants, both organic and inorganic, in order to help environmental policy-makers in detecting early possible critical issue of future oceans projections.

LIST OF PAPERS

This Thesis is based on the following papers, which will be reffered to in the text by their Roman numerals:

I. Benedetti M, Lanzoni I, Nardi A, d'Errico G, Di Carlo M, Fattorini D, Nigro M, Regoli F (2016). Oxidative responsiveness to multiple stressors in the key Antarctic species, *Adamussium colbecki*: Interactions between temperature, acidification and cadmium exposure. Marine Environmental Research, 121, 20-30.

II. Nardi A, Mincarelli LF, Benedetti M, Fattorini D, d'Errico G, Regoli F (2017). Indirect effects of climate changes on cadmium bioavailability and biological effects in the Mediterranean mussel *Mytilus galloprovincialis*. *Chemosphere*, 169, 493-502.

III. Nardi A, Benedetti M, Fattorini D, Regoli F (in submission) Seasonal effects of ocean warming and acidification on cadmium accumulation and responsiveness in mussels *Mytilus galloprovincialis.*

IV. Nardi A, Giuliani ME, Regoli F (in submission) Interactive effect of temperature, pH and Cd of transcriptional responsiveness toward oxidative stress in the Mediterranean mussel *Mytilus galloprovincialis.*

V. Nardi A, Fattorini D, Regoli F (in submission) Oxidative and interactive challenge of cadmium and ocean acidification on the smooth scallop, *Flexopecten glaber*

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7. SUPPLEMENTARY MATERIALS

7.1. SUPPLEMENTATY MATERIAL 1

In this section detailed protocols for the analyzed biomarkers and respective bibliography are given.

SM1.1 Metallothioneins

Metallothioneins (MTs) were analyzed in gills and digestive gland samples homogenized (1:3 and 1:5 w/v respectively) in 20 mM Tris–HCl buffer, pH 8.6, 0.5M sucrose, 0.006mM leupeptin, 0.5 mM PMSF (phenylmethylsulphonylfluoride), and 0.01% b-mercaptoethanol. After acidic ethanol/chloroform fractionation of the tissue homogenate, MTs were quantified by the spectrophotometric assay as described in Viarengo *et al.* (1997) using GSH as standard.

SM1.2 Single antioxidant defenses and Total Oxyradical Scavenging Capacity (TOSC) assay

For enzymatic antioxidants, digestive gland and gills samples were homogenized (1:5 and 1:3 w:v ratio respectively) in 100 mM K-phosphate buffer (pH 7.5), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mg/ml bacitracin, 0.008 TIU/ml aprotinin, 1 µg/ml leupeptin, 0.5 µg/ml pepstatin, NaCl 2.5%, and centrifuged at 100 000 × g for 70 min at 4 °C. Activities were measured with a Varian (model Cary 100 Scan) spectrophotometer at a constant temperature of 18 °C (Bocchetti *et al.*, 2008).

Catalase (CAT) was measured by the decrease in absorbance at 240 nm (extinction coefficient, ε = 0.04 mM−1 cm−1) due to the consumption of hydrogen peroxide, H_2O_2 (12 mM H_2O_2 in 100 mM K-phosphate buffer pH 7.0).

Glutathione S-transferases were determined at 340 nm using 1-chloro-2,4-dinitrobenzene as substrate (CDNB). The assay was carried out in 100 mM K-phosphate buffer pH 6.5, 1.5 mM CDNB, 1 mM GSH (ε = 9.6 mM−1 cm−1).

Glutathione peroxidases activities were assayed in a coupled enzyme system where NADPH is consumed by glutathione reductase to convert the formed GSSG to its reduced

form (GSH). The decrease of absorbance was monitored at 340 nm (ε = −6.22 mM−1 cm−1) in 100 mM K-phosphate buffer pH 7.5, 1 mM EDTA, 1 mM sodium azide (NaN3) (for hydrogen peroxide assay), 2 mM GSH, 1 unit glutathione reductase, 0.24 mM NADPH, and 0.5 mM hydrogen peroxide or 0.8 mM cumene hydroperoxide as substrates, respectively, for the selenium-dependent and for the sum of Se-dependent and Se-independent forms. Glutathione reductase (GR) was determined from NADPH oxidation during the reduction of oxidized glutathione, GSSG (λ = 340 nm, ε = −6.22 mM−1 cm−1). The final assay conditions were 100 mM K-phosphate buffer pH 7.0, 1 mM GSSG, and 60 μM NADPH.

Total glutathione was analyzed in samples homogenized (1:5 w:v ratio) in 5% sulfosalicilic acid with 4 mM EDTA, maintained for 45 min on ice and centrifuged at 37.000 × g for 15 min, before being enzymatically assayed (Akerboom & Sies, 1981).

The total oxyradical scavenging capacity (TOSC) assay measures the overall capability of cellular antioxidants to absorb different forms of artificially generated oxyradicals, thus inhibiting the oxidation of 0.2 mM α-keto-γ-methiolbutyric acid (KMBA) to ethylene gas (Winston *et al.*, 1998; Regoli & Winston, 1999). Peroxyl radicals (ROO•) were generated by the thermal homolysis of 20 mM 2-2'-azo-bis-(2-methylpropionamidine)-dihydrochloride (ABAP) in 100 mM K-phosphate buffer, pH 7.4. Hydroxyl radicals (HO•) were produced by the Fenton reaction of iron-EDTA (1.8 μM Fe^{3+}, 3.6 μM EDTA) plus ascorbate (180 μM) in 100 mM K-phosphate buffer. Ethylene formation in control and sample reactions was analyzed at 10–12 min time intervals by gas-chromatographic analyses and the TOSC values are quantified from the equation: TOSC = 100 − (∫SA/∫CA × 100), where ∫SA and ∫CA are the integrated areas calculated under the kinetic curves for samples (SA) and control (CA) reactions. For all the samples, a specific TOSC (normalized to content of protein) was calculated by dividing the experimental TOSC values by the relative protein concentration contained in the assay, measured by the spectrophotometric method of Lowry *et al.*, 1951 with bovine serum albumin (BSA) as standard.

SM1.3 Malondialdehyde

The content of malondialdehyde (MDA) was measured in samples homogenized (1:3 and 1:5 w/v respectively for gills and digestive gland) in 20 mM Tris–HCl pH 7.4, centrifuged at 3000 × g for 20 min and then derivatized in 1 ml reaction mixture containing 10.3 mM 1-metyl-2-phenylindole (dissolved in acetonitrile/methanol 3:1), HCl 32%, 100 µl water and an equal volume of sample or standard (standard range 0–6 µM 1,1,3,3-tetramethoxypropane, in 20 mM Tris–HCl, pH 7.4). The tubes were vortexed and incubated at 45 °C for 40 min. Samples were cooled on ice, centrifuged at 15 000 × g for 10 min and read spectrophotometrically at 586 nm; levels of MDA were calibrated against a malondialdehyde standard curve and expressed as nmol/g wet weight (Shaw *et al.*, 2004).

SM1.4 Lipofuscin and Neutral Lipids

Lipofuscin content was determined using the Schmorl reaction (Moore, 1988) on cryostat sections (8 µm thick) of digestive glands. Slides were fixed in calcium-formol for 15 min at 4 °C, rinsed in distilled water and immersed in the reaction medium containing an aqueous solution of 1% ferric chloride and 1% potassium ferricyanide. Sections were stained for 5 min, rinsed in 1% acetic acid and washed in distilled water before mounting with Eukitt. For analyses of neutral lipids, cryostat sections (8 µm thick) of digestive glands were fixed as above and washed in 60% isopropilic alcohol solution (Moore, 1988). Sections were stained for 20 min in a saturated oil red O solution (1% in isopropyl alcohol 60%), washed in isopropyl alcohol and then in distilled water before mounting in glycerine gelatine. For both lipofuscin and neutral lipids, four measurements were made on digestive tubules of each section. Quantification of staining intensity was performed with Image-Pro® Plus 6.2 Analysis Software and then normalized to the area of digestive tubules.

SM1.5 Immune system responses

For the analysis of immune system responses, heamolymph was withdrawn from each mussel and then splitted for the analysis of lysosomal membrane stability, granulocytes–hyalinocytes ratio and phagocytosis rate.

The cationic probe Neutral Red (NR) was used to evaluate the capability of the lysosomal membranes to retain the dye (Lowe *et al.*, 1995). Haemocytes were incubated on a glass slide with a freshly prepared NR working solution (2 µl/ml filtered seawater) from a stock solution of 20 mg neutral red dye dissolved in 1 ml of dimethyl sulfoxide, and microscopically examined at 15 min intervals, to determine the time at which 50% of cells had lost to the cytosol the dye previously taken up by lysosomes.

Phagocytosis rate assay was performed following previous methods (Gorbi *et al.*, 2013); 50 µl of haemolymph were dispersed on glass slides and allowed to adhere incubating them for 15 min at 15 °C in the dark. Fluorescein-labelled Zymosan A bioparticles (Invitrogen) were added at 10:1 target:haemocyte ratio. After 2 h incubation at 15 °C in the dark, uninternalized particles were removed by washing with physiological solution and slides were finally fixed in Beker's fixative (+2.5% NaCl) and mounted in Eukitt. Phagocytosis was expressed as percentage of cells that internalized at least 3 fluorescent particle (positive cells), observed under a fluorescence microscope, after counting at least 200 cells for each sample.

Granulocytes versus hyalinocytes ratio was assessed on 50 µl of haemolymph dispersed on glass slides, dryed and fixed in Baker's fixative (+2.5% NaCl). The slides were washed with water, stained with H&E and mounted in Eukitt. Observations were carried out with a light microscope (1000X) and the ratio was evaluated after counting almost 200 cells for each sample (Gorbi *et al.*, 2013).

SM1.6 Genotoxic damage

The onset of DNA damage was evaluated at molecular level as single strand breaks (SB) by the Comet assay, and at chromosomal level by the frequency of micronuclei.

The comet assay was carried out on haemocytes collected from the adductor muscle of organisms according to Gorbi *et al.*, 2008. Cells were diluted in Ca^{2+}/Mg^{2+}-free buffer at 4 °C in the dark (20 mM Hepes, 500 mM NaCl, 12.5 mM KCl, 10 mM EDTA, pH 7.3), centrifuged at 1000 rpm for 1 min at 4 °C, resuspended in 0.6% low-melting-point agarose, and added with a sandwich stratification to glass slides coated with 1% normal-melting-

point agarose. After gel solidification, slides were placed into the lysing solution (2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, and 10% DMSO, pH 10) at 4 °C in the dark for 90 min. DNA was unwound in 75 mM NaOH, 10 mM EDTA (pH 13), and the electrophoretic migration was carried out in the same buffer at 1 V/cm for 10 min. Slides were then neutralized for 10 min in 0.4 M Tris, pH 7.5, fixed in cold methanol for 3 min at −20 °C, and dried. After staining with DAPI, 100 randomly selected "nucleoids" per slide, and two replicates per sample, were examined under fluorescence microscopy (200 × magnification; Olympus BX-51), and the captured images (Image-Pro-Plus package) were analyzed through the software Comet Score. The percentage of DNA in the tail was used to estimate the level of DNA fragmentation.

For the frequency of micronuclei, an aliquot of haemolymph collected from the adductor muscle of organisms was rapidly washed in saline buffer (20 mM Hepes, 500 mM NaCl, 12.5 mM KCl, 10 mM EDTA, pH 7.3) and centrifuged at 1000 rpm for 1 min at 4 °C, following the procedure described in Nigro *et al.*, 2006. Cells were immersed in a fixative solution (3:1 methanol, acetic acid) and washed twice. Suspended cells were dispersed on glass slides, air dried and stained with the fluorescent dye 4',6-diamidino-2-phenylindole DAPI (100 ng/ml). For each specimen, 2000 cells with preserved cytoplasm were scored to determine the frequency of micronuclei, defined as round structures, smaller than 1/3 of the main nucleus diameter on the same optical plan and clearly separated from it.

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7.2. SUPPLEMENTARY MATERIAL 2

Detailed protocols of RNA extraction and RT-PCR are given in this section along to cited references.

SM2.1 RNA isolation and cDNA synthesis

Total RNA was purified from *M. galloprovincialis* digestive glands an gills, using the Hybrid-RTM kit (GeneAll Biotechnology) according to the manufacturer's protocol. Total RNA concentrations and purity were measured using Nano-Drop ND-1000 UV-Visible Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA quality was verified on an agarose-formaldehyde gel. Total cDNA was generated by RT-PCR (Reverse Transcription-Polymerase Chain Reaction) from 1 µg of total RNA for each sample using combined oligo(dT) and random hexamer primers (iScript cDNA Synthesis Kit, Bio-Rad).

SM2.2 Quantitative (real-time) polymerase chain reaction (PCR)

Absolute quantitative real-time PCR with gene-specific primer pairs (Table xxx) was performed for evaluating the mRNA levels of individual target genes, using SYBR green method in StepOnePlus® Real-Time PCR System (Applied Biosystems). Each 15 µl DNA amplification reaction contained 7.5 µl of SYBR Select Master Mix (Life Technologies), 5 µl of total cDNA (synthesized as described above and diluted 1:5) and 200 nM of each forward and reverse primers. The real-time PCR program included an enzyme activation step at 95 °C (2 min) and 40 cycles each composed by 15 s at 95 °C and 1 min at the annealing temperature (Table SM2). The specificity of target cDNA amplification was checked by including controls lacking cDNA template and by a melting analysis (95 °C for 1 min, 65 °C for 10 s and fluorescence detection at increasing temperature between 65 and 95 °C).

For each target gene, serial dilutions of known amounts of plasmid containing the amplicon of interest were used as standards. Samples and standards were run in duplicate in the same run. Cycle threshold (Ct) values of unknown samples were converted into mRNA copy number interpolating the standard plot of Ct versus log copy number. Obtained data from

the same experimental group (n = 6), mRNA copy number per µg of total RNA, were averaged and expressed as $\log_2$ of the fold change relative to control.

Table SM2 - Primer pair sequences, amplicon size, annealing temperatures and accession numbers of genes investigated in quantitative PCR.

Gene	Primer pair sequences	Amplicon size	Accession n.	Annealing T, time	References
Cu/Zn-SOD	Fwd: AGCCAATGCAGAGGGAAAAGCAGA Rev: CCACAAGCCAGACGACCCCC	177 bp	FM177867	65°C, 1 min	Koutsogiannaki *et al.*, 2014
CAT	Fwd: CGACCAGAGACAACCCACC Rev: GCAGTAGTATGCCTGTCCATCC	132 bp	AY743716	55°C, 15 sec 72°C, 1 min	Canesi *et al.*, 2007
Gpx1	Fwd: AGCCTCTCTCTGAGGAACAACTG Rev: TGGTCGAACATGCTCAAGGGC	166 bp	HQ891311	55°C, 15 sec 72°C, 1 min	Giuliani *et al.*, 2013
GSTpi	Fwd: TCCAGTTAGAGGCCGAGCTGA Rev: CTGCACCAGTTGGAAACCGTC	172 bp	AF527010	55°C, 15 sec 72°C, 1 min	Canesi *et al.*, 2008
Hsp70	Fwd: GGTGGTGAAGACTTTGACAACAG Rev: CTAGTTTGGCATCGCGTAGAGC	295 bp	AY861684	65°C, 1 min	Cellura *et al.*, 2006
MT-20	Fwd: TGTGAAAGTGGCTGCGGA Rev: GTACAGCCACATCCACACGC	80 bp	AY566247	55°C, 15 sec 72°C, 1 min	Dondero et al., 2005

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Oxidative responsiveness to multiple stressors in the key Antarctic species, *Adamussium colbecki*: Interactions between temperature, acidification and cadmium exposure

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ABSTRACT

High-latitude marine ecosystems are ranked to be among the most sensitive regions to climate change since highly stenothermal and specially adapted organisms might be seriously affected by global warming and ocean acidification. The present investigation was aimed to provide new insights on the sensitivity to such environmental stressors in the key Antarctic species, *Adamussium colbecki*, focussing also on their synergistic effects with cadmium exposure, naturally abundant in this area for upwelling phenomena. Scallops were exposed for 2 weeks to various combinations of Cd (0 and 40 μgL-1), pH (8.05 and 7.60) and temperature (−1 and +1 °C). Beside Cd bioaccumulation, a wide panel of early warning biomarkers were analysed in digestive glands and gills including levels of metallothioneins, individual antioxidants and total oxyradical scavenging capacity, onset of oxidative cell damage like lipid peroxidation, lysosomal stability, DNA integrity and peroxisomal proliferation. Results indicated reciprocal interactions between multiple stressors and their elaboration by a quantitative hazard model based on the relevance and magnitude of effects, highlighted a different sensitivity of analysed tissues. Due to cellular adaptations to high basal Cd content, digestive gland appeared more tolerant toward other prooxidant stressors, but sensitive to variations of the metal. On the other hand, gills were more affected by various combinations of stressors occurring at higher temperature.

1. Introduction

The concentration of CO_2 in the atmosphere has continuously increased from 278 ppm in pre-industrial time up to 400 ppm nowadays, representing one of the most important drivers of global climate change (http://www.sciencedirect.com/science/article/pii/S002209811400077XIPCC, 2013). Approximately 30% of total CO_2 emissions has been absorbed by seawater, causing the well known process of ocean acidification, OA (Raven et al., 2005). Progressive changes in carbonate chemistry determined a decrease of 0.1 pH units compared to the pre-industrial levels, with a further expected reduction of 0.3–0.5 units by the end of the 21st century (Raven et al., 2005).

The Southern Ocean accounts for about 4% of the global uptake of CO_2 by the world oceans due to the high solubility of CO_2 at low temperature and mixing patterns from upwellings and deep water formation (Fabry et al., 2009). Similar characteristics lead to an increased rate of acidification and a more rapid shoaling of the saturation horizons (Fabry et al., 2009). In this respect, polar organisms, which have evolved in environmentally stable conditions, might be more vulnerable to climate change, in particular regarding calcification processes and variations of fundamental pathways like energy metabolism, growth, reproduction, larval development and oxidative stress. Compared to temperate models, however, only a few studies have investigated the possible effects of climate change on Antarctic marine species (Cubillos et al., 2007; McClintock et al., 2009; Moy et al., 2009; Seibel et al., 2012; Walker et al., 2013; Constable et al., 2014; Collard et al., 2015; Flynn et al., 2015).

In addition, while future scenarios of temperature and ocean acidification can be simulated from CO_2 emission models (IPCC, 2013), at this moment it is virtually impossible to predict the biological impact and synergistic effects of multiple stressors, which

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can be indirectly modulated by variations of environmental factors due to climate change. In this respect, it has been widely postulated that increased temperature and ocean acidification could influence chemical speciation and bioavailability of environmental pollutants, bioaccumulation processes, responsiveness of detoxification mechanisms, sensitivity and consequences on organisms health condition, but clear evidences of similar modulations are still lacking.

In the Ross Sea, local upwelling phenomena are responsible for a natural enrichment and elevated bioavailability of cadmium (Cd) which is accumulated in tissues of Antarctic invertebrates and fish at values typically 10–50 folds higher than those of similar temperate species (Nigro et al., 1997). Although this element does not apparently cause adverse effects on the organisms, in Antarctic fish it was shown to influence the responsiveness of fundamental metabolic pathways to other stressors, i.e. the cytochrome P450 biotransformation mechanisms, male vitellogenin expression and the antioxidant network (Regoli et al., 2005; Canapa et al., 2007; Benedetti et al., 2007, 2009). In the scallop *Adamussium colbecki* (Smith, 1902), another key sentinel species of the Antarctic environment, the enhanced accumulation of Cd during algal bloom is paralleled by a general increase of antioxidant defences (Regoli et al., 2000, 2002). Beside the role in counteracting a natural increase of prooxidant pressure during phytoplankton blooms, oxyradical metabolism and antioxidant defences have a fundamental role for polar organisms in adaptation mechanisms to high solubility of oxygen in cold seawater, elevated content of oxidizable poly-unsatured fatty acids (PUFAs) in membranes, high cellular mitochondrial densities, and the need of long-term protection of proteins and RNAs due to their low turnover rate (Abele and Puntarulo, 2004).

Based on previous issues, the main aim of this study was to investigate whether variations of temperature and pH may singularly or synergistically affect the sensitivity of *A. colbecki* to Cd, highlighting a potentially reciprocal modulation of key cellular responses by multiple stressors. Scallops were exposed to various combinations of treatments including two different levels of temperature, pH and Cd concentrations, opportunely chosen as reflecting environmentally realistic or future scenarios for Antarctic marine environment.

Analyses of Cd bioaccumulation were integrated with a wide panel of early warning biomarkers and results were elaborated within a quantitative model (Sediqualsoft) which, based on biological relevance and magnitude of observed variations, summarize an hazard index for biomarkers results (Piva et al., 2011; Benedetti et al., 2012).

Overall, this study was expected to provide new insights on mechanisms underlying the responsiveness of a model Antarctic species to variations of temperature and acidification, interactions occurring between multiple stressors, and potential consequences of climate change in areas characterized by elevated environmental pollution or geochemical anomalies.

2. Materials and methods

2.1. Experimental design

Scallops, *A. colbecki*, were sampled during the XXIX Italian Antarctic Expedition (2013–2014) from Terra Nova Bay (Ross Sea) and acclimatized to laboratory conditions for 10 days with running, unfiltered seawater at the controlled temperature of −1 °C and pH 8.05. A total of 240 organisms were randomly distributed in eight tanks (150 L each) and exposed to one of the following experimental conditions: 1) control (CTRL), at environmental temperature (−1 °C) and environmental pH (8.05); 2) Cd exposure (Cd), at 40 μg/L of Cd, −1 °C, pH 8.05; 3) acidified water condition (Ac), at pH 7.60 and −1 °C; 4) warm exposure (Warm), at +1 °C and pH 8.05; 5) acidified and Cd exposure (Ac + Cd), at pH 7.60, 40 μg/L of Cd and −1 °C; 6) warm and Cd exposure (W + Cd), at +1 °C, 40 μg/L of Cd and pH 8.05; 7) warm and acidified condition (W + Ac), at +1 °C and pH 7,6; 8) warm, acidified and Cd exposure (W + Ac + Cd), at +1 °C, pH 7.6 and 40 μg/L of Cd. After 14 days, organisms were sacrificed, haemolymph, gills and digestive glands were rapidly dissected, frozen in liquid nitrogen and stored at-80 °C until analyses. A portion of haemolymph and gills was maintained in Carnoy's solution (3:1 methanol:acetic acid) for micronuclei frequency analyses. No mortality was observed during the experiments. For both chemical and biochemical analyses, 5 pools, each constituted by tissue of 6 specimens, were prepared for digestive glands, gills and haemolymph.

2.2. Chemical analyses

Cd concentration in scallops tissues was analysed according to previously described methods (Regoli et al., 2005). For every treatment, digestive glands and gills were dried at 70 °C until constant weight and digested under pressure with nitric acid in microwave digestor systems (CEM, Mars Systems). Quality assurance and quality control was assessed by processing blank samples and reference standard material (Mussel Tissue Standard Reference Material SRM 2977, National Institute of Standards and Technology). Cd was analysed by atomic absorption spectrophotometry with electrothermal atomization. The concentrations obtained for the standard reference material were always within the 95% confidence interval of certified values. Data are expressed as μg/g dry weight (mean values ± standard deviations, n = 5).

2.3. Biomarker analyses

Sample preparation and analytical protocols have been fully detailed elsewhere (Regoli et al., 2000). Metallothioneins were analysed in digestive glands and gills homogenized in 20 mM Tris-HCl buffer (pH 8.6), 0.5 M sucrose, 0.006 mM phenylmethylsulfonyl fluoride (PMSF), and 0.01% β-mercaptoethanol and centrifuged at 30,000 g for 45 min. After acidic ethanol/chloroform fractionation of tissue supernatants, metallothioneins were quantified by a spectrophotometric assay using reduced glutathione (GSH) as standard.

For measurement of enzymatic antioxidants, tissues (digestive gland and gills) were homogenized (1:5 and 1:3 w:v ratio respectively) in 100 mM K-phosphate buffer (pH 7.5), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mg mL^{-1} bacitracin, 0.008 TIU mL−1 aprotinin, 1 mg mL^{-1} leupeptin, 0.5 mg/mL pepstatin, NaCl 2.5%, and centrifuged at 110,000 g for 1 h at 4 °C. Measurements were made with a Varian (model Cary 3) spectrophotometer at a constant temperature of 18 °C. Catalase (CAT) was measured by the decrease in absorbance at 240 nm (extinction coefficient, $\varepsilon = 0.04$ mM^{-1} cm^{-1}) due to the consumption of hydrogen peroxide, H_2O_2 (12 mM H_2O_2 in 100 mM K-phosphate buffer pH 7.0). Glutathione reductase (GR) was determined from NADPH oxidation during the reduction of oxidized glutathione, GSSG ($\lambda = 340$ nm, $\varepsilon = 6.22$ mM^{-1} cm^{-1}). The final assay condition were 100 mM K-phosphate buffer pH 7.0, 1 mM GSSG, and 60 mM NADPH. Glutathione peroxidases (GPx) activities were assayed in a coupled enzyme system where NADPH is consumed by glutathione reductase to convert the formed GSSG to its reduced form (GSH). The decrease of absorbance was monitored at 340 nm ($\varepsilon = 6.22$ mM^{-1} cm^{-1}) in 100 mM K-phosphate buffer pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 1 mM sodium azide (NaN_3) (for hydrogen peroxide assay), 2 mM GSH, 1 unit glutathione reductase,

0.24 mM NADPH, and 0.5 mM hydrogen peroxide or 0.8 mM cumene hydroperoxide as substrates, respectively, for the selenium-dependent and for the sum of Se-dependent and Se-independent forms. The rate of the blank reaction was subtracted from the total rate. Glutathione S-transferases (GST) were determined at 340 nm using 1-chloro-2,4-dinitrobenzene as substrate (CDNB). The assay was carried out in 100 mM K-phosphate buffer pH 6.5, 1.5 mM CDNB, 1 mM GSH ($\varepsilon = 9.6$ mM^{-1} cm^{-1}).

Total glutathione was analysed in samples of digestive gland and gill homogenized (1:5 and 1:3 w:v ratio respectively) in 5% sulfosalicylic acid with 4 mM EDTA, maintained for 45 min on ice and centrifuged at 37,000 g for 15 min. The resulting supernatants were enzymatically assayed (Benedetti et al., 2007).

The total oxyradical scavenging capacity (TOSC) assay measures the overall capability of cellular antioxidants to neutralize different forms of artificially generated oxyradicals, thus inhibiting the oxidation of 0.2 mM a-keto-γ-methiolbutyric acid (KMBA) to ethylene gas (Regoli et al., 2000). Peroxyl radicals (ROO·) were generated by the thermal homolysis of 20 mM 2–2′-azo-bis-(2-methylpropionamidine)-dihydrochloride (ABAP) in 100 mM K-phosphate buffer, pH 7.4. Hydroxyl radicals (·OH) were produced by the Fenton reaction of iron-EDTA (1.8 μM Fe3+, 3.6 μM EDTA) plus ascorbate (180 μM) in 100 mM K-phosphate buffer. Ethylene formation in control and sample reactions was analysed at 10–12 min time intervals by gas-chromatographic analyses and the TOSC values quantified from the equation: TOSC = 100-($\int$SA/$\int$CA × 100), where $\int$SA and $\int$CA are the integrated areas calculated under the kinetic curves for samples (SA) and control (CA) reactions. For all the samples, a specific TOSC (normalized to content of protein) was calculated by dividing the experimental TOSC values by the relative protein concentration contained in the assay.

The content of malondialdehyde (MDA) was measured in homogenates of scallops digestive glands and gills derivatized with 1-metyl-2-phenylindole and spectrophotometrically determined after calibration against a malondialdehyde standard curve.

