

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

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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.

Keywords: ocean acidification, *Syllis prolifera*, oxidative stress, acetone, copper, CO₂ vents

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 (Rodriguez-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 $p\text{CO}_2$ exchanges at the atmosphere-water interface (Caldeira & Wickett 2005). Based on recent models (Caldeira & 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 & Cornell 2015; Asnicar et al., 2021). Besides, other pioneering studies carried out in CO_2 vent systems, which are naturally acidified by the CO_2 surplus emitted from the seafloor, focused on species able to survive and thrive in a lower pH-high $p\text{CO}_2$ ocean (Foo et al. 2018). The CO_2 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 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; Gonzalez-Delgado & Hernandez 2018), driving some species to counteract the effects of OA through a range of adaptive processes, including acclimatisation (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 CO_2 vent systems nowadays studied around the world (Gonzalez-Delgado & Hernandez 2018; Rastrik 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 CO_2 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; Auriemma et al. 2019), and show different adaptations to OA at the individual species level (Lucey et al. 2015, 2016; Ricevuto et al. 2015a,

114 2016). Therefore, polychaetes represent ideal biological models to shed light on the molecular and
 115 physiological key mechanisms which allow counteracting OA otherwise detrimental effects.

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

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

133 Among polychaetes, *Syllis prolifera* (Krohn, 1852) is a meso-herbivore species, which lives on rocky bottoms,
 134 usually associated with algal-dominated habitats in shallow waters (Giangrande 1988; Ricevuto et al. 2015a),
 135 including disturbed biotopes (Musco et al. 2009). This species is abundant in the hard bottom habitats in the
 136 Castello CO₂ vents, including the most acidified zone, where it represents one of the few abundant species
 137 (Cigliano et al. 2010; Kroeker et al. 2011; Ricevuto et al. 2012, 2014; Foo et al. 2018), and where also it can
 138 reproduce by stolonization with the production of sexual satellites (Gambi et al. 2017). There is still little
 139 evidence of the effects of elevated *p*CO₂ on *S. prolifera*, however, Calosi et al. (2013) showed that the species,
 140 although collected in the vents area, showed a significant increase in mean oxygen consumption when exposed
 141 to low *p*CO₂ conditions for 5 days transplants at the vents. Besides, a study carried out on *S. prolifera* and
 142 *Platynereis* spp. inhabiting the vent system of Castello Aragonese showed that native organisms displayed
 143 enhanced basal antioxidant efficiency compared to specimens living under normal pH conditions (Ricevuto et
 144 al. 2015a). Since the antioxidant defence system is one of the key cellular pathways to counteract the adverse
 145 effects of toxic substances (Regoli and Giuliani 2014), the increased antioxidant capability observed in those
 146 organisms could be winning also in developing tolerance to chemical contamination.

147 In this scenario, this study aimed to assess if organisms able to cope with natural acidified conditions have also
 148 an enhanced resistance against different environmental stress such as chemical contamination or whether
 149 physiological modifications, induced to cope with the acidified conditions, will reduce the capability of the
 150 organism to respond to chemical contamination, and whether the occurrence of this further challenge will

151 impact more heavily their health and survival. To answer the question, we first assessed the basal antioxidant
152 capability of individuals of *Syllis prolifera* inhabiting the CO₂ vent system of the Castello Aragonese. Then
153 we investigated the responsiveness of individuals living inside the vents compared to those collected in control
154 zones, living under normal pH conditions, to face exposure to two environmental pollutants with different
155 mechanism of action. Acetone is a widely used industrial solvent commonly found in the atmosphere, in natural
156 water bodies and in groundwater as well (Armutcu et al. 2005). This molecule is commonly used as a carrier
157 of contaminants in many ecotoxicity tests, due to its high solubility and low toxicity. Copper (Cu) is a metal,
158 widespread in the marine ecosystems as a result of mining activities, municipal and industrial effluent
159 discharges and application in antifouling paints (Corcoll et al., 2019). The most characterized mechanisms of
160 toxicity of Cu is through the overproduction of ROS and imbalance of acid-base homeostasis (Geracitano et
161 al., 2004; Viarengo et al., 1996). The combined effects of Cu and OA have been investigated under laboratory
162 conditions on different marine invertebrates, showing contrasting outcomes depending on the species'
163 physiology, since often the toxicity of Cu was increased under OA conditions (Campbell et al., 2014; Siddiqui
164 and Bielmyer-Fraser 2015; Lewis et al., 2016; Bielmyer-Fraser et al., 2018; Huagn et al., 2018; Scanes et al.,
165 2018) but also antagonistic effects have been described (Marangoni et al., 2019; Nielson et al., 2019).

