



Surviving in a changing ocean. Tolerance to acidification might affect the susceptibility of polychaetes to chemical contamination

Marco Munari^a, Antonia Chiarore^{a,d,*}, Silvia Giorgia Signorini^b, Antonio Cannavacciuolo^a, Matteo Nannini^a, Stefano Magni^b, Andrea Binelli^b, Maria Cristina Gambi^c, Camilla Della Torre^{b,**}

^a Department of Integrative Marine Ecology, Ischia Marine Centre, Stazione Zoologica Anton Dohrn, Ischia (Naples), Italy

^b Department of Biosciences, University of Milan, Milan, Italy

^c National Institute of Oceanography and Applied Geophysics, OGS, Trieste, Italy

^d Department of Biology, University of Naples Federico II, Naples, Italy

ARTICLE INFO

Keywords:

Ocean acidification
Syllis prolifera
 Oxidative stress
 Acetone
 Copper
 CO₂ vents

ABSTRACT

This study aimed to assess the combined effects of ocean acidification (OA) and pollution to the polychaete *Syllis prolifera* inhabiting the CO₂ vent system of the Castello Aragonese (Ischia Island, Italy). We investigated the basal activities of antioxidant enzymes in organisms from the acidified site and from an ambient-pH control site in two different periods of the year. Results showed a limited influence of acidified conditions on the functionality of the antioxidant system. We then investigated the responsiveness of individuals living inside the CO₂ vent compared to those from the control to face exposure to acetone and copper. Results highlighted a higher susceptibility of organisms from the vent to acetone and a different response of antioxidant enzymes in individuals from the two sites. Conversely, a higher tolerance to copper was observed in polychaetes from the acidified-site with respect to controls, but any significant oxidative stress was induced at sublethal concentrations.

1. Introduction

Human activities have a detrimental impact on ecosystems' health, especially in marine environments. Several environmental and anthropogenic stressors, such as seawater warming, acidification, pollution and overexploitation are drastically reducing the biodiversity and functionality of marine ecosystems (Pecl et al., 2017). In this global change scenario, it is extremely important to understand how stressors are interacting with each other in order to provide more realistic projections of the intensity of impacts on species populations as well as communities facing global environmental changes (Rodríguez-Romero et al., 2021).

Among the various stressors, those related to climate change, such as temperature rise and ocean acidification (OA), are receiving an increasing attention in the last two decades due to their widespread effects on a global scale (Kroeker et al., 2013). OA consists in a profound alteration of the carbonate chemistry and decreasing of the pH, due to

increasing pCO₂ exchanges at the atmosphere-water interface (Caldeira and Wickett, 2005). Based on recent models (Caldeira and Wickett, 2005) and a business-as-usual scenario, OA is going to increase with lowering of the actual pH of 0.3–0.4 units at the end of this century, with still unpredictable effects for the majority of the marine organisms.

Several studies carried out under laboratory conditions described detrimental effects due to OA across many taxa, such as reduced calcification, metabolic stress and energetic constraints, altered growth and reproduction, highlighting that predicted OA conditions will have negative consequences for marine organisms (Bressan et al., 2014; Nagelkern and Cornell, 2015; Asnicar et al., 2021). Besides, other pioneering studies carried out in CO₂ vent systems, which are naturally acidified by the CO₂ surplus emitted from the seafloor, focused on species able to survive and thrive in a lower pH-high pCO₂ ocean (Foo et al., 2018). The CO₂ vents, mainly of volcanic origin, are sites with reduced pH, representing valuable windows to mimic future ocean conditions, which help to predict changes in biodiversity and adjustment in the

* Correspondence to: A. Chiarore, Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, Ischia Marine Centre, P.ta San Pietro, 80077 Ischia Napoli, Italy.

** Correspondence to: C. Della Torre, Department of Biosciences, University of Milano, Via Celoria 26, 20133 Milano, Italy.

E-mail addresses: antonia.chiarore@szn.it (A. Chiarore), camilla.dellatorre@unimi.it (C. Della Torre).

<https://doi.org/10.1016/j.marpolbul.2022.113857>

Received 23 February 2022; Received in revised form 26 April 2022; Accepted 12 June 2022

Available online 21 June 2022

0025-326X/© 2022 Published by Elsevier Ltd.

ecophysiology of marine species associated with OA (Kroeker et al., 2011).

The studies carried out in such natural laboratories show that divergent and compensatory biological responses to OA can occur (Foo et al., 2018; González-Delgado and Hernández, 2018), driving some species to counteract the effects of OA through a range of adaptive processes, including acclimatization (phenotypic plastic adjustment) and genetic adaptation (Foo and Byrne, 2016). Some of these strategies are energy-consuming and could occur at the expense of other energy-expensive physiological functions such as reproduction and growth. Nevertheless, some adaptation mechanisms might entail stress tolerance, allowing a natural population to survive and reproduce even in more stressful conditions.

Among the various shallow CO₂ vent systems nowadays studied around the world (González-Delgado and Hernández, 2018; Rastrick et al., 2018), the Castello Aragonese vents in Ischia Island (Tyrrhenian Sea, Italy) represent the first system where research on the responses of benthic biota to OA have been addressed (Hall-Spencer et al., 2008).

At temperate CO₂ vents, benthic polychaetes represent some of the most abundant metazoans colonizing the benthic biota (Ricevuto et al., 2014; Gambi et al., 2016; Vizzini et al., 2017; Auremma et al., 2019), and show different adaptations to OA at the individual species level (Lucey et al., 2015, 2016; Ricevuto et al., 2015a, 2016). Therefore, polychaetes represent ideal biological models to shed light on the molecular and physiological key mechanisms which allow counteracting OA otherwise detrimental effects.

The ongoing climatic changes are not the only treat that marine organisms must face. Especially in coastal areas, the most subjected to anthropogenic pressures, the OA-related stress is expected to combine with pollution, posing an even major threat to marine ecosystems (Schiedek et al., 2007; Nikinmaa, 2013; Delorenzo, 2015).

Classic laboratory studies have already been conducted in mesocosms conditions to test if OA can exacerbate the negative effects of different pollutants in several marine species including bivalves (Freitas et al., 2016b; Munari et al., 2016, 2018, 2019, 2020a, 2020b), echinoderms (Dorey et al., 2018; Munari et al., 2022) and polychaetes (Lewis et al., 2013; De Marchi et al., 2019) at different stages of their life history. Regarding to polychaetes, the combined effects of OA and different types of contaminants were investigated, showing synergistic and additive effects (Campbell et al., 2014; De Marchi et al., 2019), but also no interactive effects (Freitas et al., 2017) and antagonistic behaviour have been described (Nielson et al., 2019). Moreover, all the above studies, on the possible combined effects between OA and environmental contaminants, were conducted through *in vivo* exposure experiments under controlled laboratory conditions with organisms that never experienced OA before during their life time. If in a hand this approach allows to understand the phenotypic plasticity of a natural population to certain stressors, it does not allow to highlight the influence of the mechanisms such as acclimation and adaptation which develop in individuals naturally subjected to the action of multiple stressors.

Among polychaetes, *Syllis prolifera* (Krohn, 1852) is a meso-herbivore species, which lives on rocky bottoms, usually associated with algal-dominated habitats in shallow waters (Giangrande, 1988; Ricevuto et al., 2015a), including disturbed biotopes (Musco et al., 2009). This species is abundant in the hard bottom habitats in the Castello CO₂ vents, including the most acidified zone, where it represents one of the few abundant species (Cigliano et al., 2010; Kroeker et al., 2011; Ricevuto et al., 2012, 2014; Foo et al., 2018), and where also it can reproduce by stolonization with the production of sexual satellites (Gambi et al., 2017). There is still little evidence of the effects of elevated pCO₂ on *S. prolifera*, however, Calosi et al. (2013) showed that the species, although collected in the vents area, showed a significant increase in mean oxygen consumption when exposed to low pCO₂ conditions for 5 days transplants at the vents. Besides, a study carried out on *S. prolifera* and *Platynereis* spp. inhabiting the vent system of Castello Aragonese showed that native organisms displayed enhanced

basal antioxidant efficiency compared to specimens living under normal pH conditions (Ricevuto et al., 2015a). Since the antioxidant defence system is one of the key cellular pathways to counteract the adverse effects of toxic substances (Regoli and Giuliani, 2014), the increased antioxidant capability observed in those organisms could be winning also in developing tolerance to chemical contamination.