Acetylcholinesterase activity (AChE) was analysed in haemolymph and gills: haemolymph was centrifuged at 3,000 g for 5 min, while gills were homogenized in 100 mM Tris-HCl buffer (pH 7.2), 0.55 M sucrose and centrifuged at 10,000 g for 10 min. Obtained supernatants were spectrophotometrically assayed by the Ellman's reaction at 18 ± 1 °C, λ = 412 nm, $\varepsilon = 13.6$ mM^{-1} cm^{-1}.

For the activity of Acyl CoA oxidase (AOX), samples were homogenized in 1 mM sodium bicarbonate buffer (pH 7.6) containing 1 mM EDTA, 0.1% ethanol, 0.01% Triton X-100 and centrifuged at 500 g for 15 min at 4 °C. The H_2O_2 production was measured in a coupled assay by following the oxidation of dichlorofluorescein-diacetate (DCF-DA) catalyzed by an exogenous horseradish peroxidase (HRP). The reaction medium was 0.5 M potassium phosphate buffer (pH 7.4), 2.2 mM DCF-DA, 40 μM sodium azide, 0.01% Triton X-100, 1.2 U mL^{-1} HRP in a final volume of 1 mL. After a pre-incubation at 25 °C for 5 min in the dark with an appropriate volume of sample, reactions were started adding the substrates palmitoyl-CoA at final concentrations of 30 μM and 100 μM for Acyl-CoA oxidase (AOX) and readings were carried out against a blank without the substrates at 502 nm.

Protein concentrations were measured according to Lowry method (1951), using bovine serum albumin (BSA) as standard. All biochemical biomarkers are expressed as mean values ± standard deviations (n = 5).

Lipofuscin content of tertiary lysosomes was determined on duplicate cryostat sections (8 μm thick) of digestive gland. Slides were fixed in Beker's fixative (+2.5% NaCl) and stained by Schmrol reaction before mounting in glycerol gelatine. Five measurements were made on digestive tubules of each section (two sections for mussel, 10 scallops for each experimental condition). Quantification of staining intensity was performed with Image-Pro® Plus 6.2 Analysis Software and then normalized to the area of digestive tubules.

The DNA integrity was evaluated at chromosomal level by measuring the micronucleus (MN) frequency in scallops haemocytes rapidly fixed in Carnoy's solution (3:1 methanol: acetic acid), dispersed on glass slides and stained with the fluorescent dye 4′,6-diamidino- 2-phenylindole at 100 ng mL^{-1}. For each experimental condition, 10 scallops were observed and for each specimen 2000 cells with preserved cytoplasm were scored to assess the presence of micronuclei, defined as round structures, smaller than 1/3 of the main nucleus diameter, on the same optical plan and clearly (Benedetti et al., 2014).

2.4. Statistical analyses

Analysis of variance (1-way ANOVA) was applied to test significance of effects on biochemical parameters caused by temperature, acidification, Cd exposure and their reciprocal interactions. Level of significance was set at $p < 0.05$, homogeneity of variance was checked by Cochram C and mathematical transformation applied if necessary; post-hoc comparison (Newman–Keuls) was used to discriminate between means of values. Multivariate statistical analysis (non-metric multidimensional scaling analysis) was applied to biomarkers data to discriminate various experimental treatments.

Results on biological parameters in scallops were further elaborated within a previously developed quantitative and software-assisted model (Sediqualsoft, Piva et al., 2011). Whole calculations, detailed flow-charts, rationale for weights and thresholds have been fully given elsewhere and successfully validated in filed conditions, during the characterization and classification of risk from industrial and harbour sediments, natural hydrocarbon seepage in coastal areas, the recent Costa Concordia wreck at Giglio Island and the ecotoxicological effects of microplastics (Piva et al., 2011; Benedetti et al., 2012, 2014; Regoli et al., 2014; Avio et al., 2015; Bebianno et al., 2015). Briefly, depending on species and tissue, the model assigns to each biomarker a “weight” based on the relevance of biological endpoint, and a “threshold” for changes of biological significance which consider both inductions and/or inhibitions of various responses. For every analysed biomarker, the measured variation is compared to the threshold, then corrected for the weight of the response and the statistical significance of the difference compared to controls. Depending on the magnitude of the calculated effect, each biomarker response is assigned by the model to 1 of 5 classes of effect (from Absent to Severe); the calculation of the Hazard Quotient for biomarkers (HQ_{BM}) does not consider the contribution of responses with an effect lower or equal to threshold (Absent or Slight), calculates the average for those with an effect up to two-fold compared to the threshold (Moderate), and adds the summation (Σ) for the responses more than 2 fold greater than the respective threshold, i.e. Major or Severe (Piva et al., 2011):

$$HQ_{BM} = \left(\frac{\sum_{j=1}^{N} Effect_W(j)_{1<Effect(j)\leq 2}}{num\ biomark_{1<Effect(j)\leq 2}} + \sum_{k=1}^{N} Effect_W(k)_{Effect(j)>2} \right)$$

According to variations measured for various biomarkers, the model summarizes the level of cumulative HQ_{BM} in one of five classes of hazard for biomarkers, from Absent to Severe (Piva et al., 2011).

3. Results

Cd concentrations significantly increased in both digestive glands and in gills of Cd exposed scallops with values 3 and 5 folds higher than those measured in respective control groups (Fig. 1A, B). Co-occurring variations of other factors had negligible effects on Cd accumulation with slightly lower values of this element measured only in digestive glands of organisms co-exposed in acidic conditions (Ac + Cd): higher temperature did not cause additional effects on the uptake of this element in both the tissues (Fig. 1A, B).

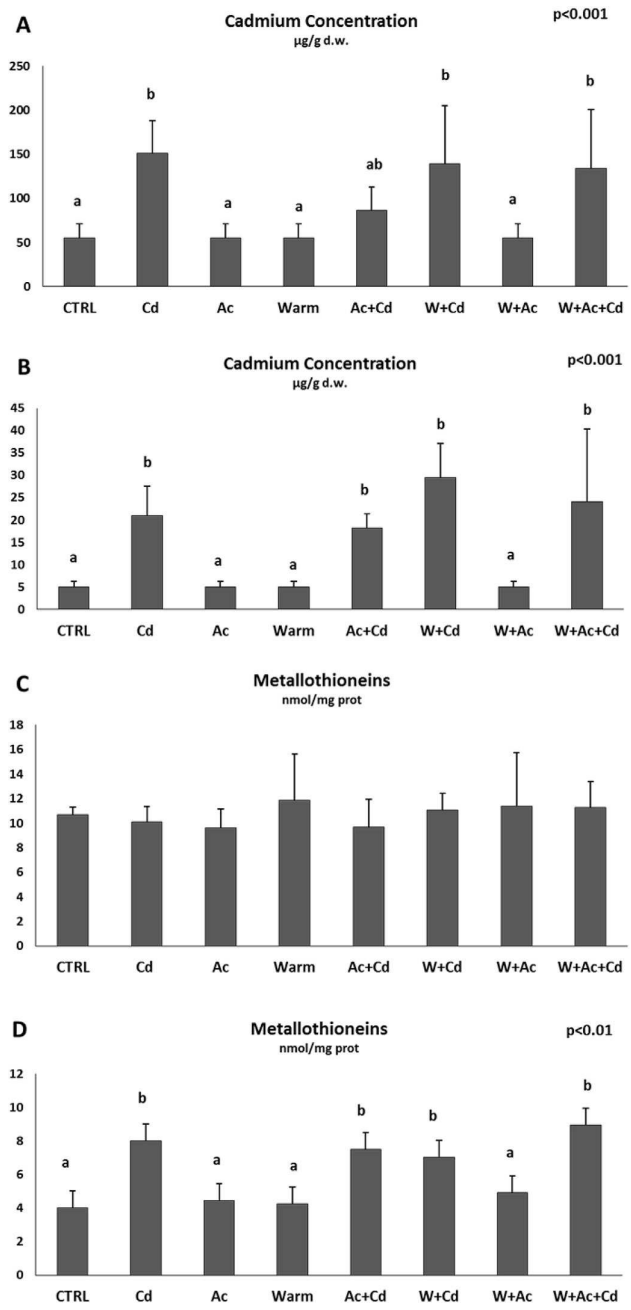


Fig. 1. Cadmium concentrations (A–B) and levels of metallothioneins (C–D) in digestive glands and gills of *A. colbecki* exposed to different experimental conditions. Letters indicate significant differences between groups of means. Data are given as mean values ± standard deviations, $n = 5$.

Levels of metallothioneins did not vary in digestive gland (Fig. 1C), while a significant increase was observed in gills after exposure to Cd, without any difference when the element was dosed alone or in combination with higher temperature and/or lower pH (Fig. 1D).

The antioxidant status was assessed by integrating analyses of individual antioxidants with the total capability to neutralize different forms of oxyradicals. In digestive gland, catalase showed a trend toward lowered enzymatic activities with significant effects after co-exposures between Cd and higher temperature, with or without acidified conditions (Fig. 2A). Increased temperature affected also the responses of GST, while no variations were observed for GR and GSH (Figs. 2B–D). Glutathione peroxidases, particularly Se-dependent forms, were slightly inhibited by co-exposures to higher temperature with Cd and/or acidification (Figs. 2E–F). Variations of individual antioxidants were not paralleled by any significant change in the TOSC values toward either ROO· or ·OH (Figs. 2G–H).

Antioxidant responses in gills exhibited lower values of catalase after exposure to Cd with increased temperature and acidification, while various combinations between Cd, acidification and/or temperature were often more effective than individual stressors in affecting responses of GST, GSH, Se-dependent GPx (Figs. 3A–F). Antioxidant variations in gills were reflected by a slight increase of TOSC toward ROO· and HO· in scallops exposed to higher temperature with or without Cd (Figs. 3G–H).

Among cellular biomarkers, malondialdehyde showed a general increase in digestive gland after treatment to multiple stressors, but no variations in gills (Figs. 4A–B). On the other hand, lipofuscin was increased by Cd, low pH and their combination, while it decreased after exposure to higher temperature with or without the other stressors (Fig. 4C). Frequency of micronuclei significantly increased in scallops exposed to all treatments with both individual and multiple stressors (Fig. 4D). Fluctuating changes of AOX were observed in digestive gland and gills with a few effects observed after exposure to treatments involving higher temperature and other stressors (Figs. 4E–F). Acetylcholinesterase was increased in digestive gland by Cd with or without acidification, while similar effects were induced in gills by higher temperature with or without Cd and acidification (Figs. 4G–H); the combination of the 3 stressors tended to reduce this enzymatic activity in both tissues.

The nMDS ordination of biomarker responses in a multidimensional space ($d = 2$) analysis is given in Fig. 5. In digestive tissues results indicated a separation between treatments involving co-exposures at higher temperature and those with Cd and/or acidification at −1 °C (environmental temperature) (Fig. 5A); in gills, a more evident difference was observed between organisms exposed to individual stressors compared to their multiple combinations (Fig. 5B). The elaboration of the overall biomarkers results through weighted criteria summarized in a single hazard index the biological significance of cellular responses observed in tissues of *A. colbecki* exposed to different experimental treatments (Fig. 6). In digestive gland, the elaborated Hazard Quotient was Moderate for scallops exposed to Cd alone and in combination with low pH and higher temperature, Slight for all the other treatments. On the other hand, gills appeared more sensitive to co-exposures between higher temperature and the other stressors (HQ Moderate), compared to individual stressors or their combination at −1 °C (HQ Slight).

4. Discussion

High-latitude marine ecosystems are ranked to be among the

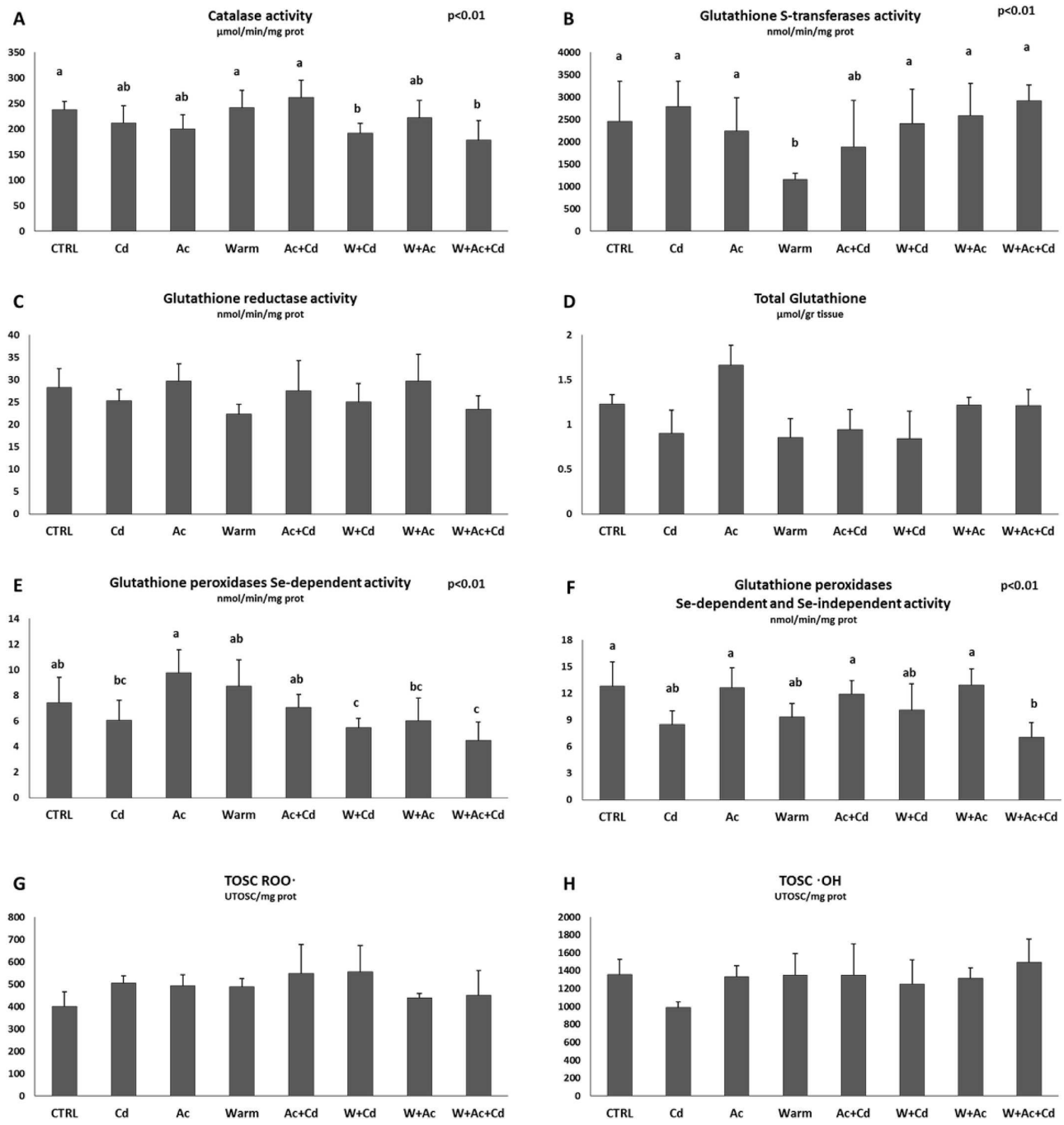


Fig. 2. Activities of individual antioxidant enzymes and Total Oxyradical Scavenging Capacity toward peroxyl and hydroxyl radicals in digestive gland of *A. colbecki* exposed to different experimental conditions. Letters indicate significant differences between groups of means. Data are given as mean values ± standard deviations, $n = 5$.

most sensitive regions to climate change since highly stenothermal and specially adapted organisms might be seriously affected by global warming and ocean acidification. The present investigation was aimed to provide new insights on the sensitivity to such environmental stressors in the key Antarctic species, *A. colbecki*, focussing also on their synergistic effects with Cd exposure. Cd accumulation is of special interest for marine organisms in the area of Terra Nova Bay due to a naturally elevated bioavailability of this metal enriched in water column by up-welling currents: during phytoplanktonic bloom, when the algae represent an important trophic source, Cd is transferred to both benthic species and pelagic food webs. As a consequence of this local peculiarity, tissue concentrations of Cd in organisms from Terra Nova Bay are up to 20 fold higher than those normally measured in similar temperate species (Nigro et al., 1997). The elevated Cd content in tissues, despite not directly toxic, was shown to influence metabolism of organic

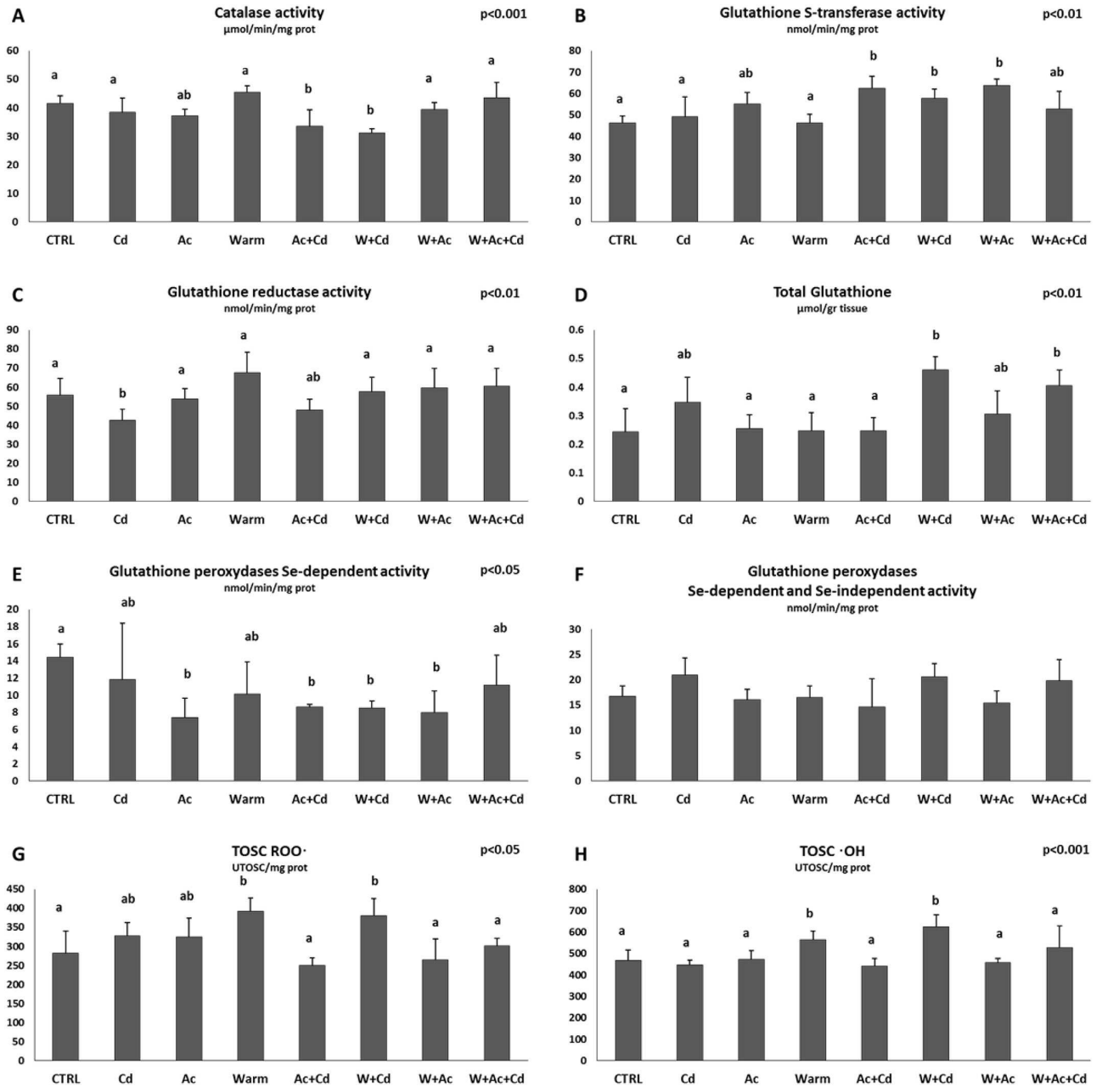


Fig. 3. Activities of individual antioxidant enzymes and Total Oxyradical Scavenging Capacity toward peroxyl and hydroxyl radicals in gills of *A. colbecki* exposed to different experimental conditions. Letters indicate significant differences between groups of means. Data are given as mean values ± standard deviations, $n = 5$.

xenobiotics (Regoli et al., 2005; Benedetti et al., 2007), and probably interfere with endocrine receptor (ER) and expression of vitellogenin (VTG) in males of fish *Trematomus bernacchii* (Canapa et al., 2007). In this respect, it was of interest to explore whether a similar peculiarity of Antarctic organisms might have reciprocal interactions with their sensitivity to the effects of climate change.

This study confirmed elevated basal concentrations of Cd in tissues of *A. colbecki*, but also the capability of this scallop to further accumulate the metal in response to enhanced bioavailability. Surprisingly, the Cd accumulation in both digestive gland and in gills was not affected by either temperature increase or acidification which were expected to influence metabolism and chemical availability respectively. In this respect, previous studies revealed that temperature increased metal uptake in some temperate and Arctic organisms (Baines et al., 2006; Sokolova and Lannig, 2008), while other investigations on temperate mussels (*Mytilus galloprovincialis*) did not support a similar modulation (Izagirre et al., 2014). Regarding acidification, PCO_2 was shown to enhance Cd and Cu accumulation in oysters and clams, but the rather subtle and tissue-specific variations suggested that CO_2-dependent effects on the metal uptake of marine bivalves are complex and not predictable from the chemical models of the metal

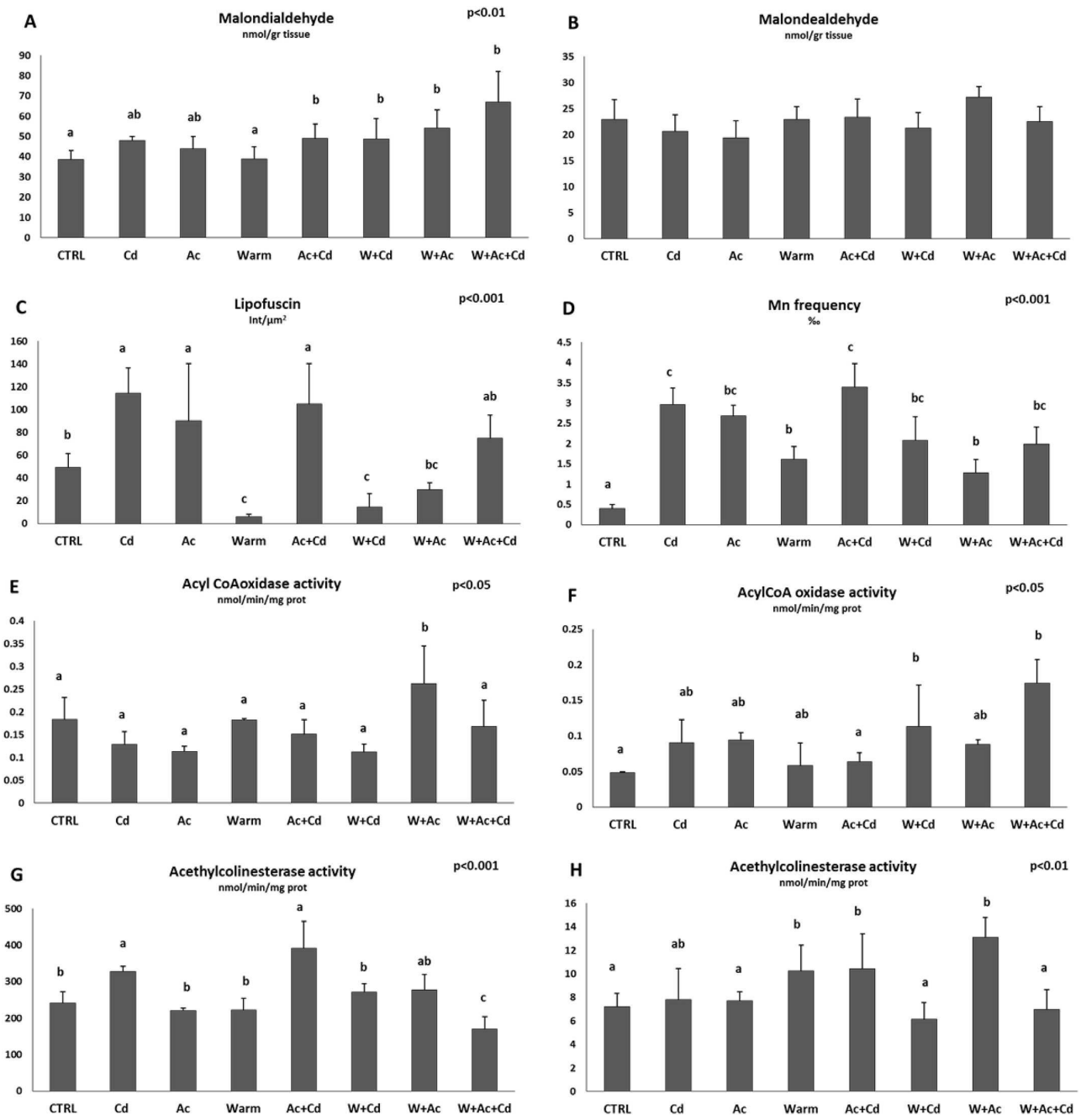


Fig. 4. Levels of malondialdehyde, lipofuscin, micronuclei, acyl CoA oxidase and acetylcholinesterase in tissues of *A. colbecki* exposed to different experimental conditions. Panels A, C, E: digestive gland; B, F: gills; D,G: haemolymph. Letters indicate significant differences between groups of means. Data are given as mean values ± standard deviations, $n = 5$.

speciation in seawater (Rodriguez-Romero et al., 2014; Ivanina et al., 2015).

The increase of Cd concentration in tissues of *A. colbecki* was not reflected by variations of metallothioneins in digestive gland of exposed scallops, while levels of these proteins increased in gills. The lack of response in digestive tissues can be related to the elevated basal levels of metallothioneins, reflecting an adaptation mechanism to high natural concentrations of Cd in these Antarctic organisms. Despite we did not analyse subcellular distribution of Cd in exposed scallops, previous studies and metallothioneins characteristics suggest this element as mostly present in a soluble form (Regoli et al., 1997, 2002): this hypothesis is also supported by the typical amino acid composition of these proteins in *A. colbecki* that confer a preferential binding capacity for Cd (Ponzano et al., 2001). High levels of metallothioneins have also been shown to provide protection against oxidative stress through reaction of sulfhydryl groups with reactive oxygen species, thus further representing an important defence in polar marine species toward the high environmental prooxidant pressure.

