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

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ABSTRACT

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

1. Introduction

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

Beside the direct effects, there is growing interest for the potential interaction of OA with other environmental stressors, such as the high levels of metal contamination in coastal environments (Ivanina and Sokolova, 2015). In this respect, OA is supposed to increase the ionic and bioavailable fraction of certain metals like copper (Cu^{2+}), which typically form strong complexes with carbonate (CO_3^{2-}) and hydroxide (OH^-) ions (Millero et al., 2009). These model predictions have been confirmed by some experimental evidence revealing that high pCO_2/low pH regimes can increase the release of metals from polluted sediments (Ardelan et al., 2009; Ardelan and Steinnes, 2010; de Orte et al., 2014a, 2014b) and enhance their bioaccumulation (Duckworth et al., 2017; Götze et al., 2014; Ivanina et al., 2014; Lacoue-Labarthe et al., 2009, 2011; López et al., 2010; Rodríguez-Romero et al., 2014). Synergistic effects of high pCO_2/low pH and metal exposure were recently reported on several cellular responses of marine invertebrates (Götze et al., 2014; Ivanina et al., 2013, Ivanina et al., 2015; Lewis et al., 2013), including the antioxidant status and the onset of oxidative stress (Benedetti et al., 2016; Nardi et al., 2017; Ricevuto et al., 2015, 2016; Siddiqui and Bielmyer-Fraser, 2015), which is one of the most relevant pathways by which trace elements exert their toxicity through a

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sophisticated array of molecular and cellular effects (Regoli and Giuliani, 2014).

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

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 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).

Scallops were then randomly assigned 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 (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 exposure was representative of a polluted but environmentally realistic scenario (Neff, 2002), while the selected target pH was based on scenario RCP 8.5 and the 2014 IPCC WGII AR5 (IPCC, 2014) where future decrease in coastal waters is predicted to be higher than in open ocean; the target pH was reached by mixing ASW (pH = 8.2) with small amounts of CO_2-saturated ASW as described elsewhere (Nardi et al., 2017). For each experimental condition temperature, pH and salinity were measured daily, while total alkalinity (A_T) was measured every three days during the experiment according to Dickson et al., 2007. Seawater carbonate parameters (pCO_2, and saturation state (Ω) for calcite and aragonite) were calculated in CO2SYS using barometric pressure values (Pierrot et al., 2006); A_T, pH, temperature, salinity values and full seawater chemistry are provided in Table 1. For calculations, we used NBS scale for seawater pH, carbonate constants from Millero (2010), KSO_4^- constant from Dickson et al. (2007) and concentration of silicate and phosphate from Instant Ocean® composition (0.21 µmol/kg and 0.05 µmol/kg, respectively). Water was changed every other day, and scallops fed 12 h prior to the water change with a commercial mixture of zooplankton (50–300 µm) for filter-feeding organisms.

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

2.2. Cadmium determination

Cadmium (Cd) concentrations in scallops were analyzed according to previously described methods (Regoli et al., 2005). For each treatment, digestive glands and gills were dried at 60 °C overnight and digested in a microwave digestion system (Mars CEM, CEM Corporation, Matthews NC). Cd was analyzed by atomic absorption spectrophotometry (AAS) using graphite furnace atomization and Zeeman effect (SpectrAA 300 Zeeman, Varian, Mulgrave, VIC, Australia). Quality assurance and quality control was assessed by processing blank samples and reference standard material (Mussel Tissue Standard Reference Material SRM NIST-2977, National Institute of Standards and Technology Gaithersburg, MD, USA). The concentrations obtained for the SRM were always within the 95% confidence interval of certified values. Data are expressed as µg/g dry weight (mean values ± standard deviation, n = 5).

2.3. Biomarker responses

Standardized protocols were used to analyze biomarkers and full methodological details are given in Supplementary Material 1 (SM1). Metallothioneins (MTs), single antioxidant defenses (catalase, glutathione S-transferases, glutathione peroxidases, glutathione reductase activities and total glutathione), total oxyradical scavenging capacity toward peroxyl radical (TOSC ROO•) and hydroxyl radical (TOSC HO•) were evaluated in digestive gland and gills. The analysis of the Total Oxyradical Scavenging Capacity (TOSC) is a reliable tool for quantitatively assessing the biological resistance to toxicity of different forms of ROS including peroxyl radicals, hydroxyl radicals and peroxynitrite decomposition products (Regoli and Winston, 1998, 1999). The assay is based on the capability of cellular antioxidants to reduce the oxidation of α-keto-ɣ-methiolbutyric acid (KMBA) in the presence of artificially generated oxyradicals. Compared to individual antioxidants, variations of TOSC have a greater biological relevance and prognostic value, indicating the capability to neutralize ROS associated to the onset of various forms of oxidative damages like lysosomal dysfunctions, lipid peroxidation and genotoxic effects (Nigro et al., 2002; Camus et al., 2003; Gorbi and Regoli, 2003; Moore et al., 2006). Lysosomal membrane stability (as Neutral Red Retention Time, NRRT) and onset of genotoxic effects as DNA strand breaks (Comet assay) and micronucleus frequency (MN) were analyzed in haemocytes.

2.4. Statistical analyses

Analysis of variance (One-way ANOVA) was used to evaluate the effects on all investigated parameters, after ensuring that all data followed the normal distribution (Shapiro-Wilk test) and that variances were homogeneous (Levene's Test). Level of significance was set to $p < 0.05$; *post-hoc* Tukey HSD tests were used to compare group of means. Multivariate principal component analysis (PCA) was applied to visualize the relationships among the different treatments and all statistical analyses were performed using RStudio (version 1.0.143).