In this scenario, this study aimed to assess if organisms able to cope with natural acidified conditions have also an enhanced resistance against different environmental stress such as chemical contamination or whether physiological modifications, induced to cope with the acidified conditions, will reduce the capability of the organism to respond to chemical contamination, and whether the occurrence of this further challenge will impact more heavily their health and survival. To answer the question, we first assessed the basal antioxidant capability of individuals of *Syllis prolifera* inhabiting the CO₂ vent system of the Castello Aragonese. Then we investigated the responsiveness of individuals living inside the vents compared to those collected in control zones, living under normal pH conditions, to face exposure to two environmental pollutants with different mechanism of action. Acetone is a widely used industrial solvent commonly found in the atmosphere, in natural water bodies and in groundwater as well (Armutcu et al., 2005). This molecule is commonly used as a carrier of contaminants in many ecotoxicity tests, due to its high solubility and low toxicity. Copper (Cu) is a metal, widespread in the marine ecosystems as a result of mining activities, municipal and industrial effluent discharges and application in antifouling paints (Corcoll et al., 2019). The most characterized mechanisms of toxicity of Cu is through the overproduction of ROS and imbalance of acid-base homeostasis (Geracitano et al., 2004; Viarengo et al., 1996). The combined effects of Cu and OA have been investigated under laboratory conditions on different marine invertebrates, showing contrasting outcomes depending on the species' physiology, since often the toxicity of Cu was increased under OA conditions (Campbell et al., 2014; Siddiqui and Bielmyer-Fraser, 2015; Lewis et al., 2016; Bielmyer-Fraser et al., 2018; Huang et al., 2018; Scanes et al., 2018) but also antagonistic effects have been described (Marangoni et al., 2019; Nielson et al., 2019).

2. Materials and methods

2.1. Sites and sampling

Individuals of the target species *Syllis prolifera* were collected in November 2019 and June 2021 from the Castello Aragonese CO₂ vents system (stations centred in 40°43'57.9" N, 13°57'51.8" E on the south side of the Castello named S3 and S2 from previous investigations) (e.g., Ricevuto et al., 2015a, 2015b; Calosi et al., 2013; Foo et al., 2018), and from the control site at San Pietro promontory 40°44'47.6" N, 13°56'40.42" E, which is located approximately 4 km from the vents (Fig. 1), a site which has been already used as reference in similar studies (Ricevuto et al., 2015b; Calosi et al., 2013). For the physico-chemical characterization of the acidified areas of the Castello we refer to multiples previous studies where pH, pCO₂ and other parameters have been intensively measured (e.g., Hall-Spencer et al., 2008; Kroeker et al., 2011) or summarized (e.g., Ricevuto et al., 2014; Foo et al., 2018). Since the acidification level and its variability are related to the intensity of the venting (bubbling) from the floor and this has not changed in the past 10 years (Gambi M.C., personal observation), we consider the characterization given by past studies still reliable of the local OA conditions. Salinity of the zone is constant at 38 PSU (Foo et al., 2018). We do not have measures of the natural levels of copper and acetone in the area, however, being both the control and the vents sites included in the Marine Protected Area of Ischia, there aren't evident source of pollution in the area.

As *Syllis prolifera* lives in association with several macroalgae species (mainly *Halopteris scoparia* and *Cladophora* spp.), the macroalgae were collected by hand by scuba divers that used fabric bags subsequently



Fig. 1. Map of the Ischia island. Location of the two sampling sites: Vent site at the Castello Aragonese ($40^{\circ}43'57.9''$ N - $13^{\circ}57'51.8''$ E), control site at San Pietro Point ($40^{\circ}44'47.6''$ N; $13^{\circ}56'40.42''$ E).

covered by plastic covers at 1–2 m depth in both sites. Individuals of *S. prolifera* were sorted from the algae and identified, worms were pooled (3 pools of 20 individuals each from each site) and stored at -80°C before biomarkers analysis.

2.2. In vivo exposure

The experiments were carried out using individuals from both the San Pietro site and the vent system of Castello Aragonese with specimens collected as previously described. The experiment with acetone was carried out in September 2020, while the one with copper in June 2021. Individuals used for *in vivo* exposures were collected from the field prior to each experiment and sorted for identification using seawater collected from the control site underneath the laboratories of the Ischia Marine Centre, conditioned at the same $p\text{CO}_2$ conditions of the site of origin. After sorting and identification, polychaetes (body length range 3–6 mm) were maintained in a thermostatic room at 25°C with 12:12 h day: night photoperiod, in glass containers (200 mL) supplied with filtered seawater ($0.22\ \mu\text{m}$) from the control site conditioned at the same $p\text{CO}_2$ conditions of the site of origin. The CO_2 was supplied and monitored through an automatic system (Touch Controller acq140 by Aquatronica S.r.l.). From previous trials and studies (Teixido et al., 2020) we know that there are no persistent differences in Total Alkalinity among different sites being open systems. During the acclimation period, polychaetes were fed *ad libitum* with grinded fresh spinach.

2.2.1. Acetone experiments

After three days of acclimation, individuals were subdivided into glass bottles (250 mL) supplied with artificial seawater (distilled water

added with Amtra Sea Complex) at $37 \pm 1\ \text{‰}$ salinity and 8.08 ± 0.01 (mean \pm S.D.) pH for control treatment and 7.73 ± 0.03 (mean \pm S.D.) pH for acidified treatment.

Organisms were subdivided in order to have homogeneous distribution in body length within groups into the following treatment groups (three replicates of 20 individuals each):

- Control seawater at pH 8.1 individuals collected from San Pietro
- Acetone at 0.05 % v/v at pH 8.1 individuals collected from San Pietro
- Control seawater at pH 7.7 individuals collected from the Castello vents
- Acetone at 0.05 % v/v at pH 7.7 individuals collected from the Castello vents.

The exposure lasted 4 days with water renewal every 48 h changing 80 % of the total volume. Polychaetes during the experiment were fed with frozen spinach finely chopped. Due to the high mortality observed in the group treated with acetone at pH 7.7, only the survival rates have been recorded at the end of the experiment.

A further experiment has been carried out with the following treatment groups:

- Control seawater at pH 8.1 individuals collected from San Pietro
- Acetone at 0.01 % v/v at pH 8.1 individuals collected from San Pietro
- Control seawater at pH 7.7 individuals collected from the Castello vents
- Acetone at 0.01 % v/v at pH 7.7 individuals collected from the Castello vents.

Samples were subdivided into glass bottles (250 mL) supplied with artificial seawater (distilled water added with Amtra Sea Complex) at 37 ± 1 ‰ (mean \pm S.D.) salinity and 8.12 ± 0.03 (mean \pm S.D.) pH for control treatment and 7.74 ± 0.01 (mean \pm S.D.) pH for acidified treatment. The exposure lasted five days with water renewal every 48 h changing 80 % of the total volume. At the end of the experiment, the survival rates were recorded and individuals from each replicate were collected and stored at -80 °C before biomarkers analysis.

2.2.2. Copper sulphate

In June 2021, after three days of acclimation, samples were divided into glass bottles (250 mL) supplied with artificial seawater (distilled water added with Amtra Sea Complex) at 37 ± 1 ‰ (mean \pm S.D.) salinity and 8.13 ± 0.03 (mean \pm S.D.) pH for control treatment and 7.67 ± 0.05 (mean \pm S.D.) pH for acidified treatment. Polychaetes during the experiment were fed with frozen spinach finely chopped.

Organisms were subdivided into eight treatment groups (three replicates of 10 individuals each), as follow:

- Control seawater at pH 8.1 individuals collected from San Pietro
- Copper sulphate at 2 mg/L at pH 8.1 individuals collected from San Pietro
- Copper sulphate at 0.2 mg/L at pH 8.1 individuals collected from San Pietro
- Copper sulphate at 0.05 mg/L at pH 8.1 individuals collected from San Pietro
- Control seawater at pH 7.7 individuals collected from the Castello vents
- Copper sulphate at 2 mg/L at pH 7.7 individuals collected from the Castello vents
- Copper sulphate at 0.2 mg/L at pH 7.7 individuals collected from the Castello vents
- Copper sulphate at 0.05 mg/L at pH 7.7 individuals collected from the Castello vents.

The Cu concentrations were selected based on LC₅₀ reported for other polychaete species (from 125 µg/L up to ≥ 500 µg/L Xie et al., 2005; Dean, 2008; Moreira et al., 2006; Bouraoui et al., 2015). Water was changed every 48 h (80 % of the total volume) and at the end of the experiment (five days of exposure), the survival rates were recorded and individuals from each replicate were collected and stored at -80 °C before biomarkers analysis.

2.3. Biomarkers analysis

Biomarkers analyses were carried out following the procedures described in Morosetti et al. (2020), properly adapted to polychaetes. Pools were homogenized in 1 mL of 100 mM potassium phosphate buffer (added with KCl 100 mM, EDTA 1 mM, dithiothreitol 1 mM and protease inhibitors, pH 7.4) using a TissueLyser II QIAGEN® set at a frequency of 30/s for 30 s each and then centrifuged at $10,000 \times g$ at 4 °C, for 10 min. The supernatant has been stored at -80 °C before the measurement of oxidative stress enzymes (glutathione-S-transferase GST, superoxide dismutase SOD, glutathione peroxidase GPx, catalase CAT) and the content of glycogen (GLY).