Oxyradical metabolism is of great importance for Antarctic

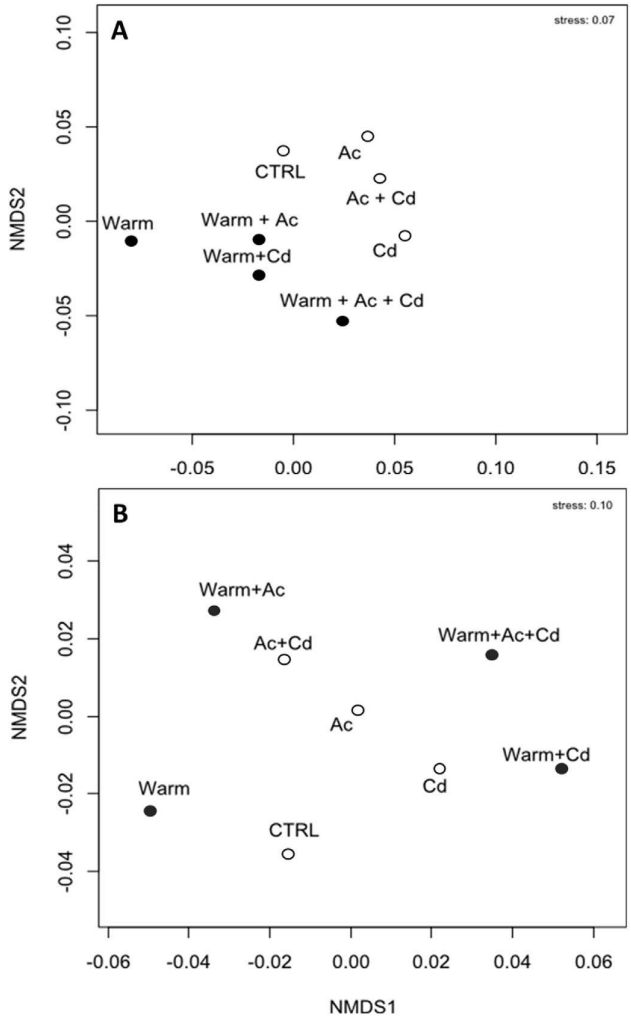


Fig. 5. Non-metric multidimensional scaling analysis (nMDS) ordination plots of biological responses in digestive glands (A) and in gills (B) of *A. colbecki* exposed to different experimental conditions. White points indicate samples exposed to environmental temperature (−1 °C) and dark points indicate samples exposed to warm conditions (+1 °C).

organisms and in our study, a wide panel of antioxidant defences was integrated with measurement of Total Oxyradical Scavenging Capacity (TOSC) towards hydroxyl and peroxyl radicals. Despite the limited effects on Cd bioaccumulation, exposure to multiple stressors appeared to modulate oxidative responsiveness to this element. In digestive gland, co-exposures to Cd and higher temperature caused a significant decrease of catalase and glutathione peroxidases, confirming the sensitivity of these enzymes in revealing a prooxidant pressure, and the importance of H_2O_2 metabolism as a possible driver (Regoli and Giuliani, 2014). Similar effects, not compensated by a varied capability to neutralize bot ROO· and HO·, resulted in a slight increase of peroxidative processes and consequent enhancement of malondialdehyde content: in this respect, the lowered values of lipofuscin after treatments involving higher temperature might be the consequence of enhanced excretion processes of oxidized membranes through tertiary lysosomes. Acidification caused generally slighter oxidative effects in digestive gland, and only when acting as co-stressor with higher temperature and/or Cd exposure.

Sensitivity to oxidative stress was highlighted also in gills where Se-dependent glutathione peroxidases confirmed decreased values in response to multiple combinations of Cd, temperature and acidification. Glutathione S-transferases and levels of GSH were upregulated in these tissues, modulating the responsiveness to Cd in combination with temperature and/or acidification. GSH can act as a direct scavenger toward H_2O_2 and, as cofactor of GST, contributes to removal of peroxidative products on damaged membranes: in this respect, no malondialdehyde accumulation was observed in gills which also exhibited a certain enhancement of the overall capability to neutralize both peroxyl and hydroxyl radicals, particularly in response to higher temperature and Cd. The different responsiveness of digestive gland and gills to investigated multiple stressors confirm that variations of antioxidants to environmental stressors can not be generalized, responding to specific signals, interactions, and pathways that differ in various tissues or exposure conditions (Regoli et al., 2011). Also for antioxidants, the more limited fluctuations observed in digestive gland compared to gills are probably related to the higher basal levels of such defences. The elevated protection in digestive tissues was shown as an important strategy of this scallop to cope with fluctuations of prooxidant pressure in the Antarctic marine environment characterized by low temperature and high levels of dissolved oxygen, marked seasonality in photochemical activation of dissolved organic matter and food availability (Viarengo et al., 1995; Regoli et al., 1997, 2000; 2002).

Overall, our results confirm previous evidences that elevated temperature and acidification may represent prooxidant stressors, as a common consequence of the metabolic and acid–base disturbance in animals (Matoo et al., 2013). Elevated temperature was shown to cause oxidative stress in marine molluscs also through a mismatch between generation and detoxification of reactive oxygen species (ROS) (Abele et al., 2001). On the other side, acidification was responsible for significant changes in gene expression and activity of antioxidant defences in the Arctic spider crab *Hyas araneus* and in the Eastern oyster, *Crassostrea virginica* (Tomanek et al., 2011; Harms et al., 2014). Different mechanisms have been suggested for prooxidant effects of acidification, including the reaction of CO_2 with peroxinitrite and formation of reactive carbonate and nitrogen species with elevated oxidizing potential (Dean, 2010). Elevated CO_2 and/or low environmental pH can also have indirect effects since molluscs have a limited capability for pH regulation, and sea water acidification would thus determine intracellular acidosis (Tomanek et al., 2011). Such intracellular conditions may negatively affect the efficiency of mitochondria by increasing the electron slip in ROS-generating mitochondrial complex I and II, and/or by partially inhibiting the flow through the downstream electron transport chain complex: in either case, these disturbances of the electron transport chain would result in elevated rates of ROS generation. Further, intracellular acidosis can cause the release of chelated transition metals such as Fe^{2+} from intracellular store, thus favouring the onset of oxidative stress through Fenton reactions and generation of hydroxyl radicals (Tomanek et al., 2011).

The responsiveness of *A. colbecki* to combinations of various stressors was confirmed by the enhanced levels of micronuclei observed after all treatments to individual and multiple stressors: considering the effects observed on antioxidants, the higher frequency of MN might confirm a certain unbalance of oxyradical metabolism but also suggest a modulation of cellular turnover: in this respect, beside an oxidative damage on DNA, micronuclei formation would be, at least partially, favoured by a higher mitotic rate. More limited variations occurred for Acyl CoA oxidase, indicating that higher temperature, in combination with Cd and/or acidification, may represent the primary stressor affecting β-

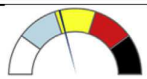
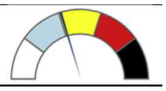
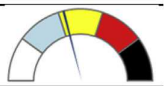
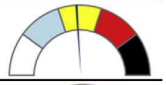
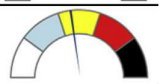
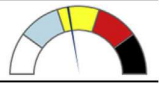
Tissues	Experimental groups	Hazard Quotients (HQ)	Class of Hazard	Level
Digestive glands	Cd	8.9	Moderate	
Digestive glands	Ac	7.99	Slight	
Digestive glands	W	5.92	Slight	
Digestive glands	Ac+Cd	8.62	Moderate	
Digestive glands	W+Cd	6.13	Moderate	
Digestive glands	W+Ac	1.9	Slight	
Digestive glands	W+Ac+Cd	5.5	Moderate	
Gills	Cd	3.53	Slight	
Gills	Ac	5.75	Slight	
Gills	w	2.35	Slight	
Gills	Ac+Cd	1.61	Slight	
Gills	W+Cd	10.58	Moderate	
Gills	W+Ac	9.03	Moderate	
Gills	W+Ac+Cd	13.15	Moderate	

Fig. 6. Weight of Evidence (WOE) classification of biomarkers data, in scallops exposed to different laboratory conditions. The quantitative Hazard Quotients (HQ) and the assigned classes of hazard are given.

oxidation of fatty acids and lipid metabolism. Finally, acetylcholinesterase was particularly modulated by Cd exposures with or without other stressors, confirming the neurotoxic potential of this element (Del Pino et al., 2014). Reported effects of Cd include block of cholinergic transmission by decrease of acetylcholine synthesis and release, inhibition of AChE activity and of postsynaptic transmission, blockage of cholinergic receptors (Del Pino et al., 2014).

When all the complex variations on biomarkers effects were elaborated through multivariate analysis or the quantitative hazard model (Sediqualsoft) interesting trends could be summarized. The multivariate scaling analysis (nMDS) revealed that temperature influence the pattern of biological responses in digestive gland where the analysis tended to separate cold from warm exposed organisms. On the other hand, considering both the biological relevance and the magnitude of observed responses, the overall quantitative effects were always higher for organisms exposed to Cd, alone and in combination with other factors: after these treatments, the elaborated HQ was assigned as Moderate, compared to Slight in organisms exposed to higher temperature, acidification or their combination. This result would further confirm that the adaptation to the high basal content of Cd, which involves specific cellular strategies like elevated antioxidant defences, makes these tissues more tolerant toward other potentially prooxidant stressors like temperature or acidification: at the same

time, however, such delicate homeostatic equilibrium, appears overwhelmed when cellular concentrations of the metal increase, not being further exacerbated by contemporary variations of temperature and pH. A different trend of responsiveness was observed in gills which, according to both nMDS and hazard model, generally appeared more sensitive to various combinations of stressors occurring at higher temperature.

In conclusion, this study provided new insights on the reciprocal, synergistic and modulatory effects of ocean warming, acidification and metals bioaccumulation in a key Antarctic invertebrate. Despite the elevated basal levels of antioxidants as adaptive mechanism to the high environmental prooxidant pressure, *A. colbecki* was sensitive to interactions between climate change and Cd bioavailability, with different sensitivity among analysed tissues toward different factors. Further studies are needed to better understand long term effects, as the onset of earlier biological responses at transcriptional levels or the link with physiological status of the organisms, as well as the different sensitivity of polar organisms in comparison with similar temperate species.

Acknowledgments:

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

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HIGHLIGHTS

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

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ABSTRACT

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

1. Introduction

Since the industrial revolution, human activities have caused a

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

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

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

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

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

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

2. Materials and methods

2.1. Animal collection and experimental design

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

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

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

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

2.2. Cadmium determination

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

2.3. Biomarkers responses

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

2.4. Statistical analyses

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

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

3. Results

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

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

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

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

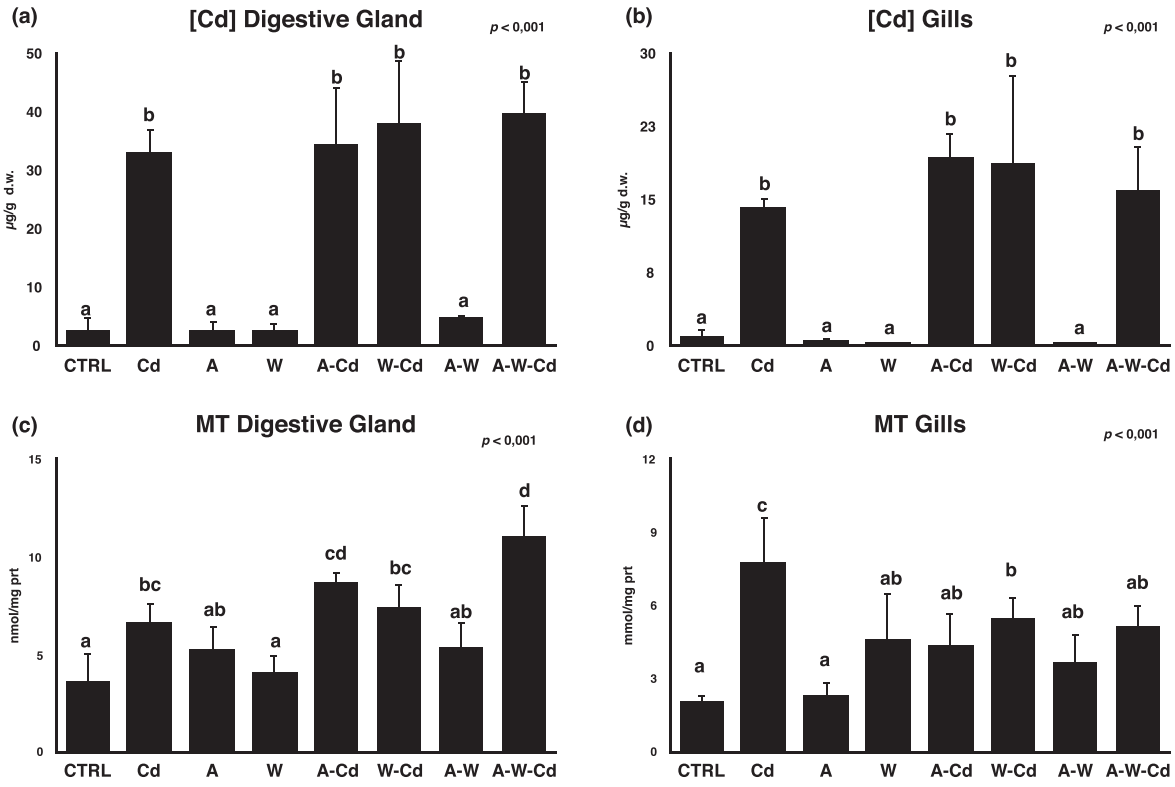


Fig. 1. Cadmium concentrations and level of metallothioneins in digestive gland (a and c) and gills (b and d) of mussels exposed to various treatments. Data are given as mean values ± standard deviations (n = 5). Different letters indicate significant differences between group of means (ANOVA and SNK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

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

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

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

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

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

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

4. Discussions

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

Although ocean acidification and warming have been suggested

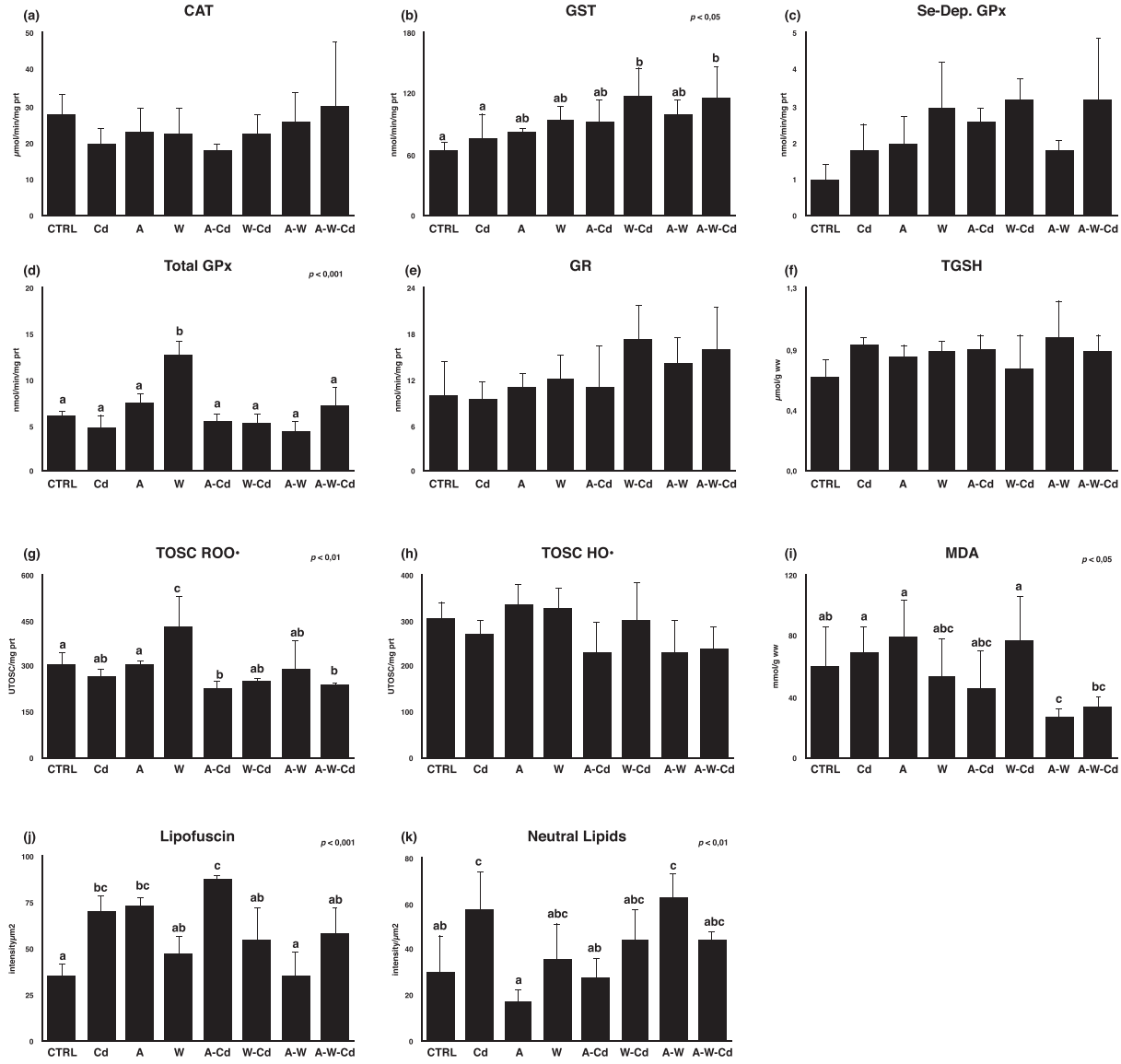


Fig. 2. Antioxidant defenses and oxidative stress biomarkers in digestive gland of mussels exposed to various treatments. CAT: catalase (a), GST: glutathione S-transferase (b), Se-Dep. GPx: Se-dependent glutathione peroxidases (c) total GPx: sum of Se-dependent and Se-independent glutathione peroxidases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

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

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

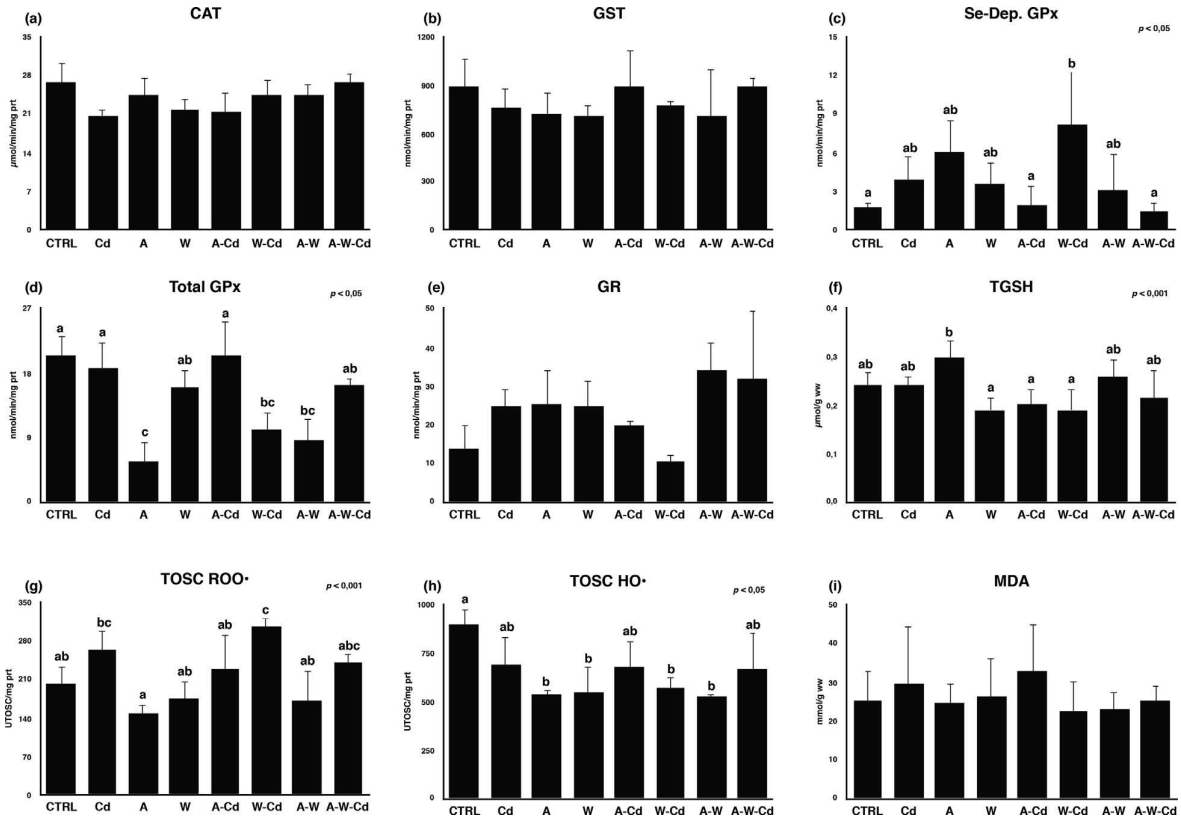


Fig. 3. Antioxidant defenses and oxidative stress biomarkers in gills of mussels exposed to various treatments. CAT: catalase (a), GST: glutathione S-transferase (b), Se-Dep. GPx: Se-dependent glutathione peroxidases (c) total GPx: sum of Se-dependent and Se-independent glutathione peroxidases (d), GR: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

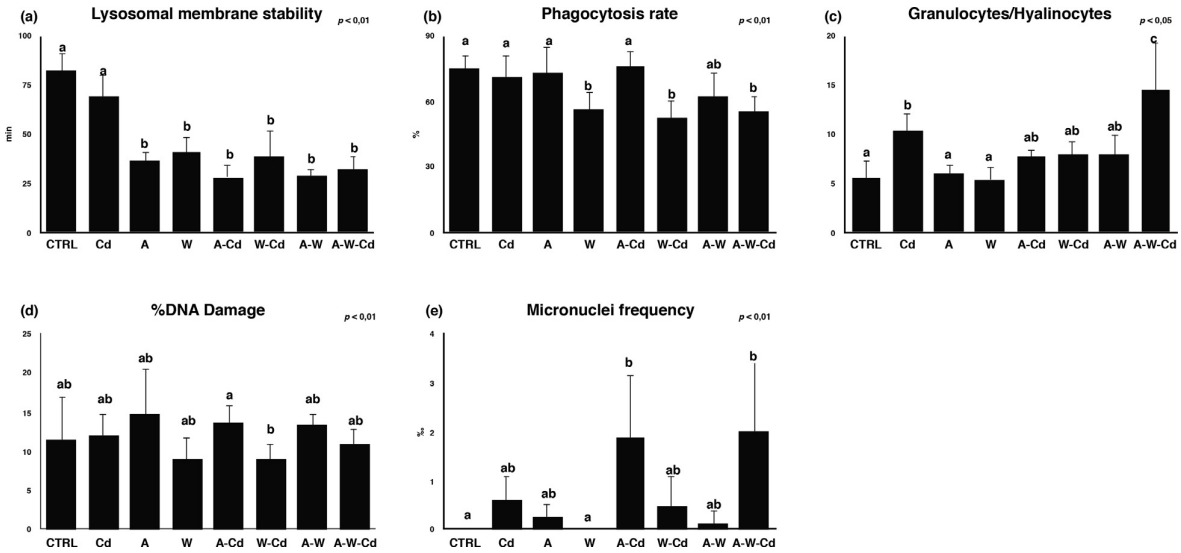


Fig. 4. Lysosomal membrane stability (a), phagocytosis rate (b), granulocytes/hyalinocytes ratio (c), DNA damage (d) and frequency of micronuclei (e) in haemocytes of mussels exposed to various treatments. Data are given as mean values ± standard deviations (n = 5). Different letters indicate significant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

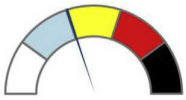
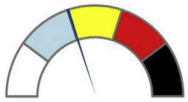
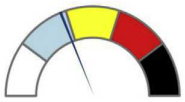
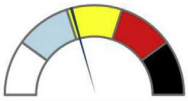
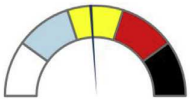
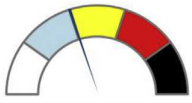
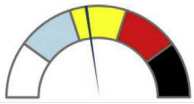
Experimental Treatment	Hazard Quotient (HQ)	Class of Hazard	Level
Cd	12.52	SLIGHT	
A	10.03	SLIGHT	
W	6.59	SLIGHT	
A-Cd	14.87	MODERATE	
W-Cd	19.34	MODERATE	
A-W	11.74	SLIGHT	
A-W-Cd	17.40	MODERATE	

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

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

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

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

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

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

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

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

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

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

Appendix A. Supplementary data

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

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III

Seasonal effects of ocean warming and acidification on cadmium accumulation and responsiveness of mussels *Mytilus galloprovincialis*

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Abstract

Ocean warming and acidification could represent additional threat to marine organisms that are already coping with other stressors, as anthropogenic pollution in coastal areas. Despite the growing interest on the interactions of these factors and their effects in terms of metals bioaccumulation and biological effects in marine organisms, clear mechanisms of action are still poorly understood due to tissue- and species-specific sensitivity. Here we investigated cellular pathways of metal detoxification and oxidative stress in the Mediterranean mussel *Mytilus galloprovincialis* 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), chosen as model inorganic pollutant. Cadmium concentration was higher in metal-exposed organisms and further enhanced by in those exposed at higher temperature. Despite this, interactive effects of temperature and/or pH affected Cd-mediated metallothioneins induction, responsiveness of antioxidant system and onset of oxidative damages to lipids, with tissue-specific effects. Metallothioneins were sensitive to interactive effects of multiple stressors mainly in the digestive gland, which exhibited also higher lipid peroxidation effects due to acidification and cadmium exposure compared to the gills, in which the higher sensitivity of single antioxidants counteracted the onset of lipid peroxidation processes. Immunitary parameters measured in haemocytes showed generalized sensitivity of lysosomal membranes stability to the investigated stressors with major effects in co-exposed organisms, while temperature significantly reduced phagocytosis efficiency and caused changes in haemocytes populations ratio. Genotoxics effect measurement showed sensitivity to Cd exposure. The elaboration of the whole biomarkers dataset showed increased sensitivity in organisms exposed to cadmium and higher temperature. The overall results confirmed the modulatory effects that climate changes can have on metals bioaccumulation and biological responsiveness, highlighting tissue-specific sensitivity and the needing of improving the knowledge on multiple stressors interaction.

1.Introduction

Ocean warming and acidification due to increasing atmospheric concentration of carbon dioxide are posing a serious threat to marine ecosystems integrity. Since the pre-industrial values, ocean pH has dropped by 0.1 units (Gattuso and Lavigne, 2009) and global mean temperature has risen by almost 1°C (Hansen *et al*., 2016); projections of future emissions scenarios assess further decrease of pH up to 7.8 by 2100 and temperature rise beyond 2°C (IPCC, 2013).

Beside direct effects of these changes on marine organisms' health status, there's growing concern on multiple stressors interactions that can occur in marine ecosystems (Crain *et al*., 2008; Burton and Johnston, 2010; Kroeker *et al*., 2013); among these, metal contamination is one of the most vexing stressor in coastal areas, where anthropogenic footprint is higher than open ocean (Doney, 2010; Bijima *et al*., 2013; Gilarranz *et al*., 2016).

Both ocean warming and acidification have been assessed as factors potentially influencing heavy metals fate in coastal ecosystems, affecting their distribution between sediment and water and their bioaccumulation in marine organisms. Temperature elevation may alter solubility of metallic compounds thus increasing metals bioavailability to marine organisms (Sokolova and Lannig, 2008). On the other hand, higher temperature may lead to increased ventilation and/or feeding due to energy demand and higher metals uptake after temperature elevation is widely reported (Baines *et al*., 2006; Cherkasov *et al*., 2007; Mubiana and Blust, 2007; Negri *et al*., 2013). On the other hand, lower availability of hydroxide and carbonate ions due to CO_2-enrichment in seawater may alter the speciation and solubility of metals that form strong complexes with these ligands (Millero *et al*., 2009; Hoffmann *et al*., 2012; Stockdale *et al*., 2016), but ocean acidification could also affect those metals that form complexes with organic ligands (Gledhill *et al*., 2015). Increased fluxes of metals from sediments to water have been showed to occur in reduced pH seawater and simulation of CO_2 leakages from sub-seabed (Ardelan *et al*., 2009; de Orte *et al*., 2014a, 2014b; Basallote *et al*., 2015). Nevertheless higher metals uptake due to acidification has been described in the tissues of bivalves *Ruditapes philippinarum*, *Crassostrea virginica*, *Mercenaria mercenaria*, *Mytilus edulis*, *Tegillarca*

75 *granosa*, *Meretrix meretrix* (López *et al*., 2010; Ivanina *et al*., 2014; Götze *et al*., 2014; Rodriguez-
76 Romero *et al*., 2014a; Shi *et al*., 2016), polychaete *Hediste diversicolor* (Rodriguez-Romero *et al*.,
77 2014b), eggs and embryos of the squid *Loligo vulgaris* and of the cuttlefish *Sepia officinalis* (Lacoue-
78 Labarthe *et al*., 2009, 2011).