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168 2. Materials and Methods

169 2.1 Sites and sampling

170 Individuals of the target species *Syllis prolifera* were collected in November 2019 and June 2021 from the
171 Castello Aragonese CO₂ vents system (stations Centred in 40°43'57.9" N, 13°57'51.8" E on the south side of
172 the Castello named S3 and S2 from previous investigations (e.g., Ricevuto et al. 2015a, 2015b; Calosi et al.
173 2013; Foo et al. 2018), and from the control site at San Pietro promontory 40°44'47.6" N, 13°56'40.42" E,
174 which is located approximately 4 km from the vents (Fig. 1), a site which has been already used as reference
175 in similar studies (Ricevuto et al. 2015b; Calosi et al. 2013). For the physico-chemical characterization of the
176 acidified areas of the Castello we refer to multiples previous studies where pH, pCO₂ and other parameters
177 have been intensively measured (e.g., Hall-Spencer et al., 2008; Kroeker et al., 2011) or summarized (e.g.,
178 Ricevuto et al. 2014; Foo et al., 2018). Since the acidification level and its variability are related to the intensity
179 of the venting (bubbling) from the floor and this has not changed in the past 10 years (Gambi M.C., personal
180 observation), we consider the characterization given by past studies still reliable of the local OA conditions.
181 Salinity of the zone is constant at 38 PSU (Foo et al., 2018). We do not have measures of the natural levels of

182 copper and acetone in the area, however, being both the control and the vents sites included in the Marine
 183 Protected Area of Ischia, there aren't evident source of pollution in the area.

184 As *Syllis prolifera* lives in association with several macroalgae species (mainly *Halopteris scoparia* and
 185 *Cladophora* spp.), the macroalgae were collected by hand by scuba divers that used fabric bags subsequently
 186 covered by plastic covers at 1-2 m depth in both sites. Individuals of *S. prolifera* were sorted from the algae
 187 and identified, worms were pooled (3 pools of 20 individuals each from each site) and stored at -80 °C before
 188 biomarkers analysis.



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190 **Fig. 1. Map of the Ischia island.** Location of the two sampling sites: Vent site at the Castello Aragonese (40°43'57.9"N
 191 - 13°57'51.8"E), control site at San Pietro Point (40°44'47.6" N; 13°56'40.42"E).
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193 2.2 *In vivo* exposure

194 The experiments were carried out using individuals from both the San Pietro site and the vent system of
 195 Castello Aragonese with specimens collected as previously described. The experiment with acetone was
 196 carried out in September 2020, while the one with copper in June 2021. Individuals used for *in vivo* exposures
 197 were collected from the field prior to each experiment and sorted for identification using seawater collected
 198 from the control site underneath the laboratories of the Ischia Marine Centre, conditioned at the same $p\text{CO}_2$
 199 conditions of the site of origin. After sorting and identification, polychaetes (body length range 3-6 mm) were
 200 maintained in a thermostatic room at 25 °C with 12:12h day: night photoperiod, in glass containers (200 mL)
 201 supplied with filtered seawater (0.22 μm) from the control site conditioned at the same $p\text{CO}_2$ conditions of the
 202 site of origin.. The CO_2 was supplied and monitored through an automatic system (*Touch Controller acq140*
 203 by Aquatronica S.r.l.). From previous trials and studies (Teixido et al., 2020) we know that there are no

204 persistent differences in Total Alkalinity among different sites being open systems. During the acclimation
205 period, polychaetes were fed *ad libitum* with grinded fresh spinach.

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207 2.2.1 Acetone experiments

208 After three days of acclimation, individuals were subdivided into glass bottles (250 mL) supplied with artificial
209 seawater (distilled water added with *Amtra Sea Complex*) at 37 ± 1 ‰ salinity and 8.08 ± 0.01 (mean \pm S.D.)
210 pH for control treatment and 7.73 ± 0.03 (mean \pm S.D.) pH for acidified treatment.

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

- 213 • Control seawater at pH 8.1, individuals collected from San Pietro
- 214 • Acetone at 0.05 % v/v at pH 8.1 individuals collected from San Pietro
- 215 • Control seawater at pH 7.7 individuals collected from the Castello vents
- 216 • Acetone at 0.05 % v/v at pH 7.7 individuals collected from the Castello vents

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

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

- 221 • Control seawater at pH 8.1, individuals collected from San Pietro
- 222 • Acetone at 0.01 % v/v at pH 8.1 individuals collected from San Pietro
- 223 • Control seawater at pH 7.7 individuals collected from the Castello vents
- 224 • Acetone at 0.01 % v/v at pH 7.7 individuals collected from the Castello vents

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

230

231 2.2.2 Copper sulphate

232 In June 2021, after three days of acclimation, samples were divided into glass bottles (250 mL) supplied with
233 artificial seawater (distilled water added with *Amtra Sea Complex*) at 37 ± 1 ‰ (mean \pm S.D.) salinity and 8.13
234 ± 0.03 (mean \pm S.D.) pH for control treatment and 7.67 ± 0.05 (mean \pm S.D.) pH for acidified treatment.
235 Polychaetes during the experiment were fed with frozen spinach finely chopped.