The GLY analysis was carried out only in samples collected from the field. We followed the sulphuric acid method described by Dubois et al. (1956), using glucose standards (0–2 mg mL⁻¹). The absorbance was read at 492 nm using an EnSight™ plate reader (Perkin Elmer), and the results were expressed in mg g⁻¹ fresh weight (FW).

The measurement of antioxidant enzymes was carried out using a 6715 UV/Vis spectrophotometer (Jenway). The protein content was determined following the method described by Bradford (1976), using bovine serum albumin (BSA) as standard (0.1–0.5 mg mL⁻¹ $r^2 > 0.98$). The SOD activity was determined by measuring the degree of inhibition of cytochrome c reduction by the superoxide anion generated by the

xanthine oxidase reaction at 550 nm. Activities were given in SOD units (1 SOD unit = 50 % inhibition of the xanthine oxidase reaction). The CAT activity was assessed by measuring the consumption of H₂O₂ at $\lambda = 240$ nm. The reading lasted 1 min and values were expressed as µmol min⁻¹ mg proteins⁻¹. The GPx activity was evaluated by measuring the NADPH consumption at 340 nm using H₂O₂ 0.2 mM as substrate with glutathione (2 mM), sodium azide (NaN₃; 1 mM), glutathione reductase (2 U/mL), and NADPH (120 µM). The activity was expressed as µmol min⁻¹ mg proteins⁻¹. The activity of GST was measured in presence of reduced glutathione (1 mM) and 1-chloro-2,4-dinitrobenzene (CDNB) as co-substrate. The spectrophotometer reading at $\lambda = 340$ nm lasted 1 min activity was expressed as mmol min⁻¹ mg proteins⁻¹.

2.4. Statistical analysis

A non-parametric PERMutational multivariate ANalysis Of Variance (PERMANOVA) applied on the Euclidean distance matrix of square root transformed data was chosen to test differences on enzyme activities including two crossed factors: site fixed with two levels (vents, control) and season fixed with two levels (November, June). In cases where results were significant, PERMANOVA was used to test for the interactive effect of site and season. PERMANOVA was also applied to test the differences in enzyme activities and survival rate related to the treatment with acetone and copper including two crossed factors: site fixed with two levels (vents, control) and treatment fixed with two levels (treatment, control). In cases where the number of unique values from permutations was too low, the Monte-Carlo procedure was used to calculate p values. Non-metric multidimensional scaling (nMDS) plots were performed on a Euclidean similarity matrix using square-root transformed data. PERMANOVA and nMDS have been performed with PRIMER v 7 Plymouth Routines in Multivariate Ecological Research. The LC₅₀ values for polychaetes exposed to Cu were calculated using LC50 calculator (AAT Bioquest).

3. Results

3.1. Effects of OA on basal activities of antioxidant enzymes and GLY content

The graphs in Fig. 2 show the variation in the basal activity of antioxidant enzymes in individuals of *S. prolifera*, according to the sampling site (San Pietro vs Castello vent) and the period (November vs June).

The activity of GST and CAT resulted significantly different as a function of the sampling period, being higher in summer compared to autumn in both sampling sites (PERMANOVA test, Table S1). Concerning the comparison between sites, any significant difference could be observed in GST SOD and CAT activities (Fig. 2; Table S1), while the activity of GPx resulted significantly higher in individuals from San Pietro compared to those from the vent, although only in November (Fig. 2; Table S1).

A similar content of GLY was observed among individuals from the two sites which was significantly higher in June than in November (Fig. 2; Table S1).

Multivariate analysis confirmed the clear separation of organisms as a function of the sampling period, more pronounced in worms from the acidified site, which resulted more clustered in the two periods than worms from San Pietro (Fig. 3).

3.2. Interactive effects of OA and pollutants

3.2.1. Effects of acetone

In the first experiment, survival showed significant differences as a function of the site, treatment and their combination (PERMANOVA test, Table S2). Indeed, a strong reduction in viability up to 90 % was found in individuals collected from the Castello vent and exposed to

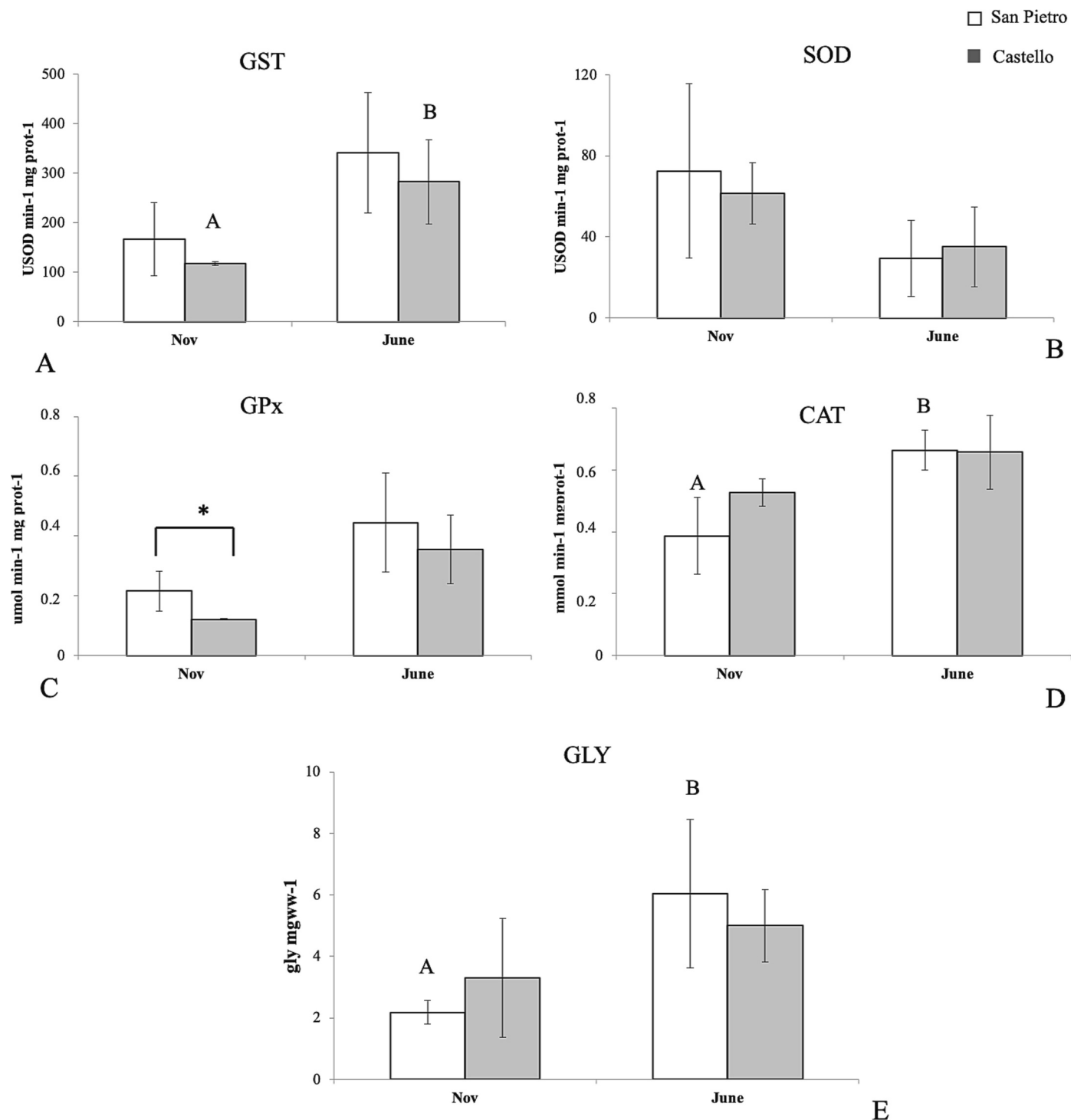


Fig. 2. Basal antioxidant activities. Mean \pm standard deviation (S.D.) of basal activity of GST (A), SOD (B), GPx (C), CAT (D) and GLY content (E) measured in individuals of *Syllis prolifera* from San Pietro and the vent system of Castello Aragonese in November and June. The asterisk (*) means statistically different activities among organisms collected in different sites within the same sampling period ($p \leq 0.05$). Different letter (A, B) means statistically significant differences among polychaetes from the same site at different collecting period ($p \leq 0.05$).

acetone (Table 1). Instead, for individuals collected at San Pietro, no variations in the survival rate were observed between controls and acetone treatments (Table 1). Similarly, in the second exposure experiment, significant differences were observed as a function of the site, treatment and their combination (PERMANOVA test, Table S2), although in general lower lethal effects have been observed in polychaetes treated with acetone (Table 1). At the end of this experiment, no differences in survival rates were found between the control group and

the acetone-treated group for individuals from the San Pietro site. On the contrary, in individuals derived from the vent site, an overall decrease up to 60 % in the survival rate when exposed to acetone was observed (Table 1; Fig. S1).

The variation in the activity of antioxidant enzymes due to acetone exposure is shown in Fig. 4, where we can observe as most enzymatic activities showed statistically significant differences as regards site and treatment (PERMANOVA test, Table S2).