79 Variation of environmental factors as temperature and pH/pCO_2 are also suggested to affect
80 biological responsiveness to metal exposure and their toxicity. The interaction between temperature
81 elevation and metal exposure it has been demonstrated to cause impairment of mitochondrial
82 function, pro-oxidative mechanisms, accumulation of lipid peroxidation products and damages to
83 lysosomes and DNA (Cherkasov *et al*., 2007; Mubiana and Blust, 2007; Sokolova & Lannig, 2008;
84 Negri *et al*., 2013; Attig *et al*., 2014; Banni *et al*., 2014; Gomiero and Viarengo, 2014; Izagirre *et al*.,
85 2014; Múgica *et al*., 2015); similarly, interactive effects between metals and ocean acidification have
86 been addressed in impaired larval development, onset of DNA damage, pro-oxidative mechanisms
87 and lower antioxidant efficiency and alteration of the immune function (Lewis *et al*., 2016; Campbell
88 *et al*., 2014; Ivanina *et al*., 2015, 2016; Siddiqui and Bielmyer-Fraser, 2015).

89 Recently, cadmium accumulation in the digestive gland and gills of *M. galloprovincialis* and *A.*
90 *colbecki* was shown to be unaffected by variations of temperature and pH, while biological responses
91 to Cd were strongly modulated by these variables, with different effects and magnitude in different
92 tissues (Benedetti *et al*., 2016; Nardi *et al*., 2017). Beside tissue-mediated specificity, seasonality may
93 represent an additional factor modulating the capability of organisms to tolerate variations of
94 environmental variables, since several biological responses in different marine invertebrates have
95 been demonstrated to exhibit seasonal fluctuations (Ringwood *et al*., 2002; Bocchetti and Regoli,
96 2006; Farcy *et al*., 2007, Múgica *et al*., 2015).

97 In this respect, considering the complexity of the effects of such interactions, this study aimed to
98 further investigate the effects of temperature and pH variations on Cd accumulation and biological
99 effects using a wide biomarkers battery, which reflected the complex network of cellular mechanisms
100 underlying metal toxicity, from detoxification to oxyradical metabolism (Regoli and Giuliani, 2014).

101 These biomarkers included induction of metallothioneins, variation of single antioxidant defenses,
102 total oxyradical scavenging capacity and accumulation of lipid peroxidation products, evaluated in
103 both digestive gland and gills, in order to evaluate tissue specific sensitivity. In addition,
104 immunological and genotoxicity analysis were conducted on mussels haemocytes. The overall
105 biomarkers dataset has been elaborated through a quantitative hazard model (Sediqualsoft) which
106 elaborates synthetic cellular hazards index based on the toxicological importance of analyzed
107 parameters and on the magnitude of observed variations (Piva et al., 2011; Benedetti et al., 2012).
108 The obtained results are intended to contribute to the growing body of knowledge on multiple
109 stressors interactions, and to highlight possible differences due to seasonal physiological features by
110 comparing obtained results with previously published research of our team on *M. galloprovincialis*
111 exposed to the same experimental plan in summer.
112

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 acclimatized 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 to one of the following treatments, each containing 36 organisms in 20 L: 1) control condition (CTRL), at environmental temperature of 10°C, normocapnia with pH=8.20/ pCO_2=~400 μatm; 2) cadmium exposure (Cd), 10°C, pH=8.20/ pCO_2=~400 μatm and 20 μg/L cadmium; 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 cadmium; 6) warming + Cd (W-Cd), 15°C, pH=8.20/ pCO_2=~400 μatm and 20 μg/L cadmium; 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 cadmium. Cadmium dose was chosen as representative of a polluted but environmentally realistic scenario in Mediterranean coastal waters (Neff, 2002), while selected target pH was adapted from scenario RCP 8.5 and IPCC WGII AR5 (IPCC, 2014; Wong *et al*., 2014) reporting a mean pH value of 7.7 for open oceans, but predicting more pronounced variations of pH/pCO_2 in coastal areas. Reduced pH was reached by mixing ASW (pH=8.2) with small amounts of CO_2-saturated ASW, obtained by bubbling pure CO_2 in ASW for at least 24h (Nardi *et al*., 2017). pH and salinity were measured daily, while total alkalinity (A_T) was measured twice per week according to Dickson *et al*., 2007, and used for calculating seawater carbonate parameters (pCO_2, and saturation state (Ω) for calcite and aragonite) in CO2SYS (Pierrott *et al*., 2006) using barometric pressure values (full seawater chemistry is provided in Table 1). For calculations, we used NBS scale for seawater pH, constants from Millero, 2010, KSO_4^- constant from Dickson *et al*., 2007, and concentration for silicate and phosphate for Instant Ocean® seawater (0.21 μmol/kg and 0.05 μmol/kg, respectively).

139 Water was changed every other day, and mussels fed 12 hours prior the water change with a
140 commercial mixture of zooplankton (50-300 μm) for filter-feeding organisms.
141 After four weeks, animals were sampled from each tank and tissues collected for chemical and
142 biological analyses. Gills and digestive glands were excised, pooled in 12 samples, each constituted
143 by tissues of 3 individuals, rapidly frozen in liquid nitrogen and maintained at -80°C: these samples
144 were shared for analyses of cadmium content or biomarker responses, to guarantee a n value = 5 for
145 each measurement. Haemolymph was withdrawn from the adductor muscle of 5 specimens and
146 immediately used for immunity parameters and measurement of genotoxic damage.
147 *2.2 Cadmium determination*
148 Cadmium (Cd) concentration in digestive gland and gills of mussels was analyzed according to
149 previously described methods (Regoli *et al*., 2005). For each treatment, tissues were dried at 60°C
150 overnight, digested in a microwave system (Mars V, CEM) and analyzed by atomic absorption
151 spectrophotometry with graphite furnace atomization and Zeeman effect. Quality assurance and
152 quality control was assessed by processing blank samples and reference standard material (Mussel
153 Tissue Standard Reference Material SRM NIST-2977, National Institute of Standards and
154 Technology Gaithersburg, MD, USA), which always resulted within the 95% confidence interval of
155 certified values. Data are expressed as μg/g dry weight (mean values ± standard error, n = 5).
156 *2.3 Biomarkers responses*
157 Biomarkers in mussels' tissues were analyzed through standardized methods which are
158 detailed in Supplementary Material 1, SM1. Metallothioneins (MTs), single antioxidant defenses
159 (catalase, glutathione S-transferases, glutathione peroxidases, glutathione reductase, total
160 glutathione), total oxyradical scavenging capacity toward peroxyl radicals (TOSC ROO•) and
161 hydroxyl radicals (TOSC HO•), malondialdehyde content (MDA) were evaluated in both digestive
162 gland and gills of exposed mussels; cryostat sections of digestive glands were further analyzed for
163 lipofuscin and neutral lipids content. Immunological alterations in haemocytes were evaluated in
164 terms of lysosomal membrane stability by neutral red retention time (NRRT), phagocytosis activity

and granolocytes versus hyalinocytes ratio; onset of genotoxic effects in haemocytes were assesed in terms of DNA strand breaks (Comet assay) and micronuclei frequency (MN).

2.7 Statistical analyses

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

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

3. Results

Cadmium exposure led to significant increase of Cd concentration in both digestive gland and gills of exposed mussels (Fig.1a, b). Organisms exposed to the metal at higher temperature (W-Cd and A-W-Cd) showed significantly higher accumulation of Cd than organisms exposed to the metal at control temperature (Cd and A-Cd), in both digestive gland and gills (Fig.1a, b).

Metallothioneins in the digestive gland were significantly induced by cadmium only in organisms exposed to reduced pH and/or higher temperature condition (A-Cd, W-Cd and A-W-Cd (Fig.1c). In

191 the gills, significant increase of metallothioneins was measured only in organisms exposed to Cd at
192 higher temperature and control pH (W-Cd) (Fig.1d).
193 Antioxidant defenses showed clear involvement of certain pathways with tissue-specific effects
194 of the investigated factors. In the digestive gland, an inductive effect of cadmium on the activity of
195 total GPx was observed only at control pH (CTRL vs. Cd and W vs. W-Cd), while at reduced pH
196 cadmium effect was less evident (A vs. A-Cd and A-W vs. A-W-Cd) (Fig. 2d). In addition, while
197 acidification alone significantly increase total GPx activity (A), when this was combined with higher
198 temperature (A-W) no induction of enzyme activity was measured (Fig. 2d). Total oxyradical
199 scavenging capacity toward peroxyl radical (TOSC ROO•, Fig. 2g) increased significantly in
200 organisms exposed to Cd at higher temperature and lower pH (A-W-Cd), but slight although not
201 significant increase of this parameter was measured also in organisms exposed to Cd alone and at
202 lower pH (Cd and A-Cd). Limited increase in malondialdehyde levels resulted due to Cd exposure in
203 organisms exposed to higher temperature at control pH compare to their respective control at higher
204 temperature (W-Cd vs. W) (MDA, Fig. 2i); accumulation of lipofuscin was significantly increased
205 by co-exposure to Cd, acidification and higher temperature (A-W-Cd, Fig. 2j), while neutral lipids
206 content was significantly reduced in all Cd-exposed organisms at reduced pH and/or higher
207 temperature (A-Cd, W-Cd and A-W-Cd) compared to organisms exposed to Cd alone (Fig. 2k).
208 Significant variations of GST and GPxs, both selenium-dependent and total, were observed in
209 the gills; glutathione S-transferase activity was significantly inhibited in organisms exposed to Cd
210 alone, Cd at higher temperature (W-Cd) and higher temperature at reduced pH (A-W) (Fig. 3b).
211 Cadmium effect on Se-dep. GPx activity was opposite at control al higher temperature: at control
212 temperature Cd exposure led to induction of the enzyme activity (Cd vs. CTRL and A-Cd vs. A),
213 while inhibited activity in organisms at higher temperature (W-Cd vs. W not significant, A-W-Cd vs.
214 A-W) (Fig. 3c). Significant variations were also observed for total GPx activity, which was reduced
215 in organisms exposed to Cd, acidification and higher temperature compared to organisms exposed to
216 Cd and higher temperature at control pH (A-W-Cd vs. W-Cd) (Fig. 3d). While no variations were

217 measured in the total oxyradical scavenging capacity toward peroxyl radical (TOSC ROO•) (Fig. 3g),
218 limited effects on toward hydroxyl radical (TOSC HO•) were measured in terms of higher values in
219 organisms exposed to higher temperature (W) (Fig. 3h). No variations of malondialdehyde content
220 were measured (Fig. 3i).

221 Lysosomal membrane stability in haemocytes significantly decreased in all experimental
222 treatments, with major effects in organisms exposed to Cd, acidification and reduced pH (A-W-Cd)
223 (Fig. 4a). Phagocytosis rate (Fig.4b) was reduced in all treatments at higher temperature (alone or in
224 combination with other stressors), while granulocytes vs. hyalinocytes type cells ratio (Fig. 4c) was
225 significantly reduced in organism exposed to Cd and higher temperature (W-Cd and A-W-Cd).
226 Micronuclei frequency (Fig.4e) was enhanced in all treatments with Cd with the exception of
227 organisms exposed to Cd and reduced pH (A-Cd), while DNA fragmentation…

228 Biomarkers responses observed in each experimental condition were summarized in a single
229 hazard index through the application of weighted criteria (Fig.5). The elaborated class of hazard was
230 "Slight" for organisms exposed to acidification without cadmium (A and A-W), while was
231 "Moderate" for all other treatments.

4. Discussion

This study provided clear evidences that climate changes effects can affect metal accumulation and responsiveness in marine organisms, representing additional threat to ecosystems integrity.

Our study showed that Cd accumulation in mussels tissues can be affected by the increase of temperature. This is in contrast with previously published studies (Izagirre *et al*., 2014; Nardi *et al*., 2017), in which temperature elevation did not affect cadmium concentration in *Mytilus galloprovincialis* tissues; this may be due to acceleration of metabolism: the temperature range used in this study is within the thermal optimum of mussels, and thus allows increase of metabolic increase at increasing temperature (Pörtner, 2010), with implications in differential sensitivity to the investigated factors in different seasons. On the other hand, metal uptake did not result affected by reduction of pH, confirming the low influence of these changes on Cd bioavailability and bioaccumulation obtained in other studies of our group on *M. galloprovincialis* and *A. colbecki* (Benedetti *et al*., 2016; Nardi *et al*., 2017).

Metallothioneins are known to be induced as cellular metal concentration increase. In this study, anyhow, MTs levels increased in the digestive gland only when Cd was dosed in association to acidification and/or warming, and in the gills only in organisms co-exposed to Cd and warming. This confirms previously reported hypothesis that MTs synthesis can be further influenced by interaction of multiple stressors (Viarengo, 2000; Regoli and Giuliani, 2014), and not only controlled by cadmium accumulation, in accordance with what observed in *M. galloprovincialis* exposed in summer with slight differences (Nardi *et al*., 2017).

The oxidative challenge exerted by the investigated stressors was assessed through the analysis of a wide battery of biomarkers, which included single antioxidant defenses, total oxyradical scavenging capacity and the measurement of oxidative damages. In the digestive gland, pH reduction masked the effects of Cd on the activity of total GPx: this was particularly evident in organisms exposed at reduced pH and higher temperature, suggesting the modulation of enzyme activity in response to oxidative pressure exerted by hypercapnic condition (Tomanek *et al*., 2011). This is in

contrast with increased total oxyradical scavenging capacity toward peroxyl radical in organisms exposed to Cd at reduced pH and higher temperature, but may suggest compensation of lowered efficiency through the involvement of other antioxidant pathways (Regoli and Giuliani, 2014). In addition to this, despite no variations of malondialdehyde were observed, the increased lipofuscin content in these organisms further confirms this hypothesis of increased oxidative challenge due to the co-exposure to Cd and acidification, similarly to the results obtained by our previously published research conducted in summer (Nardi *et al*., 2017). Neutral lipids content in *M. galloprovincialis* was shown to be decreased after short-term exposure to heavy metals and organic pollutants (Koukouzika and Dimitriadis, 2008); since in our study we observed a slight decrease in organisms exposed to Cd and acidification and/or warming compared to those exposed to Cd alone, this can be explained as the utilization of reserve material under increasing cellular stress, as already suggested for *M. galloprovincialis* (Regoli, 1992).

In the gills, similar but opposite trends of single antioxidants responsiveness were found, confirming the involvement of certain antioxidant pathways already observed in the digestive gland. Selenium-dependent GPx activity was induced by Cd at control temperature, while at higher temperature this lead to an inhibition of the activity, partially due to an increase of the activity in organisms exposed to warming without cadmium. Similar effects were observed also for the activity of total GPx in organisms exposed to Cd at higher temperature and reduced pH. This highlights the different effects that the investigated stressors have in different tissues due to their metabolic and physiological function: temperature increase could exert oxidative stress in the gills due to increased metabolic rates and respiration, and thus it is possible to observe higher protection toward oxidative insult; this, anyhow, becomes less effective when higher temperature is associated to Cd exposure, which is known to trigger oxidative stress by direct production of ROS or by indirect depletion of antioxidant defenses (Regoli and Giuliani, 2014). The changes of single antioxidants efficiency however were not paralleled by considerable variations of the total oxyradical scavenging capacity, except for a slight increase in organisms exposed to higher temperature without cadmium or

284 acidification; nevertheless, the absence of variations of malondialdehyde content confirms the
285 efficiency of antioxidant system, probably involving other antioxidants as suggested for digestive
286 gland.

287 Haemocytes investigation showed high sensitivity of lysosomal membrane stability to all the
288 investigated stressors, with the higher effect in organisms co-exposed to cadmium, acidification and
289 warming. This confirms previously published results on haemocytes of marine invertebrates (Beesley
290 *et al*., 2008; Matozzo *et al*., 2012) and what observed by our group on *M. galloprovincialis* in summer
291 (Nardi *et al*., 2017). Beside lysosomal membranes impairment, we observed also a reduction of the
292 phagocytosis rate due to increased temperature, in contrast with the absence of effects observed by
293 Parry and Pipe, 2014 in *M. galloprovincialis* exposed at 10°C and 15°C. Since granulocytes are the
294 haemocytes population deputy to phagocytosis (Gorbi *et al*., 2013), decreased phagocytic activity in
295 addition to the observed lower granulocytes versus hyalinocytes type cells ratio can be explained by
296 granulocytes depletion due to the increased temperature. Haemocytes sensitivity to the investigated
297 factors was further corroborated by the increased micronuclei frequency, particularly in organisms
298 exposed to Cd alone and at higher temperature. These results are in contrast with what we observed
299 in *M. galloprovincialis* in summer, and suggest seasonal specific sensitivity to acidification and
300 temperature (Nardi *et al*., 2017).

301 The generalized sensitivity was further corroborated by the elaboration of biomarkers data within
302 the quantitative model Sediqualsoft, which elaborates synthetic hazard index through weighted
303 criteria based on the magnitude of variations and the relative biological relevance of investigated
304 parameters (Piva *et al*., 2011; Benedetti *et al*., 2012). Biomarkers have been widely used in recent
305 years as a tool in early warning detection of environmental disturbance, but the difficulties in easily
306 summarizable and predictive use of these has led to the increasing necessity of integrative methods
307 and health indices (Beliaeff and Burgeot, 2002; Piva et al., 2011; Broeg et al., 2005; Dagnino et al.,
308 2008; Benedetti et al., 2012; Marigómez et al., 2013). The elaboration applied in the present study,
309 part of a Weight of Evidence model which elaborates multiple typologies of data and already

validated in several risk assessment studies (Benedetti et al., 2012 Regoli et al., 2014; Avio et al., 2015), assigns each treatment to one of five classes of hazard, from "Absent" to "Severe". In this study all experimental treatment were classified as "Moderate", with the exception of those with reduced pH without cadmium that resulted in the "Slight" class, highlighting higher sensitivity to Cd and warming; these results are in contrast with those obtained in the experiment performed in summer (Nardi et al., 2017), which showed higher sensitivity to Cd when dosed in association to warming and/or acidification, further confirming the hypothesis of involvement of seasonal-specific sensitivity to the investigated stressors.

In conclusion this study provided clear evidences that temperature and pH/pCO_2 variations can affect cellular effects of cadmium, which uptake is modulated by temperature. Our data suggest both tissue-specific mechanisms of action, probably related to tissue physiological function, and seasonal-dependent effects of investigated stressors on metal accumulation and biological effects. These could be useful to understand factors influencing tolerance to climate changes and related effects of multiple stressors interactions.

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

	measured parameters				calculated parameters		
treatment	T (°C)	S	pH (NBS scale)	A_T (µmol/kg)	*p*CO2 (µatm)	Ωc	Ωa
CTRL	10.17 ± 0.30	37 ± 0.5	8.14 ± 0.03	2453.6 ± 251.5	456.3 ± 38.8	6.1 ± 0.7	3.9 ± 0.4
Cd	10.05 ± 0.33	37 ± 0.5	8.15 ± 0.05	2390.5 ± 354.1	429.8 ± 35.9	6.1 ± 1.2	3.9 ± 0.7
A	10.20 ± 0.30	37 ± 0.5	7.38 ± 0.02	2325.4 ± 267.7	3009.85 ± 93.63	1.2 ± 0.1	0.8 ± 0.1
W	14.97 ± 0.09	37 ± 0.5	8.09 ± 0.06	2557.3 ± 183.7	492.7 ± 48.5	6.4 ± 1.1	4.1 ± 0.7
A-Cd	10.08 ± 0.23	37 ± 0.5	7.41 ± 0.04	2517.9 ± 206.9	2849.1 ± 145.4	1.2 ± 0.2	0.8 ± 0.1
W-Cd	15.01 ± 0.08	37 ± 0.5	8.12 ± 0.07	2556.7 ± 479.0	477.8 ± 73.2	6.9 ± 1.2	4.4 ± 0.8
A-W	15.01 ± 0.02	37 ± 0.5	7.38 ± 0.02	2721.4 ± 215.7	3192.6 ± 90.7	1.5 ± 0.1	0.9 ± 0.1
A-W-Cd	14.88 ± 0.09	37 ± 0.5	7.39 ± 0.02	2504.2 ± 182	3063.9.7 ± 195.5	1.6 ± 0.1	1.0 ± 0.1

521

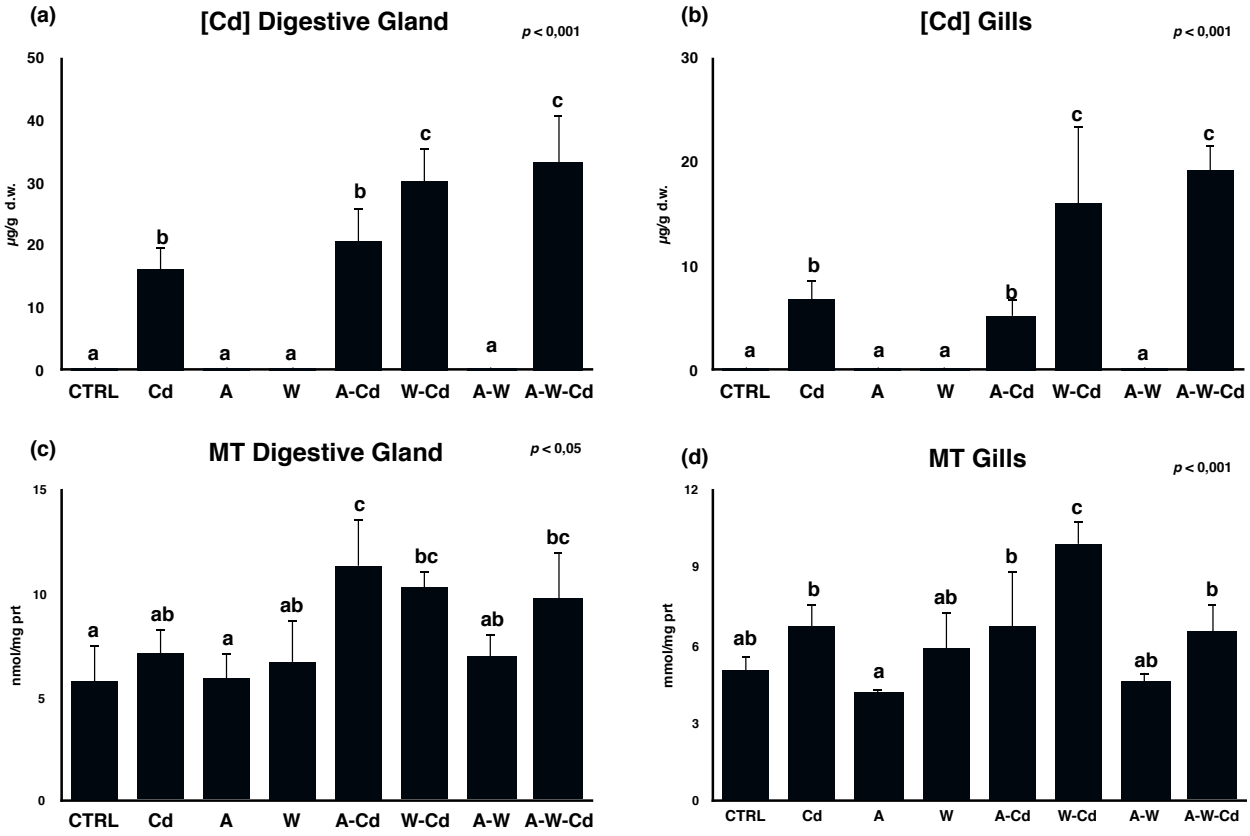


Figure 1 – Cadmium concentrations and level of metallothioneins in digestive gland (a and c) and gills (b and d) of mussels exposed to various treatments. Data are given as mean values ± standard deviations (n=5). Different letters indicate significant differences between group of means (ANOVA and SNK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

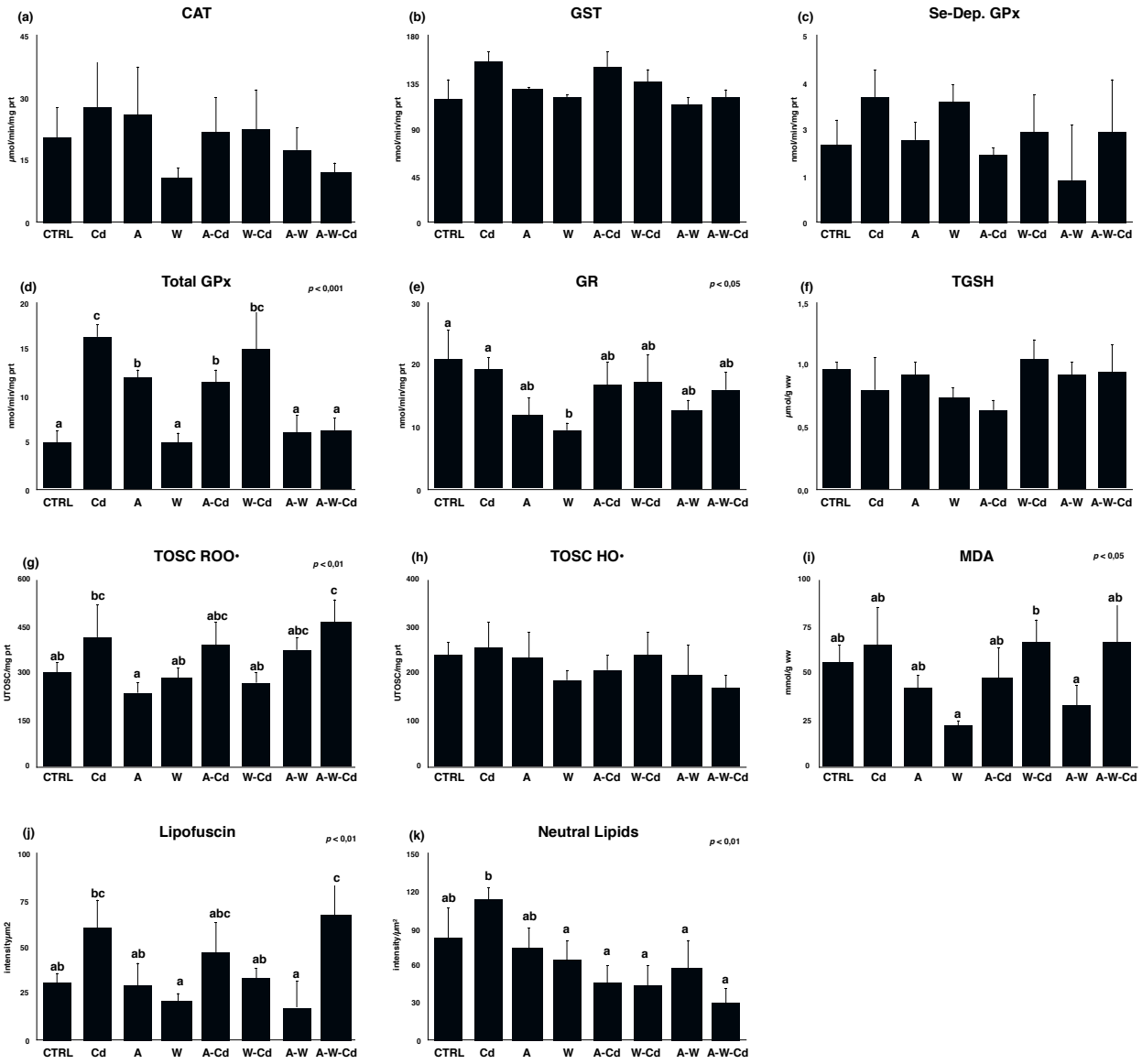


Figure 2 – Antioxidant defenses and oxidative stress biomarkers in digestive gland of mussels exposed to various treatments. CAT: catalase (a), GST: glutathione S-transferase (b), Se-Dep. GPx: Se-dependent glutathione peroxidases (c) total GPx: sum of Se-dependent and Se-independent glutathione peroxidases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