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

- 237 • Control seawater at pH 8.1, individuals collected from San Pietro
- 238 • Copper sulphate at 2 mg/L at pH 8.1 individuals collected from San Pietro
- 239 • Copper sulphate at 0.2 mg/L at pH 8.1 individuals collected from San Pietro

- 240 • Copper sulphate at 0.05 mg/L at pH 8.1 individuals collected from San Pietro
- 241 • Control seawater at pH 7.7 individuals collected from the Castello vents
- 242 • Copper sulphate at 2 mg/L at pH 7.7 individuals collected from the Castello vents
- 243 • Copper sulphate at 0.2 mg/L at pH 7.7 individuals collected from the Castello vents
- 244 • Copper sulphate at 0.05 mg/L at pH 7.7 individuals collected from the Castello vents

245 The Cu concentrations were selected based on LC₅₀ reported for other polychaete species (from 125 µg/L up
 246 to ≥500 µg/L Xie et al., 2005; Dean, 2008; Moreira et al., 2009; Bouraoui et al., 2015). Water was changed
 247 every 48 h (80% of the total volume) and at the end of the experiment (five days of exposure), the survival
 248 rates were recorded and individuals from each replicate were collected and stored at -80 °C before biomarkers
 249 analysis.

250 **2.3 Biomarkers analysis**

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

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

262 The measurement of antioxidant enzymes was carried out using a 6715 UV/Vis spectrophotometer (Jenway).
 263 The protein content was determined following the method described by Bradford (1976), using bovine serum
 264 albumin (BSA) as standard (0.1-0.5 mg mL⁻¹ r² > 0.98). The SOD activity was determined by measuring the
 265 degree of inhibition of cytochrome c reduction by the superoxide anion generated by the xanthine oxidase
 266 reaction at 550 nm. Activities were given in SOD units (1 SOD unit = 50% inhibition of the xanthine oxidase
 267 reaction). The CAT activity was assessed by measuring the consumption of H₂O₂ at λ =240 nm. The reading
 268 lasted 1 min and values were expressed as µmol min⁻¹ mg proteins⁻¹. The GPx activity was evaluated by
 269 measuring the NADPH consumption at 340 nm using H₂O₂ 0.2 mM as substrate with glutathione (2 mM),
 270 sodium azide (NaN₃; 1 mM), glutathione reductase (2 U/mL), and NADPH (120 µM). The activity was
 271 expressed as µmol min⁻¹ mg proteins⁻¹. The activity of GST was measured in presence of reduced glutathione
 272 (1 mM) and 1-chloro-2,4-dinitrobenzene (CDNB) as co-substrate. The spectrophotometer reading at λ = 340
 273 nm lasted 1 min activity was expressed as mmol min⁻¹ mg proteins⁻¹.

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275 **2.4 Statistical analysis**

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

288 **3. Results**

289 **3.1 Effects of OA on basal activities of antioxidant enzymes and GLY content**

290 The graphs in Fig. 2 show the variation in the basal activity of antioxidant enzymes in individuals of *S.*
291 *prolifera*, according to the sampling site (San Pietro vs Castello vent) and the period (November vs June).
292 The activity of GST and CAT resulted significantly different as a function of the sampling period, being higher
293 in summer compared to autumn in both sampling sites (PERMANOVA test, Tab S1). Concerning the
294 comparison between sites, any significant difference could be observed in GST SOD and CAT activities (Fig.
295 2; Tab. S1), while the activity of GPx resulted significantly higher in individuals from San Pietro compared to
296 those from the vent, although only in November (Fig. 2; Tab. S1).