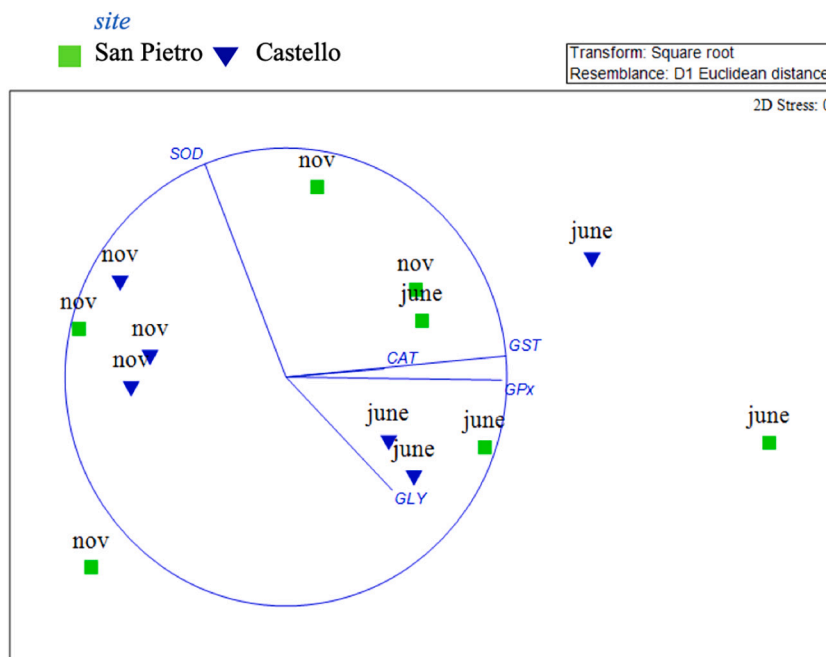


Fig. 3. Multivariate analysis. nMDS of biomarkers measured in *Syllis prolifera* collected in San Pietro and the vent system of Castello Aragonese as a function of collection site and period.

Table 1

Survival rate of individuals of *S. prolifera* from San Pietro and the Castello Aragonese vent system upon treatment with acetone and copper. The asterisk (*) means statistically different activities due to treatment within the same site of origin. Different letter (A, B) means statistically significant differences between individuals from the two sites ($p \leq 0.05$).

	San Pietro	Castello Aragonese
<i>Acetone</i>		
ctrl	91.8 ± 3.15	76.7 ± 7.64
0.01 % v/v	93.3 ± 2.89 ^a	43.3 ± 22.5 ^{b,*}
ctrl	85.0 ± 5.0	91.7 ± 7.64
0.05 % v/v	80.0 ± 15.0 ^a	38.3 ± 41.6 ^{b,*}
<i>Cu</i>		
ctrl	83.3 ± 15.3	83.3 ± 15.3
0.05 mg/L	80.0 ± 10.0	90.0 ± 17.3
0.2 mg/L	36.7 ± 47.3	83.3 ± 5.77
2 mg/L	0	0

Concerning GST, individuals from San Pietro showed very similar activity in both exposure conditions, while in individuals from the vent site the treatment with acetone determined a not significant increase in GST activity compared to controls. Statistically supported differences were observed between individuals exposed to acetone from the two sampling sites (Fig. 4). As for the SOD enzyme, a decrease in activity was observed in individuals exposed to acetone and derived from both sites, with significant differences respect to controls only in individuals from San Pietro (Fig. 4). In addition, significant differences from the two sites were observed in the SOD activity between individuals exposed to acetone, with lower values overall observed in the specimens from Castello vent.

The GPx enzyme showed a profile of activity similar to GST, with a significant increase in activity observed only in the group treated with acetone compared to the control for individuals from the Castello vent (Fig. 4), while no changes were observed in the activity of this enzyme for individuals from the San Pietro site. Finally, a slight increase of CAT activity in individuals from San Pietro was observed in acetone-treated group compared to control ones, while the acetone treatment generated

a slight activity decline in individuals from the Castello vent (Fig. 4). Due to the reduced number of individuals survived at the end of the exposure experiments, it was not possible to measure GLY content for both the copper and acetone experiments.

The multivariate analysis showed in a synoptic way the response of individuals from the two sites to the different exposure conditions: populations from San Pietro and Castello vent showed similar response when kept in artificial seawater, while individuals from both sites tended to form distinct clusters upon treatment with acetone (Fig. 4). Moreover, considering the origin site, individuals from San Pietro showed distinct clusters between control and treatment with acetone, while those from the Castello vent showed a more disperse distribution and with no clear distinction between untreated and exposed (Fig. 4).

3.2.2. Effects of copper

The highest concentration of Cu (2 mg/L) induced 100 % lethal effects in exposed polychaetes regardless the site of collection (Table 1). At 0.2 mg/L Cu determined strong reduction in viability, up to 90 % in individuals collected from the San Pietro site. Instead, for individuals collected at the Castello vent, no variations in the survival rate were observed between controls and Cu treatment (Table 1). At the lowest concentration of 0.05 mg/L no differences in survival rates were found between the control group and the Cu-treated groups for individuals from both sites. The LC₅₀ values calculated for population from the not acidified site and from the Castello vent were 0.182 mg/L and 0.313 mg/L, respectively (Fig. S2).

Unlike what was observed for acute effects, the analysis of sub-lethal parameters measured in individuals exposed to 0.05 mg/L of Cu did not show significant differences as regards site and treatment (PERMANOVA test, Table S3; Fig. 5).

4. Discussion

This study aimed to highlight if organisms able to cope with natural acidified conditions have also an enhanced resistance against different environmental stress such as chemical contamination or whether the occurrence of this further challenge will impact more heavily their health. To date, this aspect is largely unexplored, although the marine

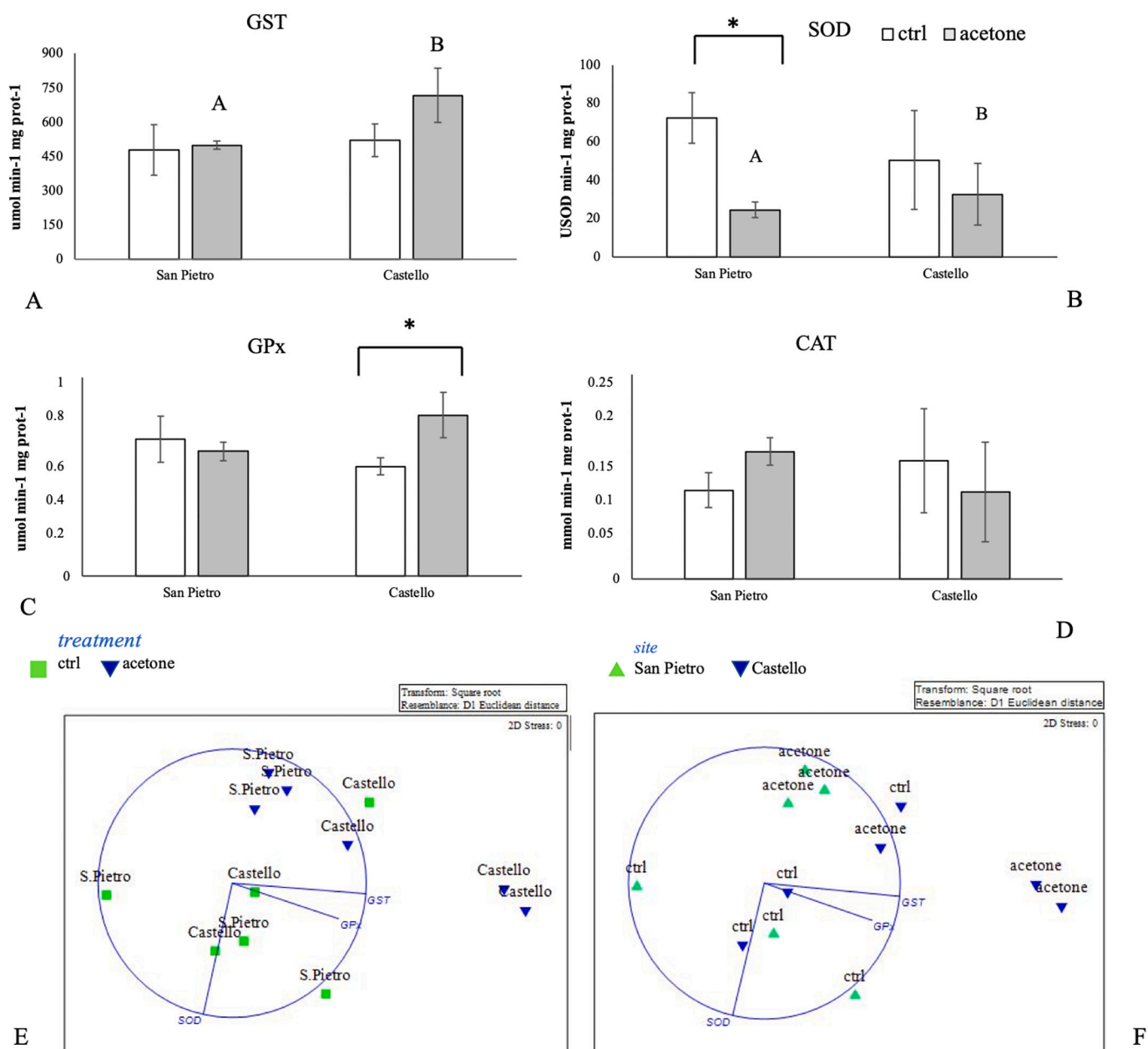


Fig. 4. Effects of acetone on antioxidant enzymes. Mean \pm S.D. of GST (A), SOD (B), GPx (C) and CAT (D) measured in *Syllis prolifera* from San Pietro and the vent system of Castello Aragonese treated with acetone 0.01 % for five days. The asterisk (*) means statistically different activities due to treatment within the same site of origin. Different letter (A, B) means statistically significant differences between individuals from the two sites treated with acetone ($p \leq 0.05$). Below, nMDS of biomarkers from *in vivo* exposure of *Syllis prolifera* to acetone, using as variable the treatment (E) or the sampling site (F).