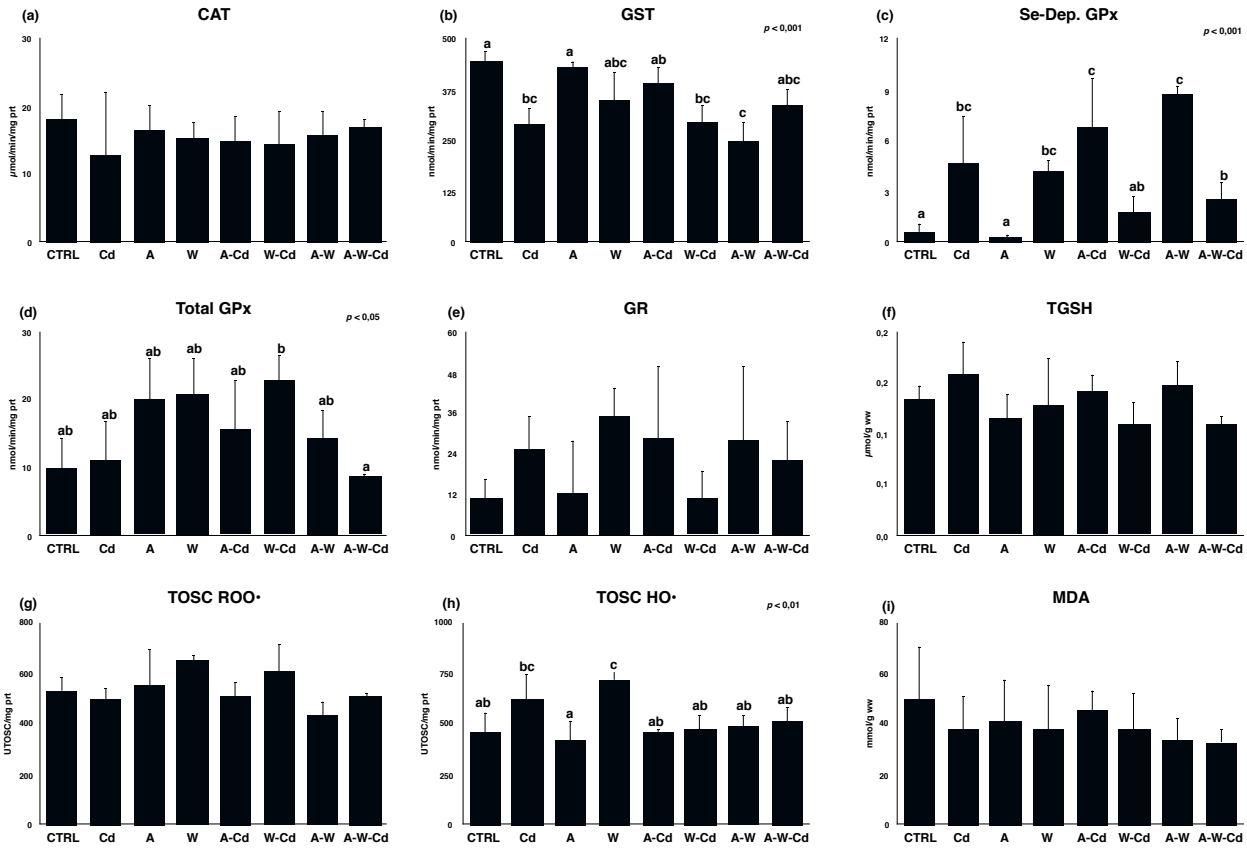


Figure 3 - Antioxidant defenses and oxidative stress biomarkers in gills of mussels exposed to various treatments. CAT: catalase (a), GST: glutathione S-transferase (b), Se-Dep. GPx: Se-dependent glutathione peroxidases (c) total GPx: sum of Se-dependent and Se-independent glutathione peroxidases (d),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

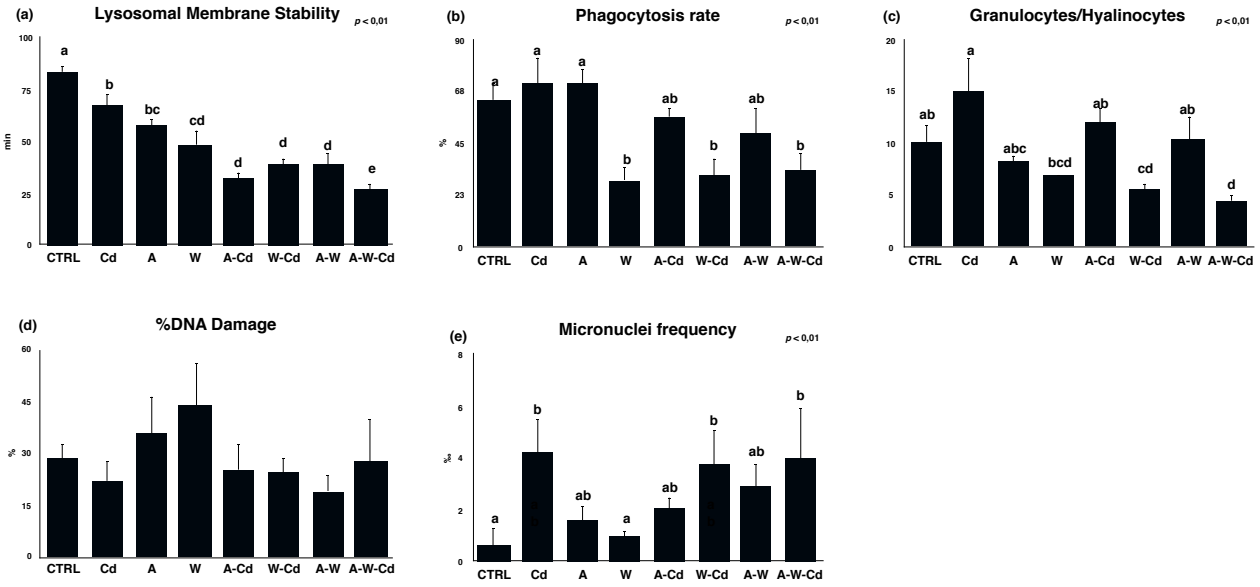


Figure 4 - Lysosomal membrane stability (a), phagocytosis rate (b), granulocytes/hyalinocytes ratio (c), DNA damage (d) and frequency of micronuclei (e) in haemocytes of mussels exposed to various treatments. Data are given as mean values ± SEM (n=5). Different letters indicate significant differences 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.

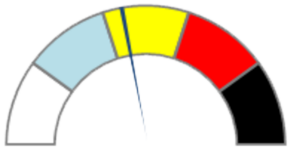
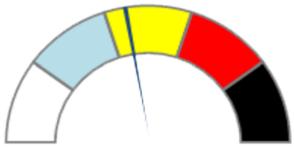
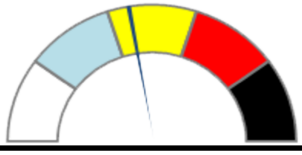
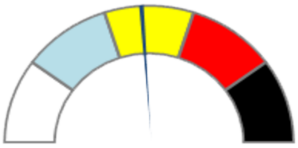
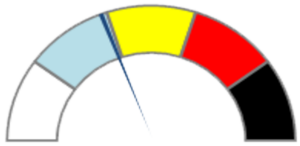
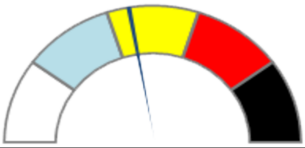
Experimental Treatment	Hazard Quotient (HQ)	Class of Hazard	Level
Cd	29.41	MODERATE	
A	14.5	SLIGHT	
W	37.39	MODERATE	
A-Cd	56.59	MODERATE	
W-Cd	22.62	MODERATE	
A-W	61.08	SLIGHT	
A-W-Cd	24.67	MODERATE	

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

Supplementary Materials SM1

Detailed protocols for biomarkers analyses

S1.1 Metallothioneins

Metallothioneins (MTs) were analyzed in gills and digestive gland samples homogenized (1:3 and 1:5 w/v respectively) in 20 mM Tris–HCl buffer, pH 8.6, 0.5M sucrose, 0.006mM leupeptin, 0.5 mM PMSF (phenylmethylsulphonylfluoride), and 0.01% b-mercaptoethanol. After acidic ethanol/chloroform fractionation of the tissue homogenate, MTs were quantified by the spectrophotometric assay as described in Viarengo *et al.* (1997) using GSH as standard.

S1.2 Single antioxidant defenses and Total Oxyradical Scavenging Capacity (TOSC) assay

For enzymatic antioxidants, digestive gland and gills samples were homogenized (1:5 and 1:3 w:v ratio respectively) in 100 mM K-phosphate buffer (pH 7.5), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mg/ml bacitracin, 0.008 TIU/ml aprotinin, 1 μg/ml leupeptin, 0.5 μg/ml pepstatin, NaCl 2.5%, and centrifuged at 100 000 × g for 70 min at 4 °C. Activities were measured with a Varian (model Cary 100 Scan) spectrophotometer at a constant temperature of 18 °C (Bocchetti *et al.*, 2008).

Catalase (CAT) was measured by the decrease in absorbance at 240 nm (extinction coefficient, $\varepsilon = 0.04$ mM−1 cm−1) due to the consumption of hydrogen peroxide, H_2O_2 (12 mM H_2O_2 in 100 mM K-phosphate buffer pH 7.0).

Glutathione S-transferases were determined at 340 nm using 1-chloro-2,4-dinitrobenzene as substrate (CDNB). The assay was carried out in 100 mM K-phosphate buffer pH 6.5, 1.5 mM CDNB, 1 mM GSH ($\varepsilon = 9.6$ mM−1 cm−1).

Glutathione peroxidases activities were assayed in a coupled enzyme system where NADPH is consumed by glutathione reductase to convert the formed GSSG to its reduced form (GSH). The decrease of absorbance was monitored at 340 nm ($\varepsilon = -6.22$ mM−1 cm−1) in 100 mM K-phosphate buffer pH 7.5, 1 mM EDTA, 1 mM sodium azide (NaN3) (for hydrogen peroxide assay), 2 mM GSH, 1 unit glutathione reductase, 0.24 mM NADPH, and 0.5 mM hydrogen peroxide or 0.8 mM cumene hydroperoxide as substrates, respectively, for the selenium-dependent and for the sum of Se-dependent and Se-independent forms.

Glutathione reductase (GR) was determined from NADPH oxidation during the reduction of oxidized glutathione, GSSG ($\lambda = 340$ nm, $\varepsilon = -6.22$ mM−1 cm−1). The final assay conditions were 100 mM K-phosphate buffer pH 7.0, 1 mM GSSG, and 60 μM NADPH.

Total glutathione was analyzed in samples homogenized (1:5 w:v ratio) in 5% sulfosalicilic acid with 4 mM EDTA, maintained for 45 min on ice and centrifuged at 37.000 × g for 15 min, before being enzymatically assayed (Akerboom & Sies, 1981).

The total oxyradical scavenging capacity (TOSC) assay measures the overall capability of cellular antioxidants to absorb different forms of artificially generated oxyradicals, thus inhibiting the oxidation of 0.2 mM α-keto-γ-methiolbutyric acid (KMBA) to ethylene gas (Winston *et al.*, 1998; Regoli & Winston, 1999). Peroxyl radicals (ROO•) were generated by the thermal homolysis of 20 mM 2-2′-azo-bis-(2-methylpropionamidine)-dihydrochloride (ABAP) in 100 mM K-phosphate buffer, pH 7.4. Hydroxyl radicals (HO•) were produced by the Fenton reaction of iron-EDTA (1.8 μM Fe^{3+}, 3.6 μM EDTA) plus ascorbate (180 μM) in 100 mM K-phosphate buffer. Ethylene formation in control and sample reactions was analyzed at 10–12 min time intervals by gas-chromatographic analyses and the TOSC values are quantified from the equation: TOSC = 100 − (∫SA/∫CA × 100), where ∫SA and ∫CA are the integrated areas calculated under the kinetic curves for samples (SA) and control (CA) reactions. For all the samples, a specific TOSC (normalized to content of protein) was calculated by dividing the experimental TOSC values by the relative protein concentration contained in the assay, measured by the spectrophotometric method of Lowry *et al.*, 1951 with bovine serum albumin (BSA) as standard.

S1.3 Malondialdehyde

The content of malondialdehyde (MDA) was measured in samples homogenized (1:3 and 1:5 w/v respectively for gills and digestive gland) in 20 mM Tris–HCl pH 7.4, centrifuged at 3000 × g for 20 min and then derivatized in 1 ml reaction mixture containing 10.3 mM 1-metyl-2-phenylindole (dissolved in acetonitrile/methanol 3:1), HCl 32%, 100 μl water and an equal volume of sample or standard (standard range 0–6 μM 1,1,3,3-tetramethoxypropane, in 20 mM Tris–HCl, pH 7.4). The tubes were vortexed and incubated at 45 °C for 40 min. Samples were cooled on ice, centrifuged at 15 000 × g for 10 min and read spectrophotometrically at 586 nm; levels of MDA were calibrated against a malondialdehyde standard curve and expressed as nmol/g wet weight (Shaw *et al.*, 2004).

S1.4 Lipofuscin and Neutral Lipids

Lipofuscin content was determined using the Schmorl reaction (Moore, 1988) on cryostat sections (8 μm thick) of digestive glands. Slides were fixed in calcium-formol for 15 min at 4 °C, rinsed in distilled water and immersed in the reaction medium containing an aqueous solution of 1% ferric chloride and 1% potassium ferricyanide. Sections were stained for 5 min, rinsed in 1% acetic acid and washed in distilled water before mounting with Eukitt. For analyses of neutral lipids, cryostat

630 sections (8 μm thick) of digestive glands were fixed as above and washed in 60% isopropilic alcohol
631 solution (Moore, 1988). Sections were stained for 20 min in a saturated oil red O solution (1% in
632 isopropyl alcohol 60%), washed in isopropyl alcohol and then in distilled water before mounting in
633 glycerine gelatine. For both lipofuscin and neutral lipids, four measurements were made on digestive
634 tubules of each section. Quantification of staining intensity was performed with Image-Pro® Plus 6.2
635 Analysis Software and then normalized to the area of digestive tubules.
636
637 S1.5 Immune system responses
638 For the analysis of immune system responses, heamolymph was withdrawn from each mussel
639 and then splitted for the analysis of lysosomal membrane stability, granulocytes–hyalinocytes ratio
640 and phagocytosis rate.
641 The cationic probe Neutral Red (NR) was used to evaluate the capability of the lysosomal
642 membranes to retain the dye (Lowe *et al.*, 1995). Haemocytes were incubated on a glass slide with a
643 freshly prepared NR working solution (2 μl/ml filtered seawater) from a stock solution of 20 mg
644 neutral red dye dissolved in 1 ml of dimethyl sulfoxide, and microscopically examined at 15 min
645 intervals, to determine the time at which 50% of cells had lost to the cytosol the dye previously taken
646 up by lysosomes.
647 Phagocytosis rate assay was performed following previous methods (Gorbi *et al.*, 2013); 50 μl
648 of haemolymph were dispersed on glass slides and allowed to adhere incubating them for 15 min at
649 15 °C in the dark. Fluorescein-labelled Zymosan A bioparticles (Invitrogen) were added at 10:1
650 target:haemocyte ratio. After 2 h incubation at 15 °C in the dark, uninternalized particles were
651 removed by washing with physiological solution and slides were finally fixed in Beker's fixative
652 (+2.5% NaCl) and mounted in Eukitt. Phagocytosis was expressed as percentage of cells that
653 internalized at least 3 fluorescent particle (positive cells), observed under a fluorescence microscope,
654 after counting at least 200 cells for each sample.
655 Granulocytes versus hyalinocytes ratio was assessed on 50 μl of haemolymph dispersed on
656 glass slides, dryed and fixed in Baker's fixative (+2.5% NaCl). The slides were washed with water,
657 stained with H&E and mounted in Eukitt. Observations were carried out with a light microscope
658 (1000X) and the ratio was evaluated after counting almost 200 cells for each sample (Gorbi *et al.*,
659 2013).
660
661 S1.6 Genotoxic damage
662 The onset of DNA damage was evaluated at molecular level as single strand breaks (SB) by the
663 Comet assay, and at chromosomal level by the frequency of micronuclei.

664 The comet assay was carried out on haemocytes collected from the adductor muscle of
665 organisms according to Gorbi *et al.*, 2008. Cells were diluted in Ca^{2+}/Mg^{2+}-free buffer at 4 °C in the
666 dark (20 mM Hepes, 500 mM NaCl, 12.5 mM KCl, 10 mM EDTA, pH 7.3), centrifuged at 1000 rpm
667 for 1 min at 4 °C, resuspended in 0.6% low-melting-point agarose, and added with a sandwich
668 stratification to glass slides coated with 1% normal-melting-point agarose. After gel solidification,
669 slides were placed into the lysing solution (2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, and 10%
670 DMSO, pH 10) at 4 °C in the dark for 90 min. DNA was unwound in 75 mM NaOH, 10 mM EDTA
671 (pH 13), and the electrophoretic migration was carried out in the same buffer at 1 V/cm for 10 min.
672 Slides were then neutralized for 10 min in 0.4 M Tris, pH 7.5, fixed in cold methanol for 3 min at
673 −20 °C, and dried. After staining with DAPI, 100 randomly selected "nucleoids" per slide, and two
674 replicates per sample, were examined under fluorescence microscopy (200 × magnification; Olympus
675 BX-51), and the captured images (Image-Pro-Plus package) were analyzed through the software
676 Comet Score. The percentage of DNA in the tail was used to estimate the level of DNA fragmentation.
677 For the frequency of micronuclei, an aliquot of haemolymph collected from the adductor
678 muscle of organisms was rapidly washed in saline buffer (20 mM Hepes, 500 mM NaCl, 12.5 mM
679 KCl, 10 mM EDTA, pH 7.3) and centrifuged at 1000 rpm for 1 min at 4 °C, following the procedure
680 described in Nigro *et al.*, 2006. Cells were immersed in a fixative solution (3:1 methanol, acetic acid)
681 and washed twice. Suspended cells were dispersed on glass slides, air dried and stained with the
682 fluorescent dye 4′,6-diamidino-2-phenylindole DAPI (100 ng/ml). For each specimen, 2000 cells with
683 preserved cytoplasm were scored to determine the frequency of micronuclei, defined as round
684 structures, smaller than 1/3 of the main nucleus diameter on the same optical plan and clearly
685 separated from it.
686
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IV

Interactive effect of temperature, pH and Cd of transcriptional responsiveness toward oxidative stress in the Mediterranean mussel *Mytilus galloprovincialis*

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

Abstract

Continuous anthropogenic inputs of carbon dioxide are driving ocean warming and acidification. The potential threat represented by these changes could be amplified in coastal areas, characterized by high levels of heavy metals inputs. Nevertheless, organisms living in temperate areas can exhibit different tolerance to environmental stressors due to seasonal fluctuations that shape physiological characteristics. In this study, we examined mRNA levels of selected genes related to metal-induced stress response in the Mediterranean mussel *Mytilus galloprovincialis* exposed to all the possible combinations of two temperatures (control and +5 °C), two pH (8.20 and 7.40) and two doses of cadmium (0 and 20 μg/L). The experiment was run with organisms collected both in summer and in winter, investigating differences due to seasonal physiological features on the transcription of metal detoxification genes (*mt-20*), heatshock and cellular stress response (*hsp70*), and selected antioxidants genes (*Cu/Zn-sod*, *cat*, *Se-dep. gpx* and *gst-pi*) both in the digestive gland and in the gills of exposed mussles, to elucidate differences related to metabolic function and physiological characteristics typical of each tissue. Tissue and seasonal differences of metallothioneins Cd-mediated induction were observed, along to *hsp70* up-regulation by acidification, both alone or in combination with other stressors. Antioxidants genes were up-regulated by cadmium and acidification in the digestive gland in summer, while in winter higher temperature and cadmium exposure led to down-regulation of certain pathways, which was even more pronounced in gills mainly due to acidification and warming, with more evident effects in winter rather than summer. The overall results were expected to further contribute to the growing knowledge on multiple stressors interactions, assessing complexity of the effects related to tissue specificities and seasonal physiological variability.

47 **1.Introduction**

48 As a consequence of increased anthropogenic carbon dioxide emissions, oceans are threatened
49 by warming and acidification (IPCC, 2013; Pörtner *et al*., 2014). From the beginning of the industrial
50 era, ocean has warmed by almost 1 °C because of the greenhouse effect and took up about 30% of
51 anthropogenic CO_2, which have caused ocean pH to drop by 0.1 units (Gattuso and Lavigne, 2009;
52 Hansen *et al*., 2016); by the end of the century, ocean changes are expected to continue and
53 temperature is projected to increase by 2°C, while ocean mean pH will further decrease by 0.3 – 0.5
54 units (IPCC, 2013; Hoegh-Guldberg *et al*., 2014), and these changes could even be more pronounced
55 in coastal areas, where large fluctuations of temperature and pH naturally occur (Wallace *et al*., 2014;
56 Wong *et al*., 2014). Nevertheless, since in these ecosystems anthropogenic footprint is higher than in
57 open ocean, these are already threatened by a wide range of stressors, among which trace metal
58 pollution is of particular interest (Bijima *et al*., 2013; Hewitt *et al*., 2016).

59 Ocean warming and acidification have been described as potential factors affecting heavy metals
60 distribution in coastal waters and their bioaccumulation in marine organisms (Sokolova and Lannig,
61 2008; Millero *et al.*, 2009; Hoffmann *et al.*, 2012; Negri *et al.*, 2013; Ivanina *et al.*, 2014a; Götze *et*
62 *al.*, 2014; Rodriguez-Romero *et al.*, 2014). In addition to this, environmental factors as temperature
63 and pH/pCO_2 are also involved in biological effects of metals and their toxicity, and their interactions
64 have been demonstrated to impair mitochondrial functioning, to affect energy metabolism, to cause
65 oxidative unbalance, accumulation of lipid peroxidation products and damages to lysosomal system
66 and DNA, as well as to impair larval development and immune function (Sokolova & Lannig, 2008;
67 Negri *et al.*, 2013; Roberts *et al.*, 2013; Banni *et al.*, 2014; Campbell *et al.*, 2014; Götze *et al.*, 2014;
68 Izagirre *et al.*, 2014; Múgica *et al.*, 2015; Ivanina *et al.*, 2016; Lewis *et al.*, 2016).

69 Trace metals toxicity can be exerted through impairment of oxidative balance, both directly
70 producing reactive oxygen species (ROS) through impairment of electron transport chains and
71 enhancement of Fenton-like and Haber-Weiss reaction, and indirectly affecting antioxidant defenses
72 efficiency (Regoli and Giuliani, 2014). Oxidizing agents and the intracellular increase of ROS levels

73 may result in the activation of antioxidant gene, through the involvement of several transcription
74 factors (Bartosz, 2009), aimed at ROS counteraction. Oxidative unbalance has been demonstrated to
75 be promoted also by thermal stress and reduced pH-hypercapnic condition in several species, both
76 vertebrates and invertebrates (Tomanek *et al*., 2011; Kaniewska *et al*., 2012; Rosa *et al*., 2012;
77 Soriano-Santiago *et al*., 2013; Vinagre *et al*., 2014; Pimentel *et al*., 2015; Wang *et al*., 2016).
78 Metal exposure is also usually associated with the induction of metallothioneins (MTs), low-
79 molecular weight metal-binding proteins involved in metal homeostasis and detoxification whose
80 transcription is up-regulated by metals but also by ROS (Viarengo *et al*., 2000; Regoli and Giuliani,
81 2014), through the metal transcription factor I (MTF-I) (Kimura *et al*., 2009). The induction of MT
82 can be affected by other factors, such as temperature, nutritional status, salinity, and hypoxia (Le *et*
83 *al*., 2016). Heat stress, but also metals, hypoxia, hypercapnia, and other environmental stressors
84 induce the expression of genes for the heat shock protein (Hsp) family (Roberts *et al*., 2010; Wang *et*
85 *al*., 2013), through the heat shock factor (HSF) transcription factor. Hsp70s, one of the most
86 conserved family, are proteins with chaperone activity, involved in folding and unfolding of damaged
87 proteins and stress response (Wang *et al*., 2013).
88 The aim of this study was to characterize the interactive effects of metal exposure, higher
89 temperature and reduced pH at transcriptional level in *M. galloprovincialis*. Mussels are widely used
90 as bioindicator organisms due their ability to concentrate pollutants and tolerate different
91 environmental stressors (Fattorini *et al*., 2008; Regoli *et al*., 2014), and have been recently addressed
92 as potentially susceptible to climate changes (Gazeau *et al*., 2014; Izagirre *et al.*, 2014). The use of
93 molecular and cellular responses represents a sensitive and early warning tool to identify biological
94 disturbance caused by various environmental stressors (Regoli *et al*., 2014; Izagirre *et al.*, 2014;
95 Benedetti *et al*., 2016). Selected target genes reflect pathways of metal-responsiveness, oxyradical
96 and cellular stress and included the Cd-inducible isoform of metallothioneins (*mt-20*), the heat shock
97 protein 70 (*hsp70*) along with antioxidants such as cytosolic superoxide dismutase, catalase, a Se-
98 dependent glutathione peroxidase and pi isoform of glutathione S-transferase (*Cu/Zn-sod*, *cat*, *Se-*

gpx, *gst-pi*). These genes were analyzed both in digestive gland and gills of exposed mussels. Nevertheless, since seasonality represents an important factor controlling cellular responses and since seasonal fluctuations of physiological parameters in *M. galloprovincialis* are well documented (Bocchetti and Regoli, 2006, Múgica *et al*., 2015), these analyses were conducted both in summer and in winter in order to investigate seasonal dependent effects of the investigated parameters. The overall results are aimed to elucidate mechanisms underlying the biochemical and cellular alterations observed in previously published researches of our group (Nardi *et al*., 2016, 2017), where oxidative and cellular effects of cadmium, temperature and pH stress were observed.

2.Materials and Methods

2.1 Animal collection and experimental design

Mussels, *Mytilus galloprovincialis* (6.0 ± 0.5 cm shell length), were obtained from a shellfish farm in an unpolluted area of Central Adriatic Sea (Regoli *et al*., 2014) in summer (June 2014) and winter (January 2015). For each season, 288 mussels were then acclimatized in 20 L tanks (36 mussels each) filled with aerated artificial seawater (ASW; Instant Ocean®) for 7 days at pH 8.20 and salinity 37 (practical salinity unit). Different acclimation temperatures were used for summer (20 °C) and winter (10°C), representative of seasonal mean surface temperature (T_{SST}) at sampling site at the time of collection. Water was changed every other day and mussels fed 12 hours prior the water change with a commercial mixture of zooplankton (50-300 μatm) for filter-feeding organisms.