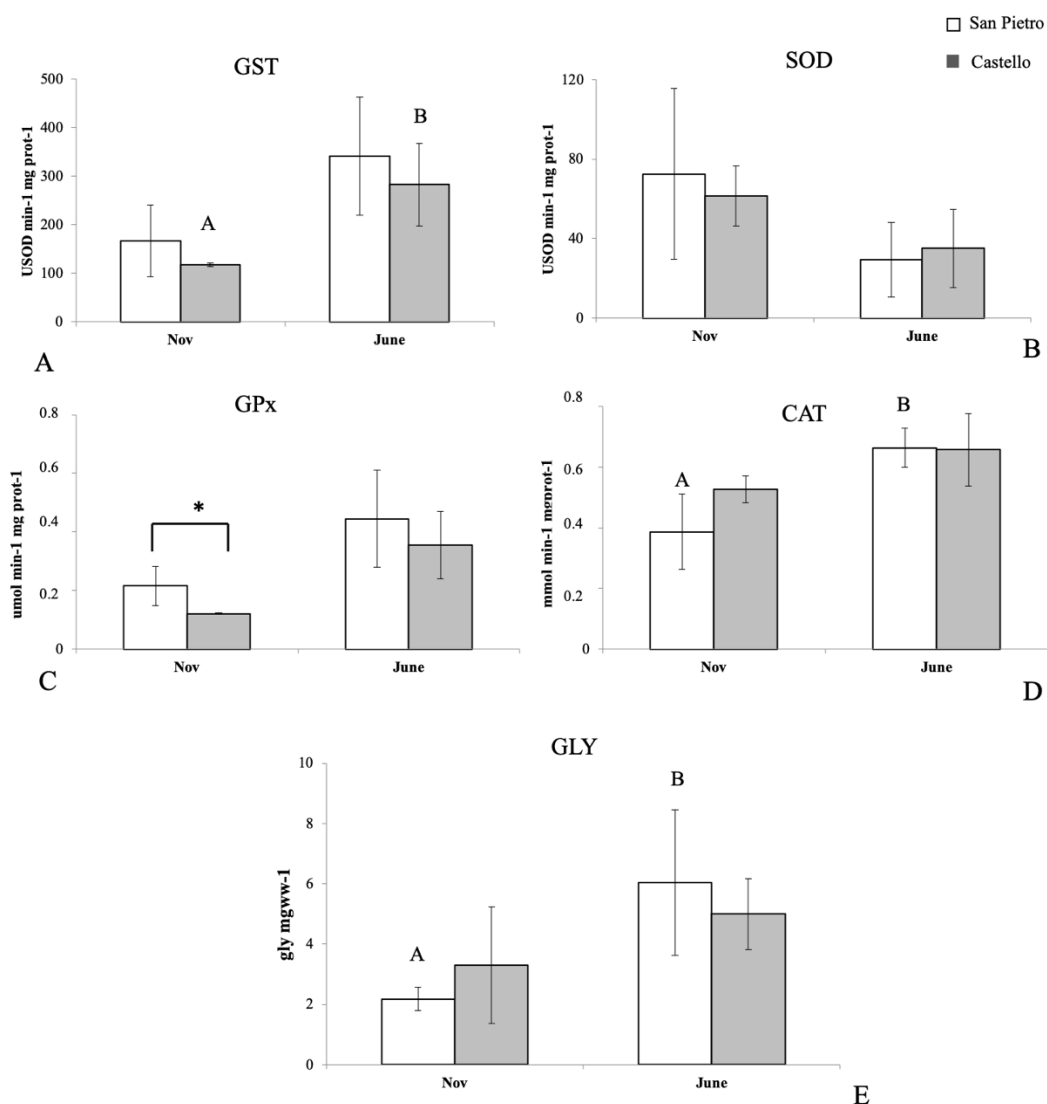


Fig. 2. Basal antioxidant activities. Mean \pm standard deviation (s.d.) of basal activity of GST (A), SOD (B), GPx (C), CAT (D) and GLY content (E) measured in individuals of *Syllis prolifera* from San Pietro and the vent system of Castello Aragonese in November and June. The asterisk (*) means statistically 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 ($p \leq 0.05$).

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

332 Table 1. Survival rate of individuals of *S. prolifera* from San Pietro and the Castello Aragonese vent system upon
 333 treatment with acetone and copper. The asterisk (*) means statistically different activities due to treatment within the
 334 same site of origin. Different letter (A, B) means statistically significant differences between individuals from the two
 335 sites ($p \leq 0.05$).
 336

	San Pietro	Castello Aragonese
acetone		
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
Cu		
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 ± 5.7
2 mg/L	0	0

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

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

356 The GPx enzyme showed a profile of activity similar to GST, with a significant increase in activity observed
 357 only in the group treated with acetone compared to the control for individuals from the Castello vent (Fig. 4),
 358 while no changes were observed in the activity of this enzyme for individuals from the San Pietro site. Finally,
 359 a slight increase of CAT activity in individuals from San Pietro was observed in acetone-treated group
 360 compared to control ones, while the acetone treatment generated a slight activity decline in individuals from
 361 the Castello vent (Fig. 4). Due to the reduced number of individuals survived at the end of the exposure
 362 experiments, it was not possible to measure GLY content for both the copper and acetone experiments.