ecosystems are affected by the combined impacts of various anthropogenic and environmental disturbances. Furthermore, most of the studies carried out on the effects of multiple stressors, using *in vivo* exposure experiments for a limited time and in controlled laboratory conditions, do not allow to differ between plastic and adaptive mechanisms, the latter induced in organisms to cope with disturbances related to global changes. This limits significantly our ability to realistically predict the impact of multiple stressors on marine ecosystems (Rodríguez-Romero et al., 2021). In this study, a different experimental approach was used, since for the first time, populations of species naturally exposed to OA during their entire life were employed.

4.1. Basal characterization of antioxidant system and GLY content

Our first objective was to characterize the basal activity of some

antioxidant enzymes in organisms from the CO₂ vent system compared to the control site. Indeed, a recent study carried out on sea urchins (Migliaccio et al., 2019) inhabiting the CO₂ vent of Castello Aragonese showed that native organisms displayed elevated basal antioxidant efficiency compared to organisms living under normal pH conditions. This suggests that an upregulation of the antioxidant machinery could occur, which might also entail tolerance to environmental pollutants. Similarly, Ricevuto et al. (2015a) observed a higher antioxidant efficiency in the nereid polychaete *Platynereis* spp. from the CO₂ vent of Castello Aragonese compared to the non-acidified site, but contradictory results were obtained in a further in-depth study on this species, in which no differences were observed in the activity of antioxidant enzymes and the antioxidant capability in organisms collected in the same sites (Valvasori et al., 2019).

Our results seem to confirm that there is a limited influence of

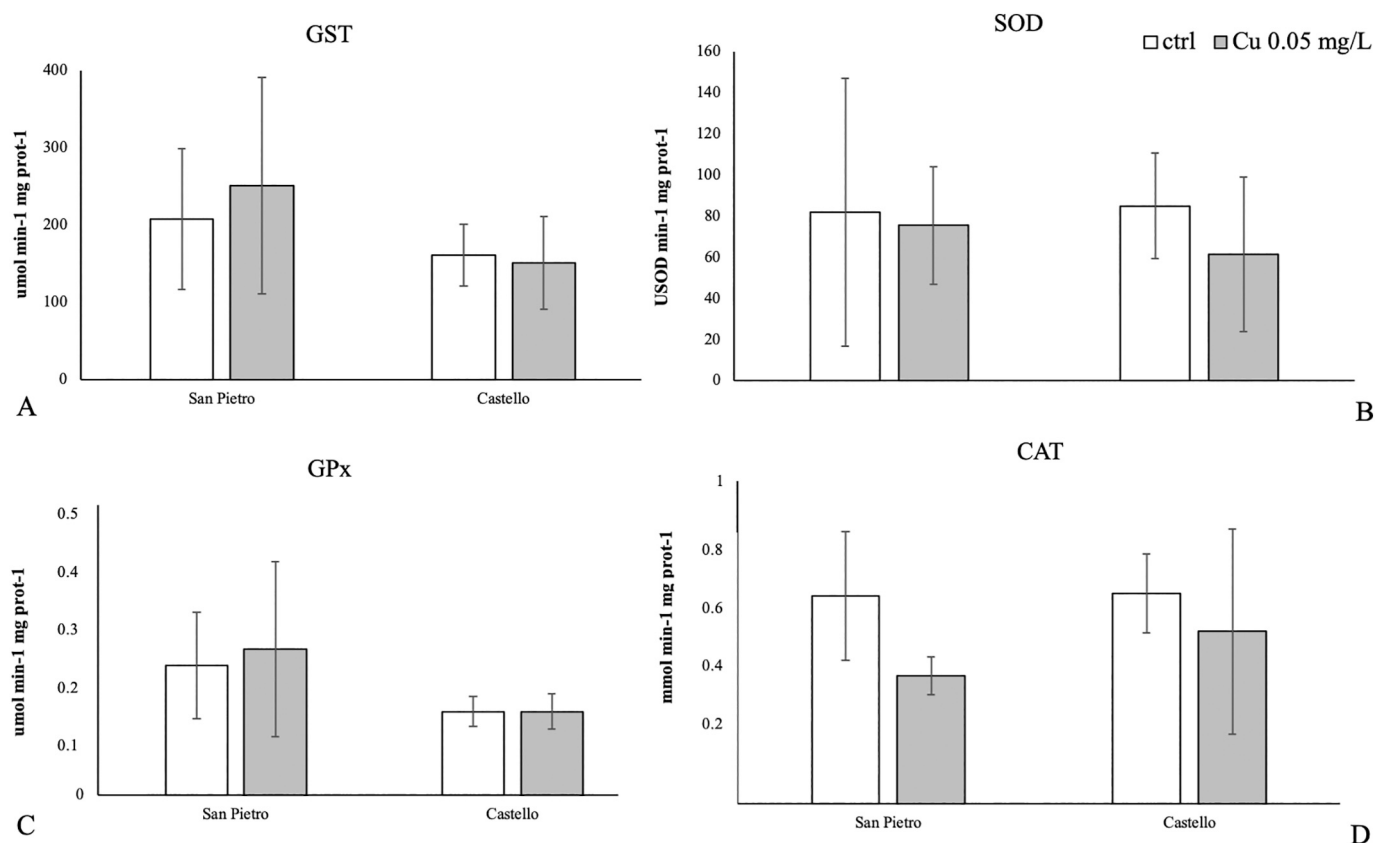


Fig. 5. Effects of copper on antioxidant enzymes. Mean \pm S.D. of GST (A), SOD (B), GPx (C) and CAT (D) measured in *Syllis prolifera* from San Pietro and the vent system of Castello Aragonese treated with Cu 0.05 mg/L for five days.

acidified conditions on the functionality of the antioxidant system also in *S. prolifera*. In fact, only the activity of the GPx was significantly different between individuals from the two sites. This observation is in contrast with the findings obtained in laboratory studies carried out on polychaetes. In fact, short-term *in vivo* exposure to low pH levels, similar to the ones observed in the CO₂ vent of Castello Aragonese, triggered an alteration of antioxidant enzymes activities in *Diopatra neapolitana* and *Hediste diversicolor*. In particular, an increase of CAT, SOD and GST activities and lipid peroxidation was detected in organisms subjected to acidified conditions (Freitas et al., 2015, 2016a). The modulation of antioxidant enzymes has been observed also in another study in which an *in situ* transplant experiment of *Sabella spallanzanii* was carried out in the CO₂ vent of Castello Aragonese (Ricevuto et al., 2016). In this case, a reduction of CAT and GPx has been observed in organisms translocated from the control site to the vents for 30 days.

Concerning the GLY content, in previous studies conducted on *H. diversicolor*, *D. neapolitana* and *Nereis virens*, decreased levels of GLY were observed as a function of pH decrease, indicating that the acidification triggers a metabolic depression and the consumption of energy reserves for the maintenance of correct physiological functions (Freitas et al., 2015, 2016a), contrary to what was observed in our study.

These discrepancies highlight how the responses to OA might be very different in the short and long term since the induction of the antioxidant system could represent an acclimatization response to a sudden increase in acidification levels, while this system does not appear to be induced in organisms living under low and fluctuating pH throughout their entire life. This seems also supported by the results of the GLY content, which suggest that in individuals from the vent an adaptation mechanism might be in place, which allows them to preserve a sufficient amount of metabolic reserves.

The nMDS analysis without showing a clear differentiation between individuals from the Castello vent compared to the San Pietro control

site, highlighted on the contrary a distinct clustering between the two sampling periods. This indicates that the activity of the antioxidant system of the species is subject to seasonal variations with higher activities (except for SOD) in summer than in autumn, regardless of the pH conditions.