After the acclimation, each group of mussels was randomly assigned to one of the eight combinations of multifactorial experimental design with two temperatures (T_{SST} and $T_{+5°C}$), two pH/pCO_2 (8.20/~400 μatm and 7.40/~3000 μatm) and two doses of cadmium (0 and 20 μg/L). The resulting experimental treatments were: 1) control condition (CTRL), seasonal mean surface temperature (T_{SST}), normocapnia (pH=8.20/ pCO_2=~400 μatm); 2) cadmium exposure (Cd), T_{SST}, normocapnia and 20 μg/L cadmium; 3) acidification (A), T_{SST}, hypercapnia (pH=7.40/ pCO_2=~3000

125 μatm); 4) warming (W), 5°C temperature increase respect the SST ($T_{+5°C}$) and normocapnia; 5)
126 acidification + Cd (A-Cd), T_{SST}, hypercapnia and 20 μg/L cadmium; 6) warming + Cd (W-Cd), $T_{+5°C}$,
127 normocapnia and 20 μg/L cadmium; 7) acidification + warming (A-W), $T_{+5°C}$ and hypercapnia; 8)
128 acidification + warming + Cd (A-W-Cd), $T_{+5°C}$, hypercapnia and 20 μg/L cadmium. The cadmium
129 concentration used herein was chosen as representative of a polluted but environmentally realistic
130 scenario in Mediterranean coastal waters (Neff, 2002), while selected target pH and temperature were
131 adapted from scenario RCP 8.5 and IPCC WGII AR5 (IPCC, 2013; Wong *et al*., 2014) predicting
132 more pronounced variations of temperature and pH/pCO_2 in coastal areas than in open ocean. The
133 hypercapnic condition was obtained mixing ASW (pH=8.2) with small amounts of CO_2-saturated
134 ASW, resulting of bubbling pure CO_2 in ASW for at least 24h (Nardi *et al*., 2017). For each
135 experimental condition temperature, pH and salinity were measured daily, while total alkalinity (A_T)
136 was measured twice per week. Seawater carbonate parameters (pCO_2, and saturation state (Ω) for
137 calcite and aragonite) were calculated in CO2SYS (Pierrott *et al*., 2006); using barometric pressure
138 values, as well as A_T, pH, temperature and salinity values for the respective samples; details of
139 calculation are described elsewhere (Nardi *et al*., 2016, 2017) and full seawater chemistry is provided
140 in Table 1). During experimental phase, water was changed every other day and mussels fed 12 hours
141 prior the water change with a commercial mixture of zooplankton (50-300 μatm) for filter-feeding
142 organisms as in the acclimation phase.
143 After four weeks, animals were sampled from each tank and tissues collected for biological
144 analyses. Gills and digestive glands were excised, pooled in 12 samples, each constituted by tissues
145 of 3 individuals, rapidly frozen in liquid nitrogen and maintained at -80°C.
146
147 *2.2 RNA isolation and cDNA synthesis*
148 Total RNA was purified from M. galloprovincialis digestive glands an gills, using the Hybrid-RTM
149 kit (GeneAll Biotechnology) according to the manufacturer's protocol. Total RNA concentrations
150 and purity were measured using Nano-Drop ND-1000 UV-Visible Spectrophotometer (NanoDrop

151 Technologies, Wilmington, DE, USA). RNA quality was verified on an agarose-formaldehyde gel.
152 Total cDNA was generated by RT-PCR (Reverse Transcription-Polymerase Chain Reaction) from 1
153 μg of total RNA for each sample using combined oligo(dT) and random hexamer primers (iScript
154 cDNA Synthesis Kit, Bio-Rad).
155
156 *2.3 Quantitative real-time PCR*
157 Absolute quantitative real-time PCR with gene-specific primer pairs (Table xxx) was performed for
158 evaluating the mRNA levels of individual target genes, using SYBR green method in StepOnePlus®
159 Real-Time PCR System (Applied Biosystems). Each 15 μl DNA amplification reaction contained 7.5
160 μl of SYBR Select Master Mix (Life Technologies), 5 μl of total cDNA (synthesized as described
161 above and diluted 1:5) and 200 nM of each forward and reverse primers. The real-time PCR program
162 included an enzyme activation step at 95 °C (2 min) and 40 cycles each composed by 15 s at 95 °C
163 and 1 min at the annealing temperature (Table 2). The specificity of target cDNA amplification was
164 checked by including controls lacking cDNA template and by a melting analysis (95 °C for 1 min, 65
165 °C for 10 s and fluorescence detection at increasing temperature between 65 and 95 °C).
166 For each target gene, serial dilutions of known amounts of plasmid containing the amplicon of interest
167 were used as standards. Samples and standards were run in duplicate in the same run. Cycle threshold
168 (Ct) values of unknown samples were converted into mRNA copy number interpolating the standard
169 plot of Ct versus log copy number.
170
171 *2. Statistical analyses*
172 Non-parametric one-way analysis of variance (Kruskal-Wallis test) was applied to evaluate the
173 effect of the different treatments. Level of significance was set to $p < 0.05$; *post-hoc* tests, Dunn's
174 test, were used to compare group of means. All statistical analyses were performed using RStudio
175 (version 0.99.491).
176

177 **3. Results**

178 In the digestive gland in summer, a significant increase of *mt-20* mRNA was observed in all
179 experimental treatments with cadmium (Cd, A-Cd, W-Cd and A-W-Cd) (Fig. 1a). Acidification
180 increased significantly *hsp70* mRNA levels (Fig. 1b), with the major effect in organisms exposed to
181 acidification and warming (A-W and A-W-Cd). Similarly, *cat* mRNA was higher in all organisms
182 exposed to reduced pH, except for those co-exposed to acidification, cadmium and warming (A-W-
183 Cd, Fig.1d). On the other hand, mRNA of *Se-dep. gpx* was significantly reduced by the exposure to
184 cadmium alone (Cd), warming (W), acidification and cadmium (A-Cd) and acidification and warming
185 (A-W) (Fig.1e). *gst-pi* mRNA was significantly increased by acidification, alone (A) or when
186 organisms were co-exposed to cadmium and warming (A-W-Cd) (Fig.1f). No significant variation of
187 *Cu/Zn-sod* expression was observed. Similar effects were not observed in winter, except for *mt-20*
188 gene expression that was upregulated by cadmium exposure (Fig. 2a). Acidification alone upregulated
189 *hsp70* (Fig. 2b), *cat* (Fig. 2d) and *gst-pi* (Fig. 2f) mRNA, with the latter significantly increased also
190 by Cd alone and co-exposure to acidification and warming (A-W). On the other hand, downregulation
191 of *Se-dep. gpx* mRNA was observed in organisms co-exposed to warming and other stressors (W-Cd,
192 A-W, A-W-Cd) (Fig. 2e), and no effect was measured on *Cu/Zn-sod* expression.

193 In the gills, in the summer experiment *mt-20* expression was upregulated by cadmium alone and
194 in combination with acidification (Cd and A-Cd) (Fig. 3a), while the latter (A-Cd) downregulated
195 *hsp70* expression (Fig. 3b). Significant lowered expression of *Se-dep. gpx* was measured in the gills
196 of organisms exposed to warming, alone or in combination with Cd (W and W-Cd) (Fig. 3e). No
197 significant variation was observed for Cu/Zn-*sod*, *cat* and *gst-pi* mRNA. In the winter experiment,
198 *mt-20* expression was upregulated by Cd alone or in combination with higher temperature (Cd and
199 W-Cd) while acidification and warming significantly reduced *mt-20* expression (Fig. 4a);
200 acidification, both at control and higher temperature, significantly increased *hsp70* expression (Fig.
201 4b). Single and interactive effects of cadmium and acidification were observed on the *cat* expression,
202 which was downregulated by Cd and acidification, both alone and in combination (Cd, A, A-Cd and

A-W-Cd, Fig. 4d). Significant reduction due to acidification and warming was observed for *Se-dep. gpx* mRNA, with further modulation in organisms exposed to acidification, warming and Cd (Fig. 4e). No significant effects were measured for *Cu/Zn-sod* and *gst-pi* mRNA.

4. Discussion

The present study showed that both acidification and warming can affect transcriptional responses associated to cadmium exposure. Nevertheless, seasonal different sensitivity was shown to modulate these effects. In *Mytilus galloprovincialis*, different isoforms of metallothioneins genes have been described, among which *mt-20* has been shown to be induced by cadmium and oxidative stress (Dondero *et al*., 2005; Aceto *et al*., 2011). Here *mt-20* gene, showed to be responsive to Cd independently to temperature and pH in the digestive gland in both summer and winter experiments, while in the gills interactive effects of warming and acidification showed to interfere with Cd-induced *mt-20* transcription respectively in summer and winter. Similar results were obtained on clams haemocytes exposed to Cd and hypercapnia (Ivanina *et al*., 2014a), and showed the differential sensitivity to environmental factors in different tissues: despite the general slight increase of this mRNA in Cd-exposed organisms, in summer temperature elevation was the main factor negatively affecting induction, while in winter this was due hypercapnia. Hsp70 is usually associated to folding of damaged proteins and to degradation of unrepairable proteins (Mayer and Bukau, 2005) and is often analyzed as marker of thermal or generic cellular stress (Wang *et al*., 2013). In this respect, our results show that after 28 days of exposure mRNA levels of *hsp70* in the digestive gland and gills of mussels are mostly sensitive to acidification, without a clear pattern. In the digestive tissue in summer, acidification, alone or in combination with other stressors, significantly upregulates *hsp70* gene transcription, while in winter this was shown only in organisms exposed to acidification alone. On the other hand, in gills a significant downregulation of this transcript was observed in organisms co-exposed to cadmium and acidification at control temperature in summer, while a slight upregulation by acidification without cadmium was observed in winter. Higher *hsp70* mRNA levels may be

229 interpreted as markers of cellular stress, but lower levels may suggest involvement of mRNA
230 degradation (Lee *et al*., 2015). Downregulation of *hsp70* mRNA was observed in the haemocytes of
231 clam *Mercenaria mercenaria* exposed to cadmium and hypercapnia (Ivanina *et al*., 2014b) and in the
232 haemocytes of oyster *Saccostrea glomerata* exposed to cadmium (Thompson *et al*., 2012). These
233 results highlight seasonal and tissue specific differences in stress response and further confirm weak
234 long-term activation of HSP70 in response to thermal stress. Nevertheless, the high sensitivity of this
235 transcript to the acidification condition may suggest mechanisms of protein damages or modulation
236 of gene expression due to changes in the intracellular milieu (Lewis *et al*., 2016; Pörtner *et al*., 2014;
237 Wang *et al*., 2016).

238 The analysis of a battery of antioxidant genes suggested onset of disturbance of the oxidative
239 balance due the investigated stressors, although clear patterns of action were not identified due to
240 seasonal and tissue specificity. In the digestive gland, acidification, alone or in combination with
241 other stressors, was the main factor promoting up-regulation of *cat* and *gst-pi* mRNA both in summer
242 and in winter with more evident variations in the former, suggesting CO_2-mediated increased
243 oxidative challenge, as already hypothesized by other authors (Tomanek *et al*., 2011; Benedetti *et al*.,
244 2016). On the other hand, *Se-dep*. *gpx* mRNA was downregulated by Cd-exposure at control
245 temperature or by warming without Cd in summer, and by higher temperature alone or in combination
246 with other stressors in winter. Cadmium negative effects on *Se-dependent gpx* expression have been
247 already reported in *Danio rerio* (Banni *et al*., 2011) and in *Oncorhynchus kisutch* (Wang *et al*., 2012),
248 while temperature increase was showed to upregulate selenium-dependent glutathione peroxidases in
249 the gastropod *Haliotis discus discus* (De Zoysa *et al*., 2009). Lower sensitivity to the investigated
250 factors was observed in the gills in both seasons. In summer, thermal stress was responsible for
251 downregulation of *Se-dep*. *gpx* at control pH as already observed in the digestive gland, while in
252 winter acidification and warming, alone or combined with cadmium significantly and negatively
253 affected *Se-dep*. *gpx* transcript levels. In addition to this, the down-regulation of *cat* mRNA due to

254 the cadmium exposure and acidification, alone or in combination, suggests lower protection toward
255 oxidative insult deriving from these stressors in the gills.
256 The complex patterns of variations observed for antioxidants genes reflect the multiple pathways
257 that regulates the transcription of each gene. Indeed, the transcriptional activation of each single
258 antioxidant genes is a complex phenomenon that can be triggered by several transcription factors,
259 binding different responsive elements on their promoter regions upon different stimuli (Miao *et al*.,
260 2005; Stoytcheva and Berry, 2009; Glorieux *et al*., 2015). Nonetheless, these results highlighted
261 frequent dissociation between mRNA levels and protein activity, as already observed in the European
262 eel *Anguilla Anguilla* and in the Mediterranean mussel *Mytilus galloprovincialis* exposed to polluted
263 sediments (Regoli *et al*., 2011; Giuliani *et al*., 2013) and in the Pacific oyster *Crassostrea gigas*
264 exposed to ibuprofen (Serrano *et al*., 2015). The functional investigations of the respective genes
265 analyzed here were previously published (Nardi *et al*., 2017a, 2017b); metallothioneins concentration
266 in the digestive gland was higher in organisms exposed to cadmium and acidification: this is not
267 reflected by *mt-20* mRNA levels, but since these is the Cd-inducible isoform, it is possible to
268 hypothesize that higher MTs levels due to cadmium and acidification may have involved other MTs
269 genes such as *mt-10*. On the other hand, MTs levels in the gills only partially reflected *mt-20*
270 expression, but the differences observed may be due to time delayed responses of translational
271 processes or more in general non-synchronous effects. Among antioxidants investigated in the
272 digestive gland, in the present study *cat* expression was up-regulated by acidification, while catalase
273 activity was not affected by investigated stressors (Nardi *et al*., 2017a, 2017b); this difference
274 between effects at transcriptional and catalytic level may be explained as a compensative mechanism
275 resulting from increased oxidative challenge (Giuliani *et al*., 2013). Non-synchronous effects were
276 also observed for *Se-dep*. *gpx* expression and Se-dep. GPx activity, with the former being down-
277 regulated by metal exposure and temperature variation in summer and by higher temperature in
278 winter, while the activity was unaffected. Similarly, the *gst-pi* expression was regulated by
279 acidification while the activity of GST was induced by warming in summer and unaffected in winter;

this may suggest both non-synchronous responses but also differential mechanisms triggering the responsiveness of genes transcription and the induction of catalytic activity. In the gills, limited variations were observed in *cat* expression in summer and similarly no effects were detected for catalytic activity. On the contrary, in winter *cat* mRNA was down-regulated by cadmium and acidification, both alone or in combination, but again no effects were found for enzyme activity. Concerning selenium-dependent glutathione peroxidases, transcriptional expression was down-regulated by warming with or without cadmium but on the contrary catalytic activity was enhanced in these organisms, suggesting once again non-synchronous effects. These time delayed effects were also observed in winter, where the effects on transcript were not paralleled at catalytic level, which was enhanced in almost all treatments with slight differences between them.

In conclusion, this study provided clear evidences that future ocean temperature and pH can interactively modulate transcriptional responses associated both directly and indirectly to metal-exposure; the observed effects are highly tissue- and season-specific, thus depending on tissue metabolic function and physiological characteristics influenced by seasonal life cycle. Nevertheless, our findings further contribute to the growing awareness on discrepancies between biological responses measured at transcriptional and catalytic level, confirming that mechanistic underlying the effects of these stressors are still to be fully comprehended.

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summer							
Treatment	measured parameters				calculated parameters		
	S	T (°C)	pH_{NBS}	A_T (µmol/kg)	*p*CO2 (µatm)	Ωc	Ωa
CTRL	37 ± 0.5	19.95 ± 0.10	8.21 ± 0.04	2453.6 ±251.5	380.8 ± 25.8	5.3 ± 0.4	3.5 ± 0.2
Cd	37 ± 0.5	20.00 ± 0.10	8.19 ± 0.04	2390.5 ± 354.1	410.6 ± 30.9	5.1 ± 0.4	3.3 ± 0.3
A	37 ± 0.5	19.98 ± 0.06	7.42 ± 0.04	2557.3 ± 183.7	2897.6 ± 183.8	1.0 ± 0.1	0.7 ± 0.1
W	37 ± 0.5	24.80 ± 0.13	8.15 ± 0.06	2325.4 ± 267.7	468.1 ± 47.9	5.4 ± 0.4	3.6 ± 0.3
A-Cd	37 ± 0.5	19.95 ± 0.06	7.41 ± 0.04	2556.7 ± 479.0	2928.2 ± 144.4	1.0 ± 0.1	0.7 ± 0.1
W-Cd	37 ± 0.5	24.83 ± 0.08	8.14 ± 0.04	2517.9 ± 206.9	477.4 ± 44.6	5.2 ± 0.4	3.5 ± 0.2
A-W	37 ± 0.5	24.76 ± 0.18	7.42 ± 0.03	2721.4 ± 215.7	3100.1 ± 241.7	1.2 ± 0.1	0.8 ± 0.1
A-W-Cd	37 ± 0.5	24.87 ± 0.16	7.43 ± 0.04	2504.2 ± 182	2993.7 ± 186.7	1.3 ± 0.1	0.9 ± 0.1

winter							
Treatment	measured parameters				calculated parameters		
	S	T (°C)	pH_{NBS}	A_T (µmol/kg)	*p*CO2 (µatm)	Ωc	Ωa
CTRL	37 ± 0.5	10.17 ± 0.30	8.14 ± 0.03	2453.6 ± 251.5	456.3 ± 38.8	6.1 ± 0.7	3.9 ± 0.4
Cd	37 ± 0.5	10.05 ± 0.33	8.15 ± 0.05	2390.5 ± 354.1	429.8 ± 35.9	6.2 ± 1.2	3.9 ± 0.7
A	37 ± 0.5	10.20 ± 0.30	7.38 ± 0.02	2325.4 ± 267.7	3009.9 ± 93.6	1.2 ± 0.1	0.8 ± 0.1
W	37 ± 0.5	14.97 ± 0.09	8.09 ± 0.06	2557.3 ± 183.7	492.7 ± 48.5	6.4 ± 1.1	4.1 ± 0.7
A-Cd	37 ± 0.5	10.08 ± 0.23	7.41 ± 0.04	2517.9 ± 206.9	2849.1 ± 145.4	1.2 ± 0.2	0.8 ± 0.1
W-Cd	37 ± 0.5	15.01 ± 0.08	8.12 ± 0.07	2556.7 ± 479.0	477.8 ± 73.2	6.9 ± 1.2	4.4 ± 0.8
A-W	37 ± 0.5	15.01 ± 0.02	7.38 ± 0.02	2721.4 ± 215.7	3192.6 ± 90.7	1.5 ± 0.1	0.9 ± 0.1
A-W-Cd	37 ± 0.5	14.88 ± 0.09	7.39 ± 0.02	2504.2 ± 182	3063.9 ± 195.5	1.6 ± 0.1	1.0 ± 0.1

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

Gene	Primer pair sequences	Amplicon size	Accession n.	Annealing T, time	References
Cu/Zn-sod	Fwd: AGCCAATGCAGAGGGAAAAGCAGA Rev: CCACAAGCCAGACGACCCCC	177 bp	FM177867	65°C, 1 min	Giuliani *et al.*, 2013
cat	Fwd: CGACCAGAGACAACCCACC Rev: GCAGTAGTATGCCTGTCCATCC	132 bp	AY743716	55°C, 15 sec 72°C, 1 min	Giuliani *et al.*, 2013
Se-dep. gpx	Fwd: AGCCTCTCTCTGAGGAACAACTG Rev: TGGTCGAACATGCTCAAGGGC	166 bp	HQ891311	55°C, 15 sec 72°C, 1 min	Giuliani *et al.*, 2013
gst-pi	Fwd: TCCAGTTAGAGGCCGAGCTGA Rev: CTGCACCAGTTGGAAACCGTC	172 bp	AF527010	55°C, 15 sec 72°C, 1 min	Giuliani *et al.*, 2013
hsp70	Fwd: GGTGGTGAAGACTTTGACAACAG Rev: CTAGTTTGGCATCGCGTAGAGC	295 bp	AY861684	65°C, 1 min	Cellura *et al.*, 2006
mt-20	Fwd: TGTGAAAGTGGCTGCGGA Rev: GTACAGCCACATCCACACGC	80 bp	AY566247	55°C, 15 sec 72°C, 1 min	Dondero *et al.*, 2005

Table 2 - Primer pair sequences, amplicon size, annealing temperatures and accession numbers of genes investigated in quantitative PCR.

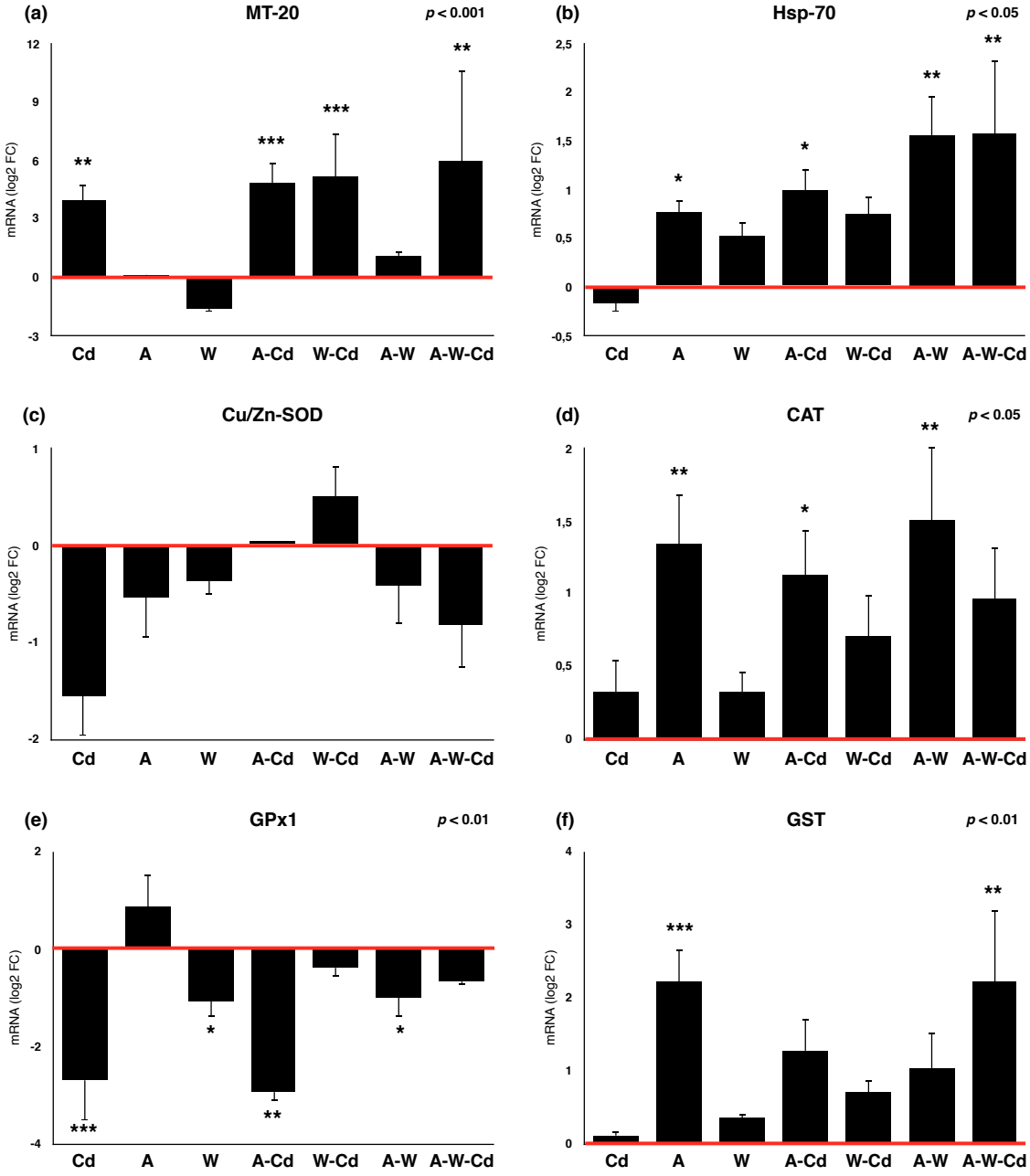


Figure 1 – mRNA levels of *mt-20* (a), *hsp-70* (b), *Cu/Zn-sod* (c), *cat* (d), *gpx1* (e) and *gst-pi* (f) in the digestive gland of mussels exposed in summer. Data are given as $\log_2$ of the fold change relative to CTRL treatment (red reference line) ± SEM (n=6). Asterisks indicate significant differences compared to CTRL treatment, *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. Cd= Cadmium; A= acidification; W= warming; A-Cd= acidification + Cd; W-Cd= warming + Cd; A-W= acidification + warming; A-W-Cd= acidification + warming + Cd.

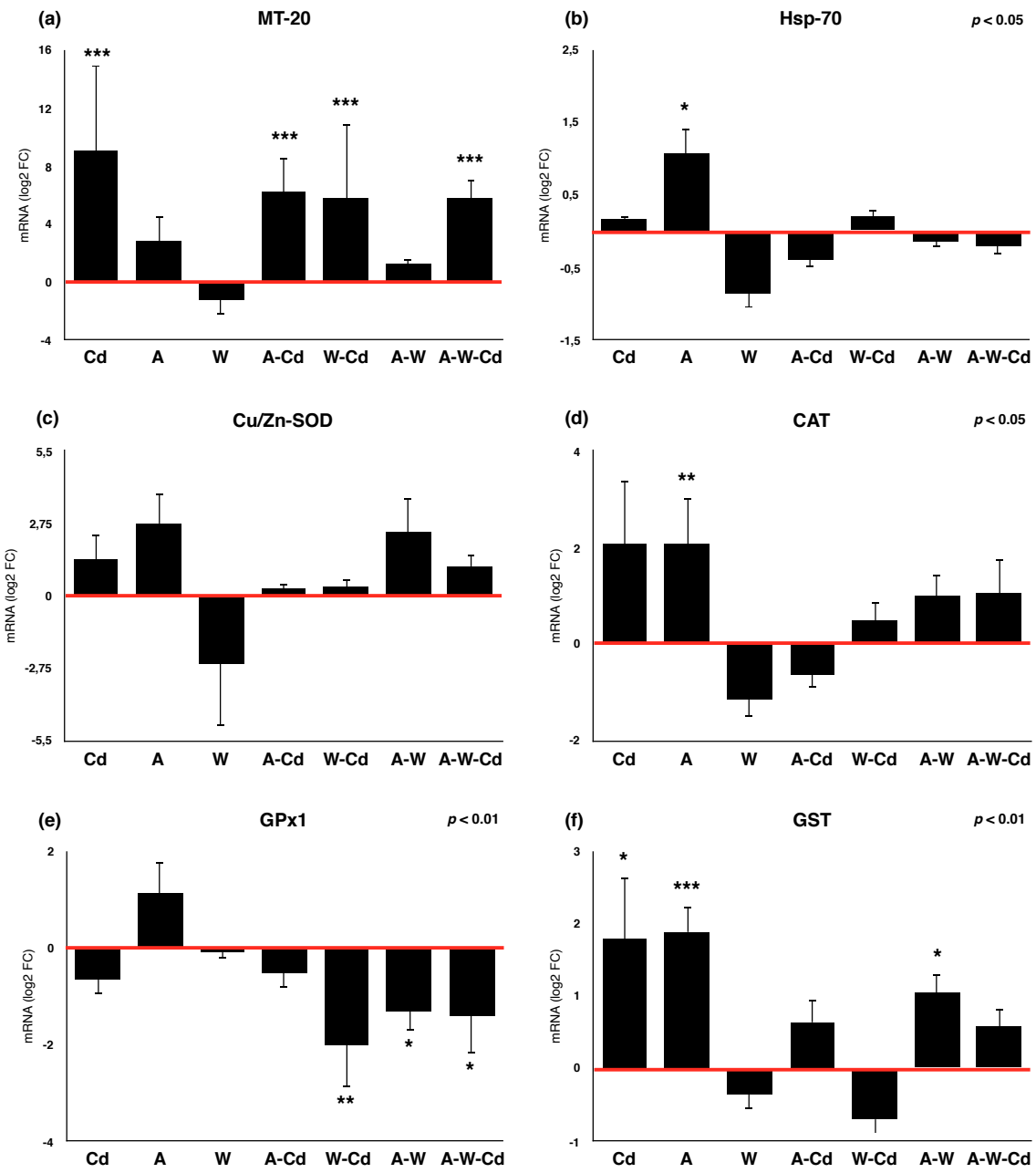


Figure 2 - mRNA levels of *mt-20* (a), *hsp-70* (b), *Cu/Zn-sod* (c), *cat* (d), *gpx1* (e) and *gst-pi* (f) in the digestive gland of mussels exposed in winter. Data are given as $\log_2$ of the fold change relative to CTRL treatment (red reference line) ± SEM (n=6). Asterisks indicate significant differences compared to CTRL treatment, *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. Cd= Cadmium; A= acidification; W= warming; A-Cd= acidification + Cd; W Cd= warming + Cd; A-W= acidification + warming; A-W-Cd= acidification + warming + Cd.