Seasonal fluctuations of antioxidant enzymes have been described in several marine invertebrates (Chainy et al., 2016). Indeed, factors such as the intensity of solar radiation, temperature, salinity and oxygen concentration, might trigger prooxidant conditions for the organisms, which therefore increase the activity of antioxidant enzymes to maintain their oxidative status (Bocchetti and Regoli, 2006). Notwithstanding, the seasonal effect was different in the two sampling sites, with CAT increasing in individuals from San Pietro, while for vent polychaetes it was GST that increased, and thus suggesting that in organisms tolerant to OA the mechanisms induced to face the response to environmental fluctuations are different to those of individuals which are not subjected to stressful conditions.

Concerning the profile of GLY content, the increase observed in the summer season is likely related to the increase in food availability. Also in this case we found differences between the two sites since a significant increase was observed only in individuals from San Pietro. This seems to suggest that there might be greater energy consumption in organisms living in the CO₂ vent to maintain homeostatic conditions during the summer season. On the hand, the macroalgae at the vents, including the two species where *S. prolifera* lives, seem to have a higher energetic values due to higher N content in their tissues (Ricevuto et al., 2015b).

4.2. Combined effects of OA and pollutants

Although living inside the vents does not seem to affect dramatically the efficiency of the antioxidant system, the response to harmful chemicals may be altered. To test this hypothesis, the organisms from

the two sites were exposed *in vivo* to acetone. The results showed the absence of lethal effects for individuals from the control site, in line with what was found in aquatic species in previous studies (Hutchinson et al., 2006; Leoni et al., 2008). On the contrary, a high mortality rate was observed in individuals from the vent exposed to acetone (0.05 % v/v). Also in the further experiment, with lower acetone concentrations (0.01 % v/v), a greater lethality for individuals from the vent compared to those from San Pietro control was observed, thus suggesting that this substance has a higher toxic effect on the vent population.

Regarding the effects of acetone treatment on the antioxidant machinery, the results suggest that acetone may interfere with the antioxidant system of polychaetes, although the enzymatic response of individuals from San Pietro was different than that of individuals from the vent, with the SOD inhibited in the former and instead with GPx and GST induced in the latter.

The results of polychaetes from San Pietro are in line with what was observed in previous studies carried out on rats, in which the effects of acetone exposure led to a decrease in the hepatic activity of SOD and GPx and an increase in the activity of CAT without altering the GSH content (Orellana et al., 2001).

Instead, a different response was observed in individuals from the vent, in which the induction of the two glutathione-dependent enzymes GST and GPx was observed. This might suggest an imbalance of GSH levels and, as a consequence, of the oxidative status, which might have detrimental consequences for organisms, since GSH is the most abundant soluble cellular thiol (Meister and Anderson, 1983), which plays a central role in several functions such as antioxidant and redox activities and protein folding and therefore is essential for cell survival (Toledano and Huang, 2017). The observation is also supported by results of the basal characterization where GPx was the only enzyme showing significant differences between the vent and the non-acidified, control site, and that GST was significantly modulated as a function of seasonality in organisms from the vent. Further studies focused on GSH metabolism in polychaetes living in OA conditions are therefore recommended, to understand the potential role of this important molecule in influencing the resilience of organisms towards OA and other environmental disturbances.

A different scenario has been observed upon exposure to copper. The LC₅₀ measured in the two groups upon exposure to Cu (0.182 mg/L vs 0.313 mg/L) and the high mortality observed in the organisms from San Pietro site treated with 0.2 mg/L acetone, in comparison with organisms from the vent site, seem to suggest that organisms from the vent could be more tolerant to this metal compared to those from the non-acidified, control site. Further experiments carried out for longer exposure times and treating polychaetes with Cu concentrations approaching LC₅₀, the measured in this study, are needed to confirm this first observation.

Our results are in accordance with the recent findings reported by Nielson et al. (2019) on the polychaete *Arenicola marina*. In this species the acid-base disturbance induced by Cu were buffered under OA conditions. Besides, the treatment with Cu did not induce the activity of the antioxidant enzyme SOD and the occurrence of lipid peroxidation, either under normal and acidified conditions. This result is in line with the absence of modulation of the antioxidant enzymes observed in *S. prolifera* upon Cu exposure. The authors suggested that the ability of the species to regulate the acid base and pH of extracellular fluids might be a key physiological feature to determine the combined effects of OA and toxic pollutants such as Cu. Further studies aimed at investigating this specific mechanism also in *S. prolifera* will be necessary to confirm this hypothesis.

The results of the two exposure experiments suggest that the combined effects of OA and environmental pollutants are dependent on their specific mechanisms of actions and also on the metabolic and physiological performances of the species. These observations highlight the need to increase our understanding of the molecular, cellular and physiological mechanisms that underlie the species tolerance towards OA, which is currently overlooked. This is essential for predicting the

vulnerability of organisms such as *S. prolifera* to further environmental disturbances.

5. Conclusions

Our study provided the first information on the antioxidant system of the polychaete *Syllis prolifera* living inside the CO₂ vents of Castello Aragonese, helping to broaden the current knowledge on the mechanisms that might promote tolerance to environmental stressors related to climate change, such as OA.

We provided first evidence that the tolerance to OA developed in the *S. prolifera* population living in the CO₂ vent of Castello Aragonese could affect other important physiological processes, influencing the individuals' ability to cope with environmental contaminants.

Our innovative approach will contribute to understand the mechanisms underpinning the capability of organisms to face multiple challenges occurring in the future oceans of the Anthropocene.

CRedit authorship contribution statement

MM, CDT, MCG conceptualization; MN, ACh, MM performed the field samplings; SGS, MN, ACh, Aca, MM, MCG, CDT performed the experimental work; ACh, data elaboration; SGS, CDT, SM performed the laboratory analyses; AB provided resources; all Authors contributed to writing and revision of the text and figures.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The project was financed by the grant Line 2 of the Research Support Plan from the University of Milan project PSR2015-1719LCOLO_07. During this study MN was supported by a fellowship from the Stazione Zoologica Anton Dohrn, ACh was supported by a fellowship from the Stazione Zoologica Anton Dohrn in collaboration with the University of Naples Federico II – AFRIMED project, and MCG was senior investigator at the Ischia Marine Center of the Stazione Zoologica Anton Dohrn, Napoli.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marpolbul.2022.113857>.

References

- Armutcu, F., Coskun, O., Gürel, A., Sahin, S., Kanter, M., Cihan, A., Numanoglu, K.V., 2005. Vitamin E protects against acetone-induced oxidative stress in rat red blood cells. *Cell Biol. Toxicol.* 21, 53–60.
- Asnicar, D., Novoa-Abelleira, A., Minichino, R., Badocco, D., Pastore, P., Finos, L., Munari, M., Marin, M.G., 2021. When site matters: metabolic and behavioural responses of adult sea urchins from different environments during long-term exposure to seawater acidification. *Mar. Environ. Res.* 169, 105372.
- Auriemma, R., De Vittor, C., Esposito, V., Gaglioti, M., Gambi, M.C., 2019. Motile Fauna associated to *Cystoseira brachycarpa* along a gradient of ocean acidification at a vent system off Panarea (Aeolian Islands, Italy). *Biol. Mar. Med.* 26, 216–219.
- Bielmyer-Fraser, G., Patel, P., Capo, T., Grosell, M., 2018. Physiological responses of corals to ocean acidification and copper exposure. *Mar. Pollut. Bull.* 133, 781–790.
- Bocchetti, R., Regoli, F., 2006. Seasonal variability of oxidative biomarkers, lysosomal parameters, metallothioneins and peroxisomal enzymes in the Mediterranean mussel *Mytilus galloprovincialis* from Adriatic Sea.
- Bourauoi, Z., Ghediraa, J., Boussetaa, H., 2015. Biomarkers responses in different body regions of the polychaete *Hediste diversicolor* (Nereidae, Polychaete) exposed to copper. *J. Integr. Coast. Zone Manag.* 15, 371–378.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.