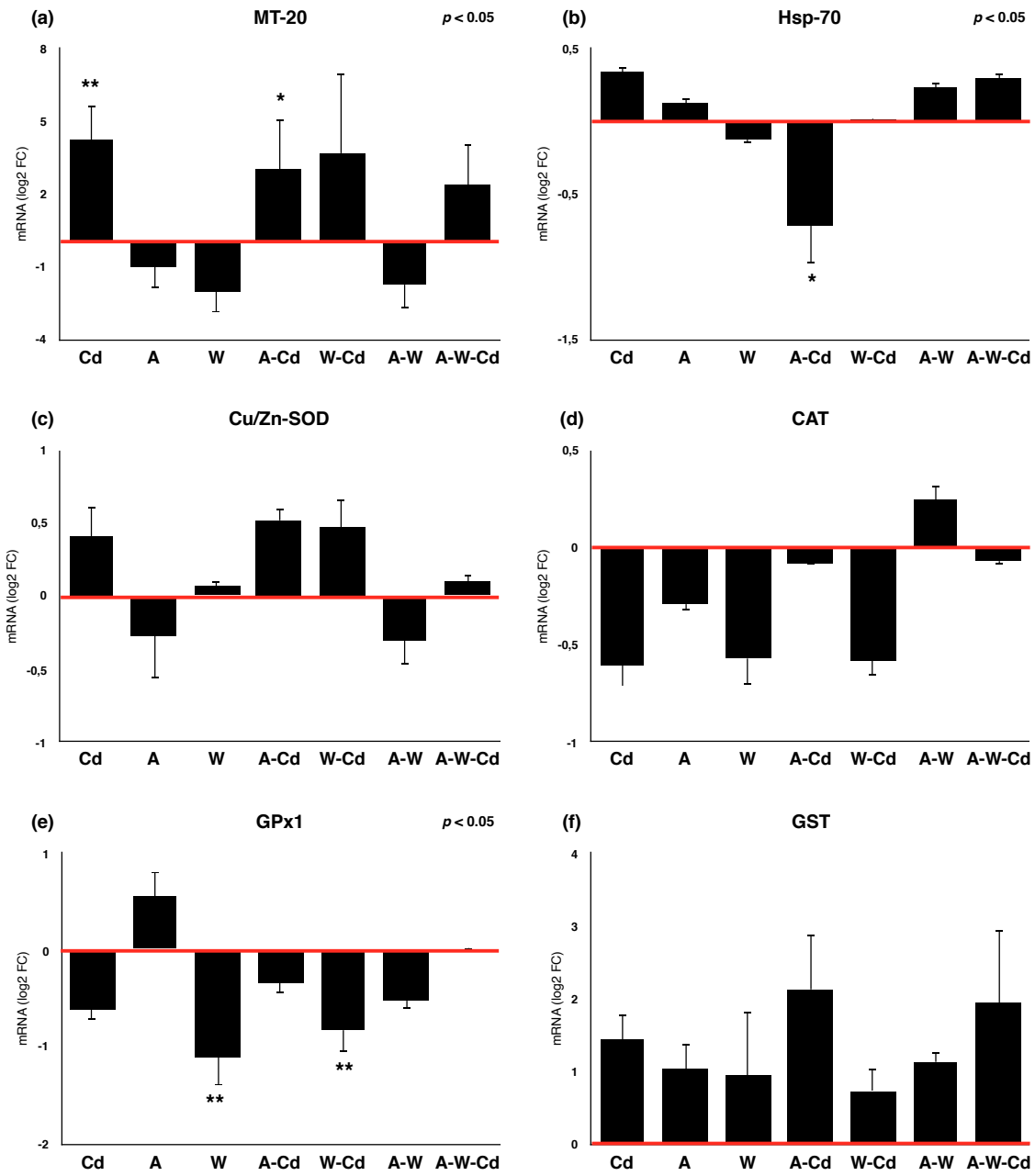
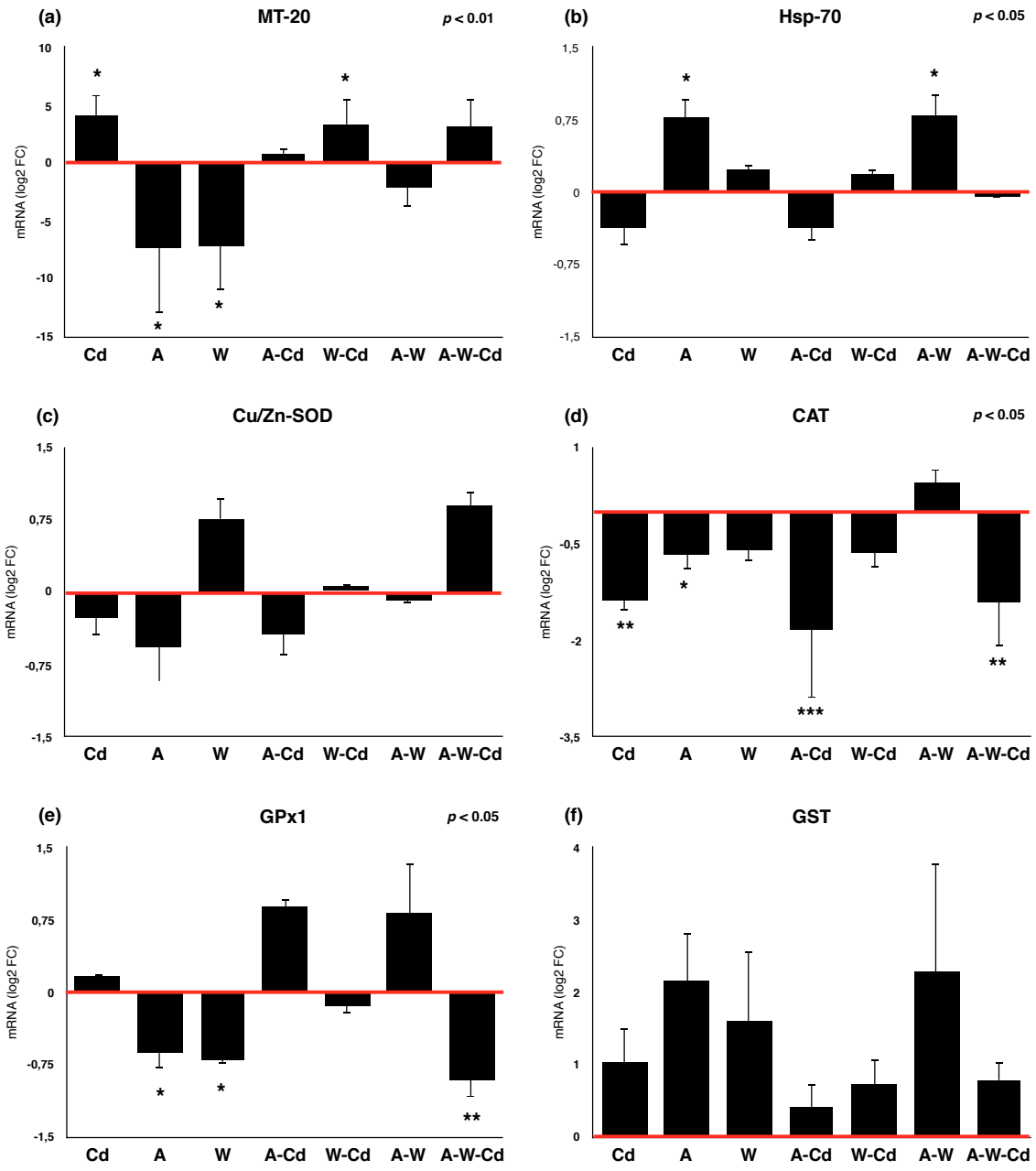


Figure 3 – mRNA levels of *mt-20* (a), *hsp-70* (b), *Cu/Zn-sod* (c), *cat* (d), *gpx1* (e) and *gst-pi* (f) in the gills of mussels exposed in summer. Data are given as $\log_2$ of the fold change relative to CTRL treatment (red reference line) ± SEM (n=6). Asterisks indicate significant differences compared to CTRL treatment, *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. Cd= Cadmium; A= acidification; W= warming; A-Cd= acidification + Cd; W-Cd= warming + Cd; A-W= acidification + warming; A-W-Cd= acidification + warming + Cd.



514

515 **Figure 4 -** mRNA levels of *mt-20* (a), *hsp-70* (b), *Cu/Zn-sod* (c), *cat* (d), *gpx1* (e) and *gst-pi* (f) in the gills of mussels

516 exposed in winter. Data are given as $\log_2$ of the fold change relative to CTRL treatment (red reference line) ± SEM

517 (n=6). Asterisks indicate significant differences compared to CTRL treatment, *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

518 Cd= Cadmium; A= acidification; W= warming; A-Cd= acidification + Cd; W-Cd= warming + Cd; A-W=

519 acidification + warming; A-W-Cd= acidification + warming + Cd.

520

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

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Abstract

Ocean acidification (OA) is expected to affect marine organisms in coastal polluted ecosystems through affecting metals bioaccumulation and indirectly modulating biological responsiveness to metals. In this study are presented the effects of reduced pH (pH/pCO_2 7.4/~3000 μatm) on cadmium uptake and on a wide battery of biomarkers in the smooth scallop *Flexopecten glaber* exposed to Cd (20 μg/L). Since both OA and metal exposure are suggested as pro-oxidant factors, the analyzed biomarkers reflected a complex network of responses linked to metal-exposure both directly, as metallothioneins, and indirectly, as single antioxidant defenses and total oxyradical scavenging capacity toward peroxyl and hydroxyl radicals. These were analyzed in both digestive gland and gills in order to investigate possible differences due to specific physiological function, while lysosomal membranes stability and onset of genotoxic damage were assessed in scallops haemocytes. Reduced pH slightly enhanced metal concentration in scallops' tissues, but no additional effects were measured in terms of metallothioneins induction. Induction of single antioxidant defenses by Cd and/or low pH exposure in the digestive gland was not reflected in variations of the total oxyradical scavenging capacity, while in the gills the exposure to these stress factors led to inhibition of certain single antioxidant defenses and reduction of the total oxyradical scavenging capacity toward peroxyl radical. Lysosomal membrane stability and onset of genotoxic damages measured in the haemocytes showed high sensitivity to the investigated factors, both alone and in combination, with possible synergistic effects. Available results allow to hypothesize that indirect effects of ocean acidification on metals accumulation and toxicity are tissue-specific and affect oxidative balance through different mechanisms; nevertheless this study confirmed the species-specific effects of multiple stressors interactions with implications in environmental protection of a key-commercial species.

1. Introduction

World oceans have absorbed about the 30% of anthropogenic emissions of carbon dioxide (CO_2) in the atmosphere (Le Quéré *et al.*, 2009). This is causing ocean acidification (OA), changes in the inorganic carbon system equilibriums in seawater and consequent reduction of ocean pH, which has dropped by 0.1 units since the beginning of industrial era (Gattuso and Lavigne, 2009) and is projected to further decrease by 0.14 to 0.35 units depending on CO_2 emissions scenarios (Caldeira and Wickett, 2005). Scientific literature now provides a wide body of evidences that future projections of ocean pH/pCO_2 could affect marine organisms health status by altering many physiological processes, *i.e.* calcification rates of marine shelled mollusks (Gazeau *et al.*, 2007; Jokiel *et al.*, 2008; Dupont *et al.*, 2010; Cerrano *et al.*, 2013), acid-base balance (Miles *et al.*, 2007; Spicer *et al.*, 2007; Gutowska *et al.*, 2010), metabolism (Lannig *et al.*, 2010; Stumpp *et al.*, 2012; Pan *et al.*, 2015), immune response (Bibby *et al.*, 2008; Hernroth *et al.*, 2011, 2012, 2016), larval development (Kurihara *et al.*, 2007; Dupont *et al.*, 2008; Ellis *et al.*, 2009; Stumpp *et al.*, 2011) and oxidative balance (Tomanek *et al.*, 2011; Rokitta *et al.*, 2012; Soriano-Santiago *et al.*, 2012; Pimentel *et al.*, 2015; Freitas *et al.*, 2016).

Even though direct impacts of OA on marine organisms physiology and health status have been deeply investigated, there's growing concern on the potential of OA to interact with other environmental stressors, as high levels of metal contamination that characterize coastal waters and sediments (Ivanina and Sokolova, 2015). OA is expected to increase the bioavailable fraction of certain metals, *i.e.* copper (Cu^{2+}), which form strong complexes with carbonate (CO_3^{2-}) and hydroxide (OH^-) ions, enhancing the ionic form of these metals which is the more bioavailable for marine organisms (Millero *et al.*, 2009). These predictions, based on chemical models, are supported by experimental evidences that high pCO_2/low pH regimes increased metals release from polluted sediments (Ardelan *et al.*, 2009; Ardelan and Steinnes, 2010; de Orte *et al.*, 2014a, 2014b) and increased metals bioaccumulation (Lacoue-Labarthe *et al.*, 2009, 2011; López *et al.*, 2010; Götze *et al.*, 2014; Ivanina *et al.*, 2014; Rodríguez-Romero *et al.*, 2014). To date, additive and synergistic

effects of high pCO_2/low pH and metal exposure have been reported also on biological effects in several invertebrates (Pascal *et al.*, 2010; Ivanina *et al.*, 2013, 2015; Lewis *et al.*, 2013; Basallote *et al.*, 2014; Campbell *et al.*, 2014; Götze *et al.*, 2014). Nonetheless, recently OA it has been proposed to affect the antioxidant status of different marine species and to interact with metal exposure in oxidative stress (Ricevuto *et al.*, 2015, 2016; Siddiqui *et al.*, 2015; Benedetti *et al.*, 2016; Nardi *et al.*, 2017), which is one of the most relevant mechanisms by which metals exert their toxicity by directly producing reactive oxygen species (ROS) or indirectly by altering the efficiency of cellular antioxidants (Regoli and Giuliani, 2014).

Since this growing concern on the interaction of ocean acidification and metal contamination suggested high levels of complexity of the effects, this study investigated the possible effects in terms of bioaccumulation and sub-lethal effects of cadmium in the smooth scallop *Flexopecten glaber.* Scallops are bivalve mollusks widely used in ecotoxicological studies due their ability to concentrate metals from the environment (Mauri *et al.*, 1990; Regoli *et al.*, 1998, 2000, 2002; Bustamante *et al.*, 2002) and have been recently addressed as potentially highly vulnerable to the effects of ocean acidification (Andersen *et al.*, 2013; Schalkhausser *et al.*, 2013; White *et al.*, 2013; Cooley *et al.*, 2015); *Flexopecten glaber* is widely distributed throughout the Mediterranean Sea and it has been recently suggested as a key commercial species (Marčeta *et al.*, 2016), especially in Northwestern Adriatic Sea where represents about 74% of shellfish fishery (Pujolar *et al.*, 2010; Mazzoldi *et al.*, 2014). These organisms are very common in shallow coastal and estuarine waters (Marčeta *et al.*, 2016) where larger CO_2 and pH fluctuations than open ocean are expected to occur in future years due to freshwaters inputs which influence carbonate chemistry and increase nutrients introduction, organic matter degradation and pollutants concentration (Nikinmaa, 2013; Wallace *et al.*, 2014; Wong *et al.*, 2014). Selected cellular responses investigated were intended to elucidate effects on a complex network of defenses against pollutants and oxidative insult (Regoli and Giuliani, 2014) and evaluated in both digestive gland and gills in terms of metallothioneins induction, variations of individual antioxidant defenses and total antioxidant capacity; nevertheless, lysosomal alterations and

onset of genotoxic damages were evaluated on haemocytes. The overall results of biomarkers responses were thus synthetized in a cellular hazard index through a quantitative hazard model (Sediqualsoft) which gives a different weight to various biological endpoints and magnitude of observed variations (Piva *et al.*, 2011; Benedetti *et al.*, 2012). Results obtained in the present study aimed to further contribute to the growing body of knowledge on the interactive effects of ocean acidification and metals focusing on a widely distributed and potentially vulnerable species still poorly investigated, evaluating possible differences in between different tissues and thus providing clear findings on multiple stressors effects.

2. Materials and Methods

2.1 Animal collection and experimental design

Scallops, *Flexopecten glaber* (4.5 ± 0.5 cm shell length), were obtained in June 2015 from a shellfish farm in an unpolluted area of Venice lagoon, Chioggia, Italy. Organisms were rapidly transported to the laboratory facilities and acclimatized for 7 days in aquaria with aerated artificial seawater (ASW; Instant Ocean®) at local environmental conditions of salinity (30 practical salinity units), temperature (20 °C) and pH_{NBS} (8.20) (measured *in situ* and confirmed by data obtained at www.chioggia.biologia.unipd.it/staz-idrobiolog/parametri-laguna/parametri-2015/).

At the end of the acclimation phase, scallops were then exposed to one of the following treatments, each containing 20 organisms in 20 L: 1) control condition (CTRL), at 20°C pH=8.20/ pCO_2=~400 µatm; 2) cadmium exposure at control condition (Cd), 20°C, pH=8.20/ pCO_2=~400 µatm and 20 µg/L cadmium; 3) acidification (A), 20°C, pH=7.40/ pCO_2=~3000 µatm; 4) acidification + Cd (A - Cd), 20°C, pH=7.40/ pCO_2=~3000 µatm and 20 µg/L cadmium. Cadmium concentration was chosen as representative of a polluted but environmentally realistic scenario in Mediterranean coastal waters (Neff, 2002), while selected target pH was based on scenario RCP 8.5 and the 2014 IPCC WGII AR5 (IPCC, 2014) where future pH of coastal waters is predicted to decrease further than what expected for the open ocean; target pH was reached by mixing ASW (pH=8.2) with small amounts

127 of CO_2-saturated ASW as described elsewhere (Nardi *et al.*, 2017). For each experimental condition
128 temperature, pH and salinity were measured daily, while total alkalinity (A_T) was measured twice
129 during the experiment according to Dickson *et al.*, 2007. Seawater carbonate parameters (pCO_2, and
130 saturation state (Ω) for calcite and aragonite) were calculated in CO2SYS (Pierrott *et al.*, 2006) using
131 barometric pressure values, as well as A_T, pH, temperature and salinity values for the respective
132 samples and full seawater chemistry is provided in Table 1. For calculations, we used NBS scale for
133 seawater pH, constants from Millero, 2010, KSO_4^- constant from Dickson *et al.*, 2007, and
134 concentration for silicate and phosphate for Instant Ocean® seawater (0.21 µmol/kg and 0.05
135 µmol/kg, respectively). Water was changed every other day, and scallops fed 12 hours prior the water
136 change with a commercial mixture of zooplankton (50-300 µm) for filter-feeding organisms.
137 After ten days, animals were sampled from each tank and tissues collected for chemical and
138 biological analyses. Gills and digestive glands were excised, pooled in 5 samples (each constituted
139 by tissues of 4 individuals), rapidly frozen in liquid nitrogen and maintained at -80°C until further
140 analyses of cadmium or biomarker responses. Haemolymph was withdrawn from the adductor muscle
141 of 5 specimens and immediately used for the measurement of lysosomal membranes stability and
142 onset genotoxic damages.
143
144 *2.2 Cadmium determination*
145 Cadmium (Cd) concentration in scallops' tissues was analyzed according to previously described
146 methods (Regoli *et al.*, 2005). For each treatment, digestive glands and gills were dried at 60°C
147 overnight and digested in a microwave digestion system (Mars CEM, CEM Corporation, Matthews
148 NC). Cd was analyzed by atomic absorption spectrophotometry (AAS) using graphite furnace
149 atomization and Zeeman effect (SpectrAA 300 Zeeman, Varian, Mulgrave, VIC, Australia). The
150 concentrations obtained for the standard reference material were always within the 95% confidence
151 interval of certified values. Data are expressed as µg/g dry weight (mean values ± standard deviation,
152 n = 5).

153 *2.3 Biomarkers responses*

154 Standardized protocols were used to analyze biomarkers in tissues of scallops (Nardi *et al.*,
155 2017), and have been fully detailed in Supplementary Material 1 (SM1). Briefly, metallothioneins
156 (MTs), single antioxidant defenses (catalase, glutathione S-transferases, glutathione peroxidases,
157 glutathione reductase activities and total glutathione), total oxyradical scavenging capacity toward
158 peroxyl radical (TOSC ROO•) and hydroxyl radical (TOSC HO•) were evaluated both in digestive
159 gland and gills of exposed scallops. Haemocytes were used to evaluate lysosomal membrane stability
160 (NRRT) and onset of genotoxic effects in terms of DNA strand breaks (Comet assay) and micronuclei
161 frequency (MN).

162

163 *2.4 Statistical analyses*

164 Analysis of variance (One-way ANOVA) was used to evaluate the effects of the treatments for
165 all investigated parameters, after checking the normal distribution (Shapiro-Wilk test) and
166 homogeneity of variances (Levene's Test). Level of significance was set to $p < 0.05$; *post-hoc* tests,
167 Student – Newman – Keuls (SNK), were used to compare group of means. All statistical analyses
168 were performed using RStudio (version 0.99.491). For biomarkers data, multivariate principal
169 component analysis (PCA) was applied to visualize the relationships among the different treatments.

170 The whole dataset of biomarkers results was summarized in a hazard index for each treatment
171 elaborated through a previously developed quantitative model which applies weighted criteria to
172 discriminate different endpoints and the magnitude of effects (Sediqualsoft, Piva *et al.*, 2011). The
173 general mechanism of the model is to compare variations of biomarkers to a specific threshold, which
174 consider the possibility of biphasic responses and the different responsiveness among various species
175 and tissues. The calculated Hazard Quotient (HQ) does not include biomarkers with variations lower
176 or equal to their threshold, averages or adds the summation (Σ) respectively for those biomarkers with
177 variations up to 2-fold or more than 2-fold greater than the specific threshold (Piva *et al.*, 2011;
178 Benedetti *et al.*, 2012; Regoli *et al.*, 2014; Avio *et al.*, 2015). The model finally assigns the elaborated

179 HQ in one of five classes of hazard, from Absent to Severe (Piva *et al.*, 2011). Whole calculations
180 and assumptions have been fully given elsewhere (Piva *et al.*, 2011; Benedetti *et al.*, 2012).

3. Results

Cd exposure led to higher concentration of the metal in both digestive gland and gills of exposed scallops (Fig.1a and 1b). We observed a marked, although not significant, higher accumulation of Cd in digestive gland of organisms exposed to the metal at lower pH compared to those exposed at control pH. In the gills, significant Cd accumulation occurred only in organisms exposed al lower pH (A-Cd). Neither Cd-exposure nor pH-reduction influenced metallothioneins levels (Fig.2a) in the digestive gland of scallops. Similarly, in the gills metallothioneins (Fig.3a) were not affected by Cd-exposure or pH-reduction.

Antioxidant system showed high sensitivity to the investigated factors, with tissue-specificities. In the digestive gland, catalase activity (Fig.2b) was enhanced in organisms exposed to lower pH, and similar effects was observed also for GST activity and Se-Dep. GPx (Fig.2c and 2d), but for these enzymes we also found out interactive effects of Cd-exposure and pH-reduction. On the other hand, total glutathione (Fig.2g) was highly enhanced by both Cd-exposure and pH reduction. Beside these punctual variations, we did not observe any significant difference between the experimental treatments concerning the TOSC against both ROO• and HO• (Fig.2h and 2i), although a slight but not significant reduction of the former was observed due to Cd and acidification, both alone or in combination.

Interactive effects of Cd and acidification on the antioxidant system were found on GST and Se-Dep. GPx activity in the gills (Fig. 3c and 3d). GST activity was inhibited by additive effects of Cd and acidification. Se-Dep GPx activity, was significantly inhibited by Cd alone, but slighter effects were observed also due to acidification. The variations observed for the single antioxidant defenses were consistent with a significant reduction of the TOSC toward ROO• (Fig.3h), while no variation was observed for the TOSC toward HO• (Fig.3i).

Haemocytes lysosomal membranes stability (Fig.4a) was significantly reduced by co-exposure to Cd and acidification, with stronger role of the latter. No variations in terms of DNA fragmentation

206 (Fig.4b) were observed, while on the other hand, MN frequency (Fig.4c) was strongly enhanced by
207 both Cd and acidification, alone or in combination.

208 The principal component analysis conducted on the whole biomarkers dataset provided a two-
209 dimensional pattern explaining almost 84% of the total variance (Fig.5). This showed clear separation
210 between control and treated organisms, with further discrimination between Cd-exposure and
211 acidification condition, both with and without cadmium. The parameters determining the separation
212 along PC1 axis, mainly between control pH vs. acidification-exposed organisms, were some
213 antioxidant parameters both in the digestive gland (CAT, GST, GPx and TOSC HO•) and in the gills
214 (GST and TOSC ROO•) and micronuclei frequency, while along the PC2 axis the distribution, the
215 separation was determined by Cd-exposure related parameters, as MTs in both tissues, certain
216 antioxidants measured in the gills (both GPxs, TGSH) and in the digestive gland (TGSH, TOSC
217 ROO•).

218 The elaboration of biomarkers dataset within a quantitative model provided synthetic hazard
219 quotients (HQs) for each experimental condition, which was classified as "Slight" for organisms
220 exposed to Cd alone and "Moderate" for organisms exposed to the acidification condition; despite
221 the same classification of hazard, higher HQ was measured in scallops exposed to acidification alone
222 respect to those exposed to acidification and cadmium (Fig.6).

223

224 **4. Discussions**

225 This study provided clear evidences that future projections of pH under ocean acidification
226 condition can modulate cadmium bioaccumulation and biological effects at cellular level in temperate
227 scallops *Flexopecten glaber*.

228 Ocean acidification effects on metals bioaccumulation is controversial: some authors described
229 increased metal uptake in marine organisms' tissues under reduced pH conditions (Lacoue-Labarthe
230 *et al.*, 2009, 2011; López *et al.*, 2010; Ivanina *et al.*, 2014; Götze *et al.*, 2014; Rodriguez-Romero *et*
231 *al.*, 2014; Shi *et al.*, 2016) while others did not observe this effect (Benedetti *et al.*, 2016; Ivanina *et*

al., 2016; Ricevuto *et al.*, 2016; Nardi *et al.*, 2017). In the present study we observed a slight but not significant increase of cadmium concentration in scallops' digestive gland, while significant accumulation of the metal occurred in the gills of organisms exposed to pH reduction, suggesting that pH effects on bioaccumulation of metals are difficult to predict, are metal-, tissue- and species-specific and exposure duration could have played an important role in determining Cd uptake.

Despite cadmium is known to induce metallothioneins, in the present study MTs levels were not affected by metal-exposure nor in digestive gland and in the gills; this may be due the high basal levels of cadmium in the digestive gland and gills of *F. glaber*, and more in general in scallops (Mauri *et al.*, 1990; Regoli *et al.*, 1998, 2000, 2002; Bustamante *et al.*, 2002), that probably leads to lower sensitivity of metallothioneins induction pathways; nevertheless, no additional effects of acidification were observed: this agree with previously published results on the Antarctic scallop *A. colbecki* exposed to cadmium and acidification (Benedetti *et al.*, 2016).

Analysis of the antioxidant system, combining single antioxidant defenses and total oxyradical scavenging capacity, revealed complex interactions between cadmium and acidification. Concerning the digestive gland, acidification enhanced catalase, glutathione S-transferase and Se-dep. GPx activity in addition to total glutathione levels. Catalase and Se-dep. GPx are involved in the detoxification of hydrogen peroxide, while GST is related to metabolism of lipid hydroperoxides (Regoli and Giuliani, 2014): this suggests that pH reduction promotes oxidative insult through the generation of peroxides as also hypothesized by previous studies on the oysters *Crassostrea virginica* and *Crassostrea gigas*, the polychaete *Sabella spallanzanii* and the scallop *Adamussium colbecki* (Tomanek *et al.*, 2011; Moreira *et al.*, 2016; Ricevuto *et al.*, 2016; Benedetti *et al.*, 2016). This inductive effect was less evident in organisms co-exposed to cadmium and acidification: significant lower enzymatic activity of GST was observed along to slight but not significant lower activity of Se-dep. GPx and level of TGSH in organisms exposed to cadmium and acidification (A-Cd) compared to those exposed to acidification (A), which can be interpreted as antagonistic effects of

257 the investigated stressors due to Cd-mediated impairment of antioxidant defenses (Regoli, 2012;
258 Regoli and Giuliani, 2014).

259 Despite these changes, no variation was observed for the TOSC toward ROO• and HO•, allowing
260 to hypothesize good capability to compensate variations in analyzed single antioxidants through the
261 involvement of other antioxidants as superoxide dismutase or peroxiredoxines, addressed as one of
262 the main pathways involved by acidification of the mantle proteasome of *Crassostrea virginica*
263 (Tomanek *et al.*, 2011).

264 In the gills, less evident effects where observed, with the exception of the co-exposure to
265 cadmium and acidification that led to inhibition of glutathione S-transferase. This may reflect
266 different capability to counteract oxidative stress in the gills compared to digestive gland and the
267 tissue-specific effects of multiple stressors, as previously observed in *Mytilus galloprovincialis*
268 exposed to cadmium and acidification (Nardi *et al.*, 2017). Nevertheless, these results are in contrast
269 with those obtained in the gills of the Antarctic scallop *A. colbecki*, in which the co-exposure to Cd
270 and acidification led to increased GST activity, thus confirming the species-specific effects probably
271 due to physiological characteristics that reflect adaptation to specific environmental features
272 (Benedetti et al., 2016). The limited variations in single antioxidant defenses were though followed
273 by a marked decrease of the total oxyradical scavenging capacity toward the peroxyl radical in
274 organisms exposed to reduced pH with or without cadmium: these results confirm previously
275 hypothesized involvement of other antioxidant pathways and the tissue-specificity of effects, and are
276 similar to what observed in the digestive gland of *M. galloprovincialis* exposed to cadmium and
277 acidification (Nardi *et al.*, 2017).

278 Haemocytes investigation confirmed the susceptibility of *F. glaber* to the investigated stressors
279 and the pro-oxidant challenge of cadmium and acidification; our results showed interactive effects of
280 acidification and cadmium exposure which reduces the stability of lysosomal membranes rather than
281 effects of single stressors. Similar effects, although mainly due to acidification, were observed in
282 haemocytes of mussels *Mytilus edulis* and *Mytilus galloprovincialis* (Beeseley *et al.*, 2008; Nardi *et*

283 *al.*, 2017); in addition, concerning the onset of genotoxic damages, we did not measure DNA
284 fragmentation, but an enhancement of micronuclei frequency after cadmium and acidification
285 exposure, with no synergistic effects of these when combined. This is in contrast with observations
286 conducted on haemocytes of *M. galloprovincialis* exposed to cadmium and acidification where
287 synergistic effects of these stressors occurred (Nardi *et al.*, 2017), but are comparable to those
288 obtained on the Antarctic scallop *A. colbecki* exposed to Cd and reduced pH (Benedetti *et al.*, 2016).
289 Principal components analysis pattern and the elaboration within the quantitative model revealed
290 clear separation between organisms exposed to Cd alone and those exposed to acidification, with or
291 without cadmium. This suggests that acidification pose a major threat compared to cadmium exposure
292 to *F. glaber*; nevertheless, the few differences observed in these data elaborations suggest that Cd
293 effects are masked under ocean acidification condition, allowing to hypothesize that metal exposure
294 effects are modulated under stress condition determined by acidification.
295 In conclusion, this study provided clear evidences that ocean acidification can modulate
296 organisms responsiveness to metal exposure. Our results indicate that reduced pH can slightly affect
297 Cd uptake and, despite this, the sub-lethal effects can be highly affected by the interactions of the
298 investigated stressors, in a tissue-specific manner.
299

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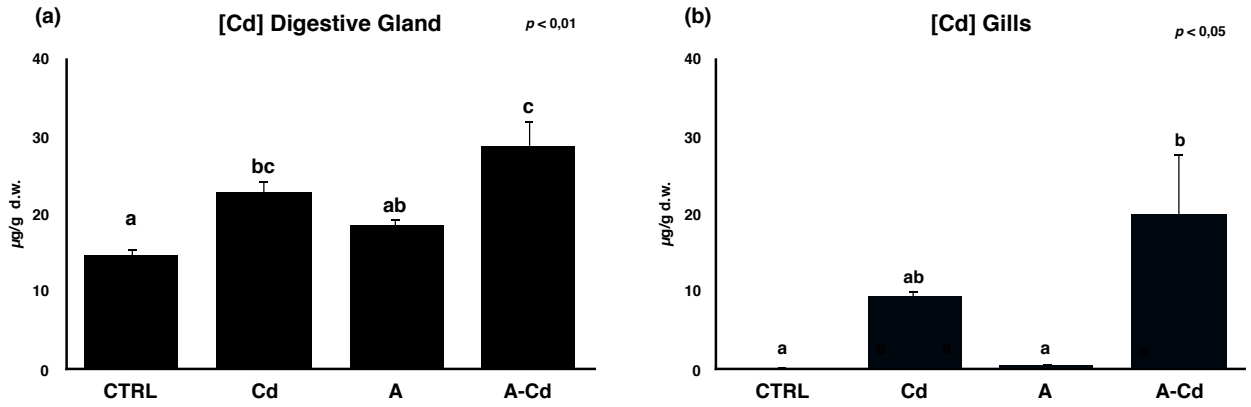

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515 Fig.1 – Cadmium bioaccumulation in digestive gland (a) and gills (b) of exposed scallops. Data are
516 expressed as mean values ± standard deviations (n=5). Different letters indicate significant
517 differences between group of means (ANOVA and SNK post-hoc). CTRL= Control; Cd= Cadmium;
518 A= acidification; A-Cd= acidification + Cd.

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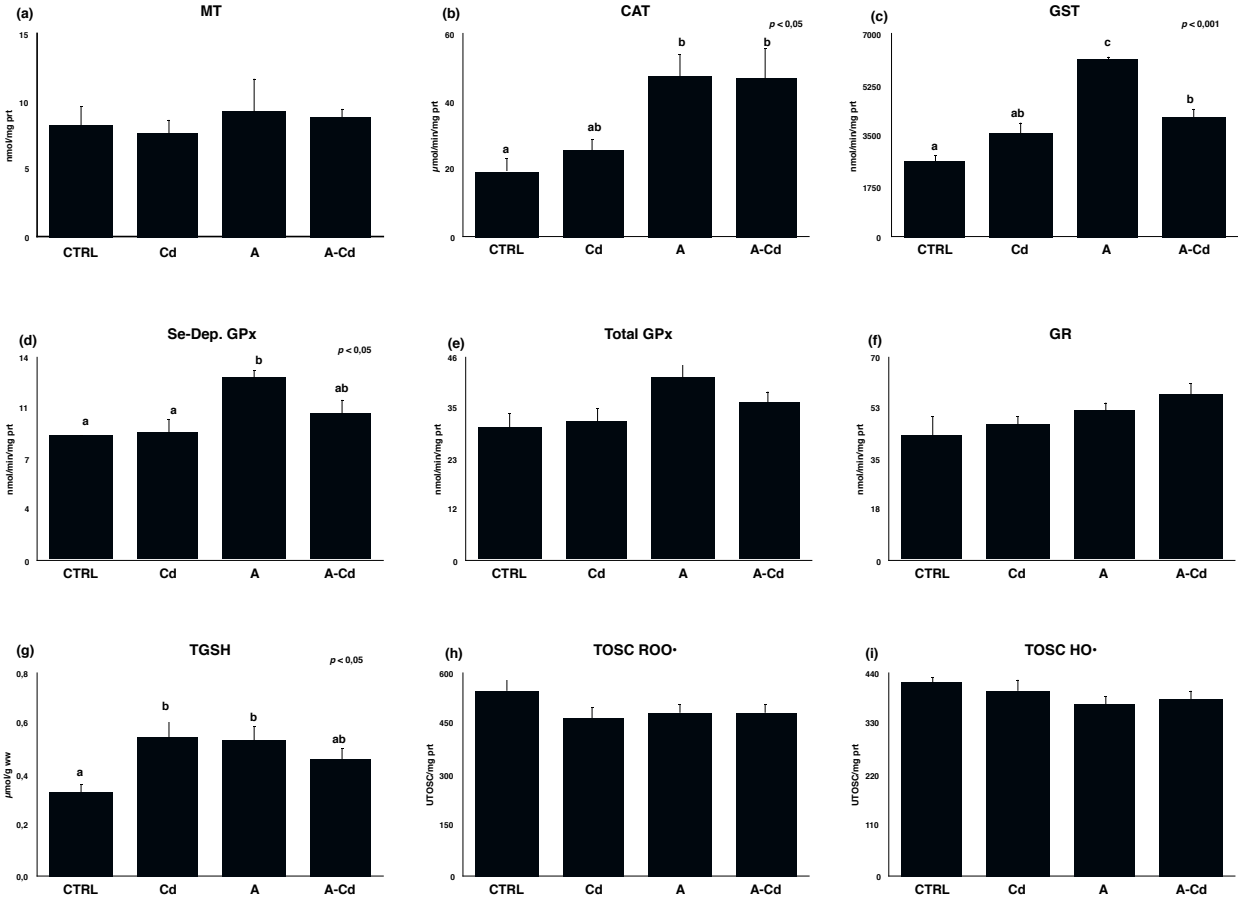


Figure 2 – Metallothioneins an antioxidant defenses in digestive gland of scallops exposed to various treatments. MT: metallothioneins (a), CAT: catalase (b), GST: glutathione S-transferase (c), Se-Dep. GPx: Se-dependent glutathione peroxidases (d) total GPx: sum of Se-dependent and Se-independent glutathione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

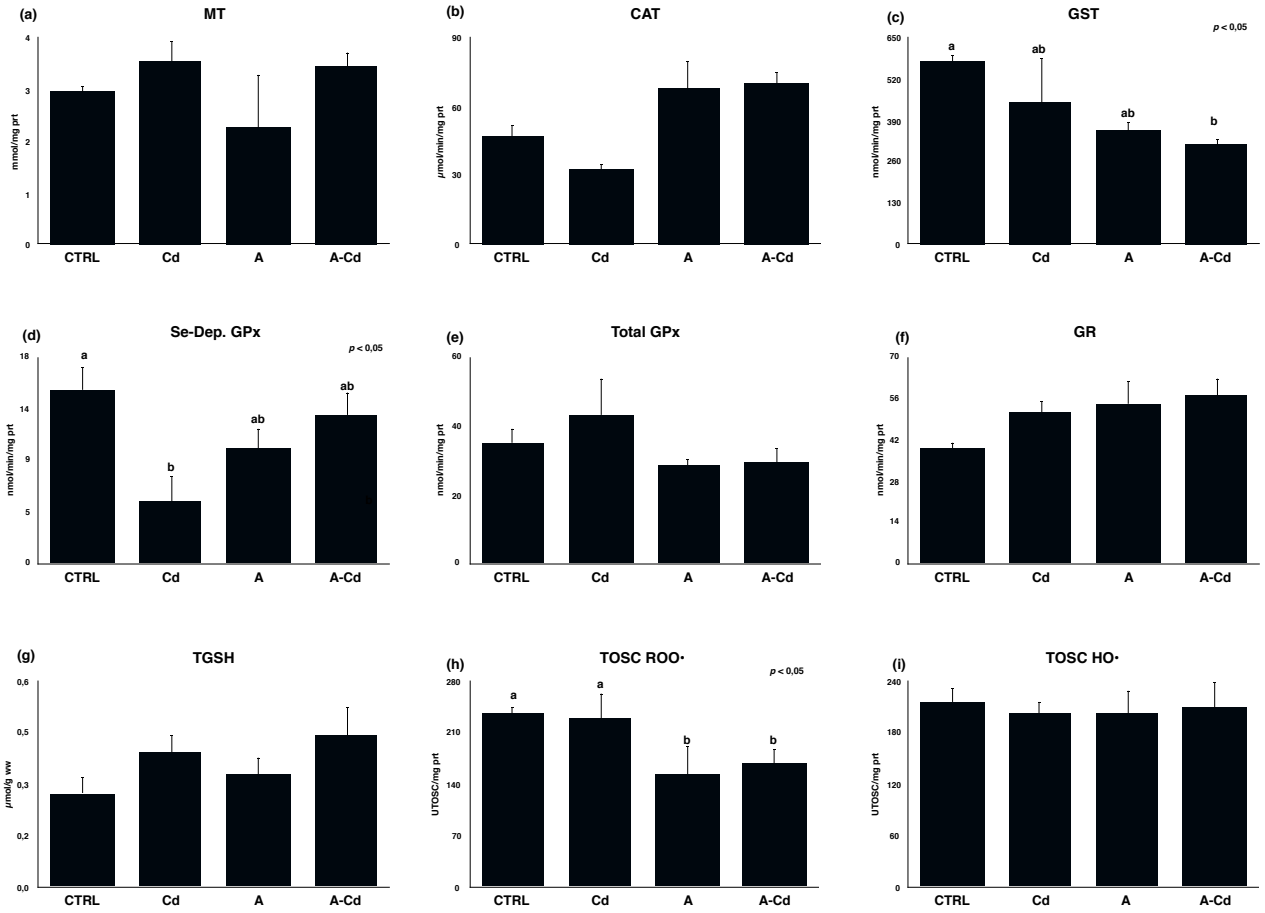


Figure 3 – Metallothioneins an antioxidant defenses in gills of scallops exposed to various treatments. MT: metallothioneins (a), CAT: catalase (b), GST: glutathione S-transferase (c), Se-Dep. GPx: Se-dependent glutathione peroxidases (d) total GPx: sum of Se-dependent and Se-independent glutathione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

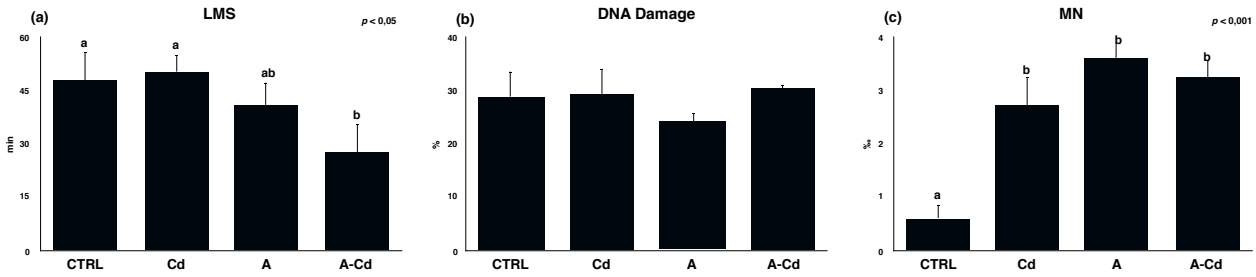
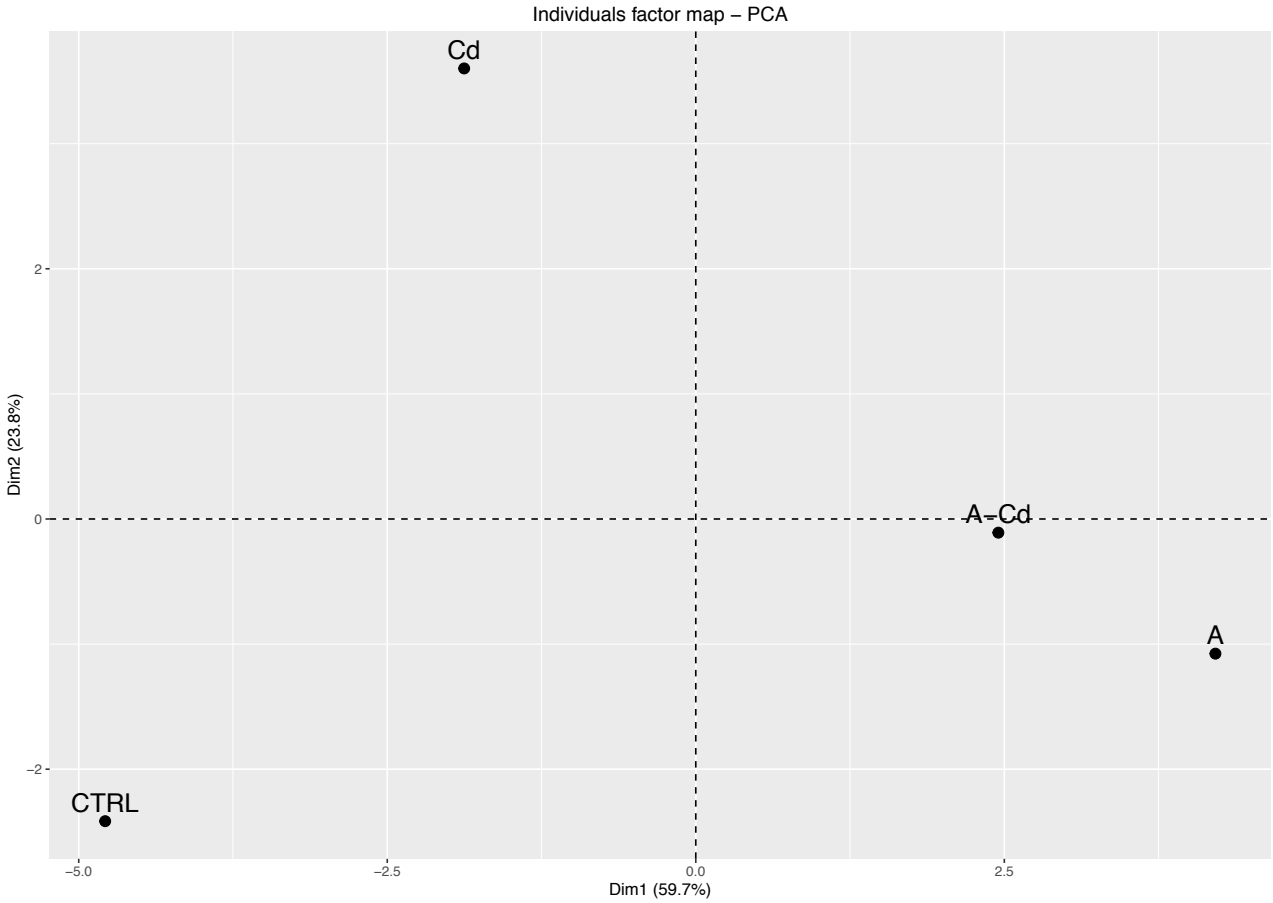


Figure 4 - Lysosomal membrane stability (a), DNA damage (b) and frequency of micronuclei (c) in haemocytes of scallops exposed to various treatments. Data are given as mean values ± SEM (n=5). Different letters indicate significant differences between group of means (ANOVA and SNK *post-hoc*). CTRL=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



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547 Figure 5 – Principal components analysis individuals factor map of scallops exposed to various
548 treatments. CTRL= Control; Cd= Cadmium; A= acidification; A-Cd= acidification + Cd.

549

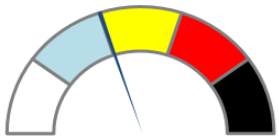
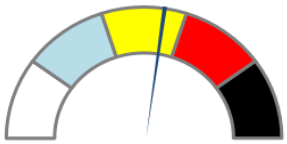
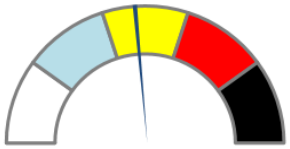
Experimental Treatment	Hazard Quotients (HQs)	Class of Hazard	Level
Cd	9.06	SLIGHT	
A	21.07	MODERATE	
A-Cd	13.13	MODERATE	

Figure 6 - Weight of Evidence (WOE) classification of biomarkers data for the whole dataset of analyzed parameters for each different laboratory condition. The quantitative hazard quotients (HQ) and the assigned class of hazard are given.

Supplementary Material SM1

Detailed protocols for biomarkers analyses

S1.1 Metallothioneins

Metallothioneins (MTs) were analyzed in gills and digestive gland samples homogenized (1:3 and 1:5 w/v respectively) in 20 mM Tris–HCl buffer, pH 8.6, 0.5M sucrose, 0.006mM leupeptin, 0.5 mM PMSF (phenylmethylsulphonylfluoride), and 0.01% b-mercaptoethanol. After acidic ethanol/chloroform fractionation of the tissue homogenate, MTs were quantified by the spectrophotometric assay as described in Viarengo *et al.* (1997) using GSH as standard.

S1.2 Single antioxidant defenses and Total Oxyradical Scavenging Capacity (TOSC) assay

For enzymatic antioxidants, digestive gland and gills samples were homogenized (1:5 and 1:3 w:v ratio respectively) in 100 mM K-phosphate buffer (pH 7.5), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mg/ml bacitracin, 0.008 TIU/ml aprotinin, 1 μg/ml leupeptin, 0.5 μg/ml pepstatin, NaCl 2.5%, and centrifuged at 100 000 × g for 70 min at 4 °C. Activities were measured with a Varian (model Cary 100 Scan) spectrophotometer at a constant temperature of 18 °C (Bocchetti *et al.*, 2008).

Catalase (CAT) was measured by the decrease in absorbance at 240 nm (extinction coefficient, $\varepsilon = 0.04$ mM−1 cm−1) due to the consumption of hydrogen peroxide, H_2O_2 (12 mM H_2O_2 in 100 mM K-phosphate buffer pH 7.0).

Glutathione S-transferases were determined at 340 nm using 1-chloro-2,4-dinitrobenzene as substrate (CDNB). The assay was carried out in 100 mM K-phosphate buffer pH 6.5, 1.5 mM CDNB, 1 mM GSH ($\varepsilon = 9.6$ mM−1 cm−1).

Glutathione peroxidases activities were assayed in a coupled enzyme system where NADPH is consumed by glutathione reductase to convert the formed GSSG to its reduced form (GSH). The decrease of absorbance was monitored at 340 nm ($\varepsilon = -6.22$ mM−1 cm−1) in 100 mM K-phosphate buffer pH 7.5, 1 mM EDTA, 1 mM sodium azide (NaN3) (for hydrogen peroxide assay), 2 mM GSH, 1 unit glutathione reductase, 0.24 mM NADPH, and 0.5 mM hydrogen peroxide or 0.8 mM cumene hydroperoxide as substrates, respectively, for the selenium-dependent and for the sum of Se-dependent and Se-independent forms.

Glutathione reductase (GR) was determined from NADPH oxidation during the reduction of oxidized glutathione, GSSG ($\lambda = 340$ nm, $\varepsilon = -6.22$ mM−1 cm−1). The final assay conditions were 100 mM K-phosphate buffer pH 7.0, 1 mM GSSG, and 60 μM NADPH.

589 Total glutathione was analyzed in samples homogenized (1:5 w:v ratio) in 5% sulfosalicilic
590 acid with 4 mM EDTA, maintained for 45 min on ice and centrifuged at 37.000 × g for 15 min, before
591 being enzymatically assayed (Akerboom & Sies, 1981).
592 The total oxyradical scavenging capacity (TOSC) assay measures the overall capability of
593 cellular antioxidants to absorb different forms of artificially generated oxyradicals, thus inhibiting the
594 oxidation of 0.2 mM α-keto-γ-methiolbutyric acid (KMBA) to ethylene gas (Winston *et al.*, 1998;
595 Regoli & Winston, 1999). Peroxyl radicals (ROO•) were generated by the thermal homolysis of 20
596 mM 2-2′-azo-bis-(2-methylpropionamidine)-dihydrochloride (ABAP) in 100 mM K-phosphate
597 buffer, pH 7.4. Hydroxyl radicals (HO•) were produced by the Fenton reaction of iron-EDTA (1.8
598 μM Fe^{3+}, 3.6 μM EDTA) plus ascorbate (180 μM) in 100 mM K-phosphate buffer. Ethylene
599 formation in control and sample reactions was analyzed at 10–12 min time intervals by gas-
600 chromatographic analyses and the TOSC values are quantified from the equation: TOSC = 100 −
601 (∫SA/∫CA × 100), where ∫SA and ∫CA are the integrated areas calculated under the kinetic curves for
602 samples (SA) and control (CA) reactions. For all the samples, a specific TOSC (normalized to content
603 of protein) was calculated by dividing the experimental TOSC values by the relative protein
604 concentration contained in the assay, measured by the spectrophotometric method of Lowry *et al.*,
605 1951 with bovine serum albumin (BSA) as standard.
606
607 S1.3 Lysosomal membranes integrity and genotoxic damages
608 For the analysis of lysosomal membrane strability, the cationic probe Neutral Red (NR) was
609 used to evaluate the capability of the lysosomal membranes to retain the dye (Lowe *et al.*, 1995).
610 Haemocytes were incubated on a glass slide with a freshly prepared NR working solution (2 μl/ml
611 filtered seawater) from a stock solution of 20 mg neutral red dye dissolved in 1 ml of dimethyl
612 sulfoxide, and microscopically examined at 15 min intervals, to determine the time at which 50% of
613 cells had lost to the cytosol the dye previously taken up by lysosomes.
614 The onset of DNA damage was evaluated at molecular level as single strand breaks (SB) by the
615 Comet assay, and at chromosomal level by the frequency of micronuclei.
616 The comet assay was carried out on haemocytes collected from the adductor muscle of
617 organisms according to Gorbi *et al.*, 2008. Cells were diluted in Ca^{2+}/Mg^{2+}-free buffer at 4 °C in the
618 dark (20 mM Hepes, 500 mM NaCl, 12.5 mM KCl, 10 mM EDTA, pH 7.3), centrifuged at 1000 rpm
619 for 1 min at 4 °C, resuspended in 0.6% low-melting-point agarose, and added with a sandwich
620 stratification to glass slides coated with 1% normal-melting-point agarose. After gel solidification,
621 slides were placed into the lysing solution (2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, and 10%
622 DMSO, pH 10) at 4 °C in the dark for 90 min. DNA was unwound in 75 mM NaOH, 10 mM EDTA

(pH 13), and the electrophoretic migration was carried out in the same buffer at 1 V/cm for 10 min. Slides were then neutralized for 10 min in 0.4 M Tris, pH 7.5, fixed in cold methanol for 3 min at −20 °C, and dried. After staining with DAPI, 100 randomly selected "nucleoids" per slide, and two replicates per sample, were examined under fluorescence microscopy (200 × magnification; Olympus BX-51), and the captured images (Image-Pro-Plus package) were analyzed through the software Comet Score. The percentage of DNA in the tail was used to estimate the level of DNA fragmentation.

For the frequency of micronuclei, an aliquot of haemolymph collected from the adductor muscle of organisms was rapidly washed in saline buffer (20 mM Hepes, 500 mM NaCl, 12.5 mM KCl, 10 mM EDTA, pH 7.3) and centrifuged at 1000 rpm for 1 min at 4 °C, following the procedure described in Nigro *et al.*, 2006. Cells were immersed in a fixative solution (3:1 methanol, acetic acid) and washed twice. Suspended cells were dispersed on glass slides, air dried and stained with the fluorescent dye 4′,6-diamidino-2-phenylindole DAPI (100 ng/ml). For each specimen, 2000 cells with preserved cytoplasm were scored to determine the frequency of micronuclei, defined as round structures, smaller than 1/3 of the main nucleus diameter on the same optical plan and clearly separated from it.

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