- Bressan, M., Chinellato, A., Munari, M., Matozzo, V., Mancini, A., Marceta, T., Finos, L., Moro, I., Pastore, P., Badocco, D., Marin, M.G., 2014. Does seawater acidification affect survival, growth and shell integrity in bivalve juveniles? *Mar. Environ. Res.* 99, 136–148.
- Caldeira, K., Wickert, M., 2005. Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. *J. Geophys. Res.* 110 <https://doi.org/10.1029/2004JC002671>.
- Calosi, P., Rastrick, S.P.S., Lombardi, C., de Guzman, H.J., Davidson, L., Jahnke, M., Giangrande, A., Hardege, J.D., Schulze, A., Spicer, J.I., Gambi, M.C., 2013. Adaptation and acclimatization to ocean acidification in marine ectotherms: an in situ transplant experiment with polychaetes at a shallow CO₂ vent system. *Phil. Trans. R. Soc. B* 368, 20120444.
- Campbell, A.L., Mangan, S., Ellis, R.P., Lewis, C., 2014. Ocean acidification increases copper toxicity to the early life history stages of the polychaete *Arenicola marina* in artificial seawater. *Environ. Sci. Technol.* 48, 9745–9753.
- Chainy, G.B.N., Paital, B., Dandapat, J., 2016. An overview of seasonal changes in oxidative stress and antioxidant defence parameters in some invertebrate and vertebrate species. *Scientifica*. <https://doi.org/10.1155/2016/6126570>.
- Cigliano, M., Gambi, M.C., Rodolfo-Metalpa, R., Patti, F.P., Hall-Spencer, J.M., 2010. Effects of ocean acidification on invertebrate settlement at volcanic CO₂ vents. *Mar. Biol.* 157, 2489–2502.
- Corcoran, N., Yang, J., Backhaus, T., Zhang, X., Eriksson, K.M., 2019. Copper affects composition and functioning of microbial communities in marine biofilms at environmentally relevant concentrations. *Front. Microbiol.* 9 <https://doi.org/10.3389/fmicb.2018.03248>.
- Dean, H.K., 2008. The use of polychaetes (Annelida) as indicator species of marine pollution: a review. *Rev. Biol. Trop.* 56, 11–38.
- Delorenzo, M.E., 2015. Impacts of climate change on the ecotoxicology of chemical contaminants in estuarine organisms. *Curr. Zool.* 61, 641–652.
- De Marchi, L., Pretti, C., Chiellini, F., Morelli, A., Neto, V., Soares, A.M.V.M., Figueira, E., Freitas, R., 2019. The influence of simulated global ocean acidification on the toxic effects of carbon nanoparticles on polychaetes. *Sci. Total Environ.* 666, 1178–1187.
- Dorey, N., Maboloc, E., Chan, K.Y.K., 2018. Development of the sea urchin *Heliocidaris crassispina* from Hong Kong is robust to ocean acidification and copper contamination. *Aquat. Toxicol.* 205, 1–10.
- Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28, 350–356.
- Freitas, R., Pires, A., Moreira, A., Wrona, F.J., Figueira, E., Soares, A.M.V.M., 2016a. Biochemical alterations induced in *Hediste diversicolor* under seawater acidification conditions. *Mar. Environ. Res.* 117, 75–84.
- Freitas, R., Pires, A., Velez, C., Almeida, A., Moreira, A., Wrona, F.J., Soares, A.M.V.M., Figueira, E., 2015. Effects of seawater acidification on *Diopatra neopolitana* (Polychaete, Onuphidae): biochemical and regenerative capacity responses. *Ecol. Indic.* 60, 152–161.
- Freitas, R., Almeida, A., Calisto, V., Velez, C., Moreira, A., Schneider, R.J., Esteves, V.I., Wrona, F.J., Figueira, E., Soares, A.M.V.M., 2016b. The impacts of pharmaceutical drugs under ocean acidification: new data on single and combined long-term effects of carbamazepine on *Scrobicularia plana*. *Sci. Total Environ.* 541, 977–985.
- Freitas, R., de Marchi, L., Moreira, A., Pestana, J.L.T., Wrona, F.J., Figueira, E., Soares, A.M.V.M., 2017. Physiological and biochemical impacts induced by mercury pollution and seawater acidification in *Hediste diversicolor*. *Sci. Total Environ.* 595, 691–701.
- Foo, S.A., Byrne, M., 2016. Chapter two - acclimatization and adaptive capacity of marine species in a changing ocean. *Adv. Mar. Biol.* 74, 69–116.
- Foo, S.A., Byrne, M., Ricevuto, E., Gambi, M.C., 2018. The carbon dioxide vents of Ischia, Italy, a natural system to assess impacts of ocean acidification on marine ecosystems: an overview of research and comparisons with other vent systems. *Oceanogr. Mar. Biol.* 56, 237–310.
- Gambi, M.C., Musco, L., Giangrande, A., Badalamenti, F., Micheli, F., Kroeker, K., 2016. Distribution and functional traits of polychaetes in a CO₂ vent system: winners and losers among closely related species. *Mar. Ecol. Prog. Ser.* 550, 121–134.
- Gambi, M.C., Manzo, M., Giangrande, A., Massa-Galluci, A., 2017. Pilot study on population features of *Syllis prolifera* (Annelida, Syllidae) in relation to ocean acidification. *Biol. Mar. Med.* 24, 114–115.
- Geracitano, L.A., Bocchetti, R., Monserrat, J.M., Regoli, F., Bianchini, A., 2004. Oxidative stress responses in two populations of *Leaenoreis acuta* (Polychaeta, Nereididae) after acute and chronic exposure to copper. *Mar. Environ. Res.* 58, 1–17.
- Giangrande, A., 1988. Polychaete zonation and its relation to algal distribution down a vertical cliff in the western Mediterranean (Italy): a structural analysis. *J. Exp. Mar. Biol. Ecol.* 120, 263–276.
- González-Delgado, S., Hernández, J.C., 2018. Chapter two - the importance of natural acidified systems in the study of ocean acidification: what have we learned? *Adv. Mar. Biol.* 80, 57–99.
- Hall-Spencer, J.M., Rodolfo-Metalpa, R., Martin, S., Ransome, E., Fine, M., Turner, S.M., Rowley, S.J., Tedesco, D., Buia, M.C., 2008. Volcanic carbon dioxide vents show ecosystem effects of ocean acidification. *Nature* 454, 96–99.
- Huang, X., Jiang, X., Sun, M., Dupont, S., Huang, W., Hu, M., Li, Q., Wang, Y., 2018. Effects of copper on hemocyte parameters in the estuarine oyster *Crassostrea rivularis* under low pH conditions. *Aquat. Toxicol.* 203, 61–68.
- Hutchinson, T.H., Shillabeer, N., Winter, M.J., Pickford, D.B., 2006. Acute and chronic effects of carrier solvents in aquatic organisms: a critical review. *Aquat. Toxicol.* 76, 69–92.
- Kroeker, K.J., Micheli, F., Gambi, M.C., Martz, T.R., 2011. Divergent ecosystem responses within a benthic marine community to ocean acidification. *Proc. Natl. Acad. Sci.* 108, 14515–14520.
- Kroeker, K.J., Kordas, R.L., Crim, R., Hendriks, I.E., Ramjo, L., Singh, G.S., Duarte, C.M., Gattuso, J.-P., 2013. Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming. *Glob. Chang. Biol.* 19, 1884–1896.
- Leoni, B., Bettinetti, R., Galassi, S., 2008. Sub-lethal effects of acetone on *Daphnia magna*. *Ecotoxicology* 17, 199–205.
- Lewis, C., Clemow, K., Holt, W.V., 2013. Metal contamination increases the sensitivity of larvae but not gametes to ocean acidification in the polychaete *Pomatoceros lamarckii* (Quatrefages). *Mar. Biol.* 160, 2089–2101.
- Lewis, C., Ellis, R.P., Vernon, E., Elliot, K., Newbatt, S., Wilson, R.W., 2016. Ocean acidification increases copper toxicity differentially in two key marine invertebrates with distinct acid-base responses. *Sci. Rep.* 6, 21554.
- Lucey, N.M., Lombardi, C., De Marchi, L., Schulze, A., Gambi, M.C., Calosi, P., 2015. To brood or not to brood: are marine invertebrates that protect their offspring more resilient to ocean acidification? *Sci. Rep.* 5, 12009.
- Lucey, N.M., Lombardi, C., Florio, M., DeMarchi, L., Nannini, M., Rundle, S., Gambi, M.C., Calosi, P., 2016. An in situ assessment of local adaptation in a calcifying polychaete from a shallow CO₂ vent system. *Evol. Appl.* 9, 1054–1071.
- Marangoni, L.F.B., Pinto, M.M.A.N., Marques, J.A., Bianchini, A., 2019. Copper exposure and seawater acidification interaction: antagonistic effects on biomarkers in the zooxanthellate scleractinian coral *Mussismilia harttii*. *Aquat. Toxicol.* 206, 123–133.
- Meister, A., Anderson, M.E., 1983. Glutathione. *Annu. Rev. Biochem.* 52, 711–760.
- Migliaccio, O., Pinsino, A., Maffioli, E., Smith, A.M., Agnisola, C., Matranga, V., Nonnis, S., Tedeschi, G., Byrne, M., Gambi, M.C., Palumbo, A., 2019. Living in future ocean acidification, physiological adaptive responses of the immune system of sea urchins resident at a CO₂ vent system. *Sci. Total Environ.* 672, 938–950.
- Moreira, S.M., Lima, I., Ribeiro, R., Guilhermino, L., 2006. Effects of estuarine sediment contamination on feeding and on key physiological functions of the polychaete *Hediste diversicolor*: laboratory and in situ assays. *Aquat. Toxicol.* 78, 186–201.
- Morosetti, B., Freitas, R., Pereira, E., Hamza, H., Andrade, M., Coppola, F., Maggioni, D., Della Torre C., 2020. Will temperature rise change the biochemical alterations induced in *Mytilus galloprovincialis* by cerium oxide nanoparticles and mercury? *Environ. Res.* 188, 109778.
- Munari, M., Chemello, G., Finos, L., Ingrosso, G., Giani, M., Marin, M.G., 2016. Coping with seawater acidification and the emerging contaminant diclofenac at the larval stage: a tale from the clam *Ruditapes philippinarum*. *Chemosphere* 160, 293–302.
- Munari, M., Matozzo, V., Gagné, F., Chemello, G., Riedl, V., Finos, L., Pastore, P., Badocco, D., Marin, M.G., 2018. Does exposure to reduced pH and diclofenac induce oxidative stress in marine bivalves? A comparative study with the mussel *Mytilus galloprovincialis* and the clam *Ruditapes philippinarum*. *Environ. Pollut.* 240, 925–937.
- Munari, M., Matozzo, V., Chemello, G., Riedl, V., Pastore, P., Badocco, D., Marin, M.G., 2019. Seawater acidification and emerging contaminants: a dangerous marriage for haemocytes of marine bivalves. *Environ. Res.* 175, 11–21.
- Munari, M., Matozzo, V., Benetello, G., Riedl, V., Pastore, P., Badocco, D., Marin, M.G., 2020a. Exposure to reduced pH and caffeine affects hemocyte parameters in the mussel *Mytilus galloprovincialis*. *J. Mar. Sci. Eng.* 8, 238.
- Munari, M., Matozzo, V., Riedl, V., Pastore, P., Badocco, D., Marin, M.G., 2020b. EAT BREATHE EXCRETE REPEAT: physiological responses of the mussel *Mytilus galloprovincialis* to diclofenac and ocean acidification. *J. Mar. Sci. Eng.* 8, 907.
- Munari, M., Devigili, A., dalle Palle, G., Asnicar, D., Pastore, P., Badocco, D., Marin, M.G., 2022. Ocean Acidification, but not environmental contaminants, affects fertilization success and sperm motility in the sea urchin *Paracentrotus lividus*. *J. Mar. Sci. Eng.* 10, 247.
- Musco, L., Terlizzi, A., Licciano, M., Giangrande, A., 2009. Taxonomic structure and effectiveness of surrogates in environmental monitoring: a lesson from polychaetes. *Mar. Ecol. Prog. Ser.* 383, 199–210.
- Nagelkern, I., Cornell, S.D., 2015. Global alteration of ocean ecosystem functioning due to increasing human CO₂ emissions. *Proc. Natl. Acad. Sci. U.S.A.* 112, 13272–13277.
- Nielson, C., Hird, C., Lewis, C., 2019. Ocean acidification buffers the physiological responses of the king ragworm *Alitta virens* to the common pollutant copper. *Aquat. Toxicol.* 212, 120–127.
- Nikinmaa, M., 2013. Climate change and ocean acidification-interactions with aquatic toxicology. *Aquat. Toxicol.* 15, 365–372.
- Orellana, M.B., Guajardo, V., Araya, J., Thieleman, L., Rodrigo, R., 2001. Oxidative stress, microsomal and peroxisomal fatty acid oxidation in the liver of rats treated with acetone. *Comp. Biochem. Physiol. Toxicol. Pharmacol.* 128, 503–509.
- Pecl, G.T., Araújo, M.B., Bell, J.D., Blanchard, J., Bonebrake, T.C., Chen, I.C., Clark, T.D., Colwell, R.K., Danielsen, F., Evengård, B., Falconi, L., Ferrier, S., Frusher, S., Garcia, R.A., Griffis, R.B., Hobday, A.J., Janion-Scheepers, C., Jarzyna, M.A., Jennings, S., Lenoir, J., Linnetved, H.I., Martin, V.Y., McCormack, P.C., McDonald, J., Mitchell, N.J., Mustonen, T., Pandolfi, J.M., Pettorelli, N., Popova, E., Robinson, S.A., Scheffers, B.R., Shaw, J.D., Sorte, C.J.B., Strugnell, J.M., Sunday, J.M., Tuanmu, M.N., Vergés, A., Villanueva, C., Wernberg, T., Wapstra, E., Williams, S.E., 2017. Biodiversity redistribution under climate change: impacts on ecosystems and human well-being. *Science* 355, eaai9124.
- Rastrick, S.P.S., Graham, H., Azetsu-Scott, K., Calosi, P., Chierici, M., Fransson, A., Hop, H., Hall-Spencer, J.M., Milazzo, M., Thor, P., Kutti, T., 2018. Using natural analogues to investigate the effects of climate change and ocean acidification on northern ecosystems. *ICES J. Mar. Sci.* 75 (7), 2299–2311.
- Regoli, F., Giuliani, M.E., 2014. Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. *Mar. Environ. Res.* 93, 106–117.
- Ricevuto, E., Lorenti, M., Patti, F.P., Scipione, M.B., Gambi, M.C., 2012. Temporal trends of benthic invertebrate settlement along a gradient of Ocean Acidification at natural CO₂ vents (Tyrrhenian Sea). *Biol. Mar. Mediterr.* 19, 49–52.

- Ricevuto, E., Kroeker, K.J., Ferrigno, F., Micheli, F., Gambi, M.C., 2014. Spatio-temporal variability of polychaete colonization at volcanic CO₂ vents indicates high tolerance to ocean acidification. *Mar. Biol.* 161, 2909–2919.
- Ricevuto, E., Benedetti, M., Regoli, F., Spicer, J.I., Gambi, M.C., 2015a. Antioxidant capacity of polychaetes occurring at a natural CO₂ vent system: results of an in situ reciprocal transplant experiment. *Mar. Environ. Res.* 11, 44–51.
- Ricevuto, E., Vizzini, S., Gambi, M.C., 2015b. Ocean acidification effects on stable isotope signatures and trophic interactions of polychaete consumers and organic matter sources at a CO₂ shallow vent system. *J. Exp. Mar. Biol. Ecol.* 468, 105–117.
- Ricevuto, E., Lanzoni, I., Fattorini, D., Regoli, F., Gambi, M.C., 2016. Arsenic speciation and susceptibility to oxidative stress in the fanworm *Sabella spallanzanii* (Annelida, Sabellidae) under naturally acidified conditions: an in situ transplant experiment in a Mediterranean CO₂ vent system. *Sci. Total Environ.* 544, 765–773.
- Rodríguez-Romero, A., Viguri, J.R., Calosi, P., 2021. Acquiring an evolutionary perspective in marine ecotoxicology to tackle emerging concerns in a rapidly changing ocean. *Sci. Total Environ.* 764, 142816.
- Scanes, E., Parker, L.M., O'Connor, W.A., Gibbs, M.C., Ross, P.M., 2018. Copper and ocean acidification interact to lower maternal investment, but have little effect on adult physiology of the Sydney rock oyster *Saccostrea glomerata*. *Aquat. Toxicol.* 203, 51–60.
- Schiedek, D., Sundelin, B., Readman, J.W., Macdonald, R.W., 2007. Interactions between climate change and contaminants. *Mar. Pollut. Bull.* 54, 1845–1856.
- Siddiqui, S., Bielmyer-Fraser, G.K., 2015. Responses of the sea anemone, *Exaiptasia pallida*, to ocean acidification conditions and copper exposure. *Aquat. Toxicol.* 167, 228–239.
- Teixido, N., et al., 2020. Ocean acidification causes variable trait-shifts in a coral species. *Glob. Change Biol.* 26, 6813–6830. <https://doi.org/10.1111/gcb.15372>.
- Toledano, M.B., Huang, M.E., 2017. The unfinished puzzle of glutathione physiological functions, an old molecule that still retains many enigmas. *Antioxid. Redox Signal.* 27, 1127–1129.
- Valvassori, G., Benedetti, M., Regoli, F., Gambi, M.C., 2019. Antioxidant efficiency of *Platynereis* spp. (Annelida, Nereididae) under different pH conditions at a CO₂ Vent's system. *J. Mar. Biol.* <https://doi.org/10.1155/2019/8415916>.
- Viarengo, A., Pertica, M., Mancinelli, G., Burlando, B., Canesi, L., Orunesu, M., 1996. In vivo effects of copper on the calcium homeostasis mechanisms of mussel gill cell plasma membranes. *Comp. Biochem. Physiol.* 113, 421–425.
- Vizzini, S., Martínez-Crego, B., Andolina, C., Massa-Gallucci, A., Connell, S.D., Gambi, M. C., 2017. Ocean acidification as a driver of community simplification via the collapse of higher-order and rise of lower-order consumers. *Sci. Rep.* 7, 4018.
- Xie, Z.C., Wong, N.C., Qian, P.Y., Qiu, J.W., 2005. Responses of polychaete *Hydroides elegans* life stages to copper stress. *Mar. Ecol. Prog. Ser.* 285, 89–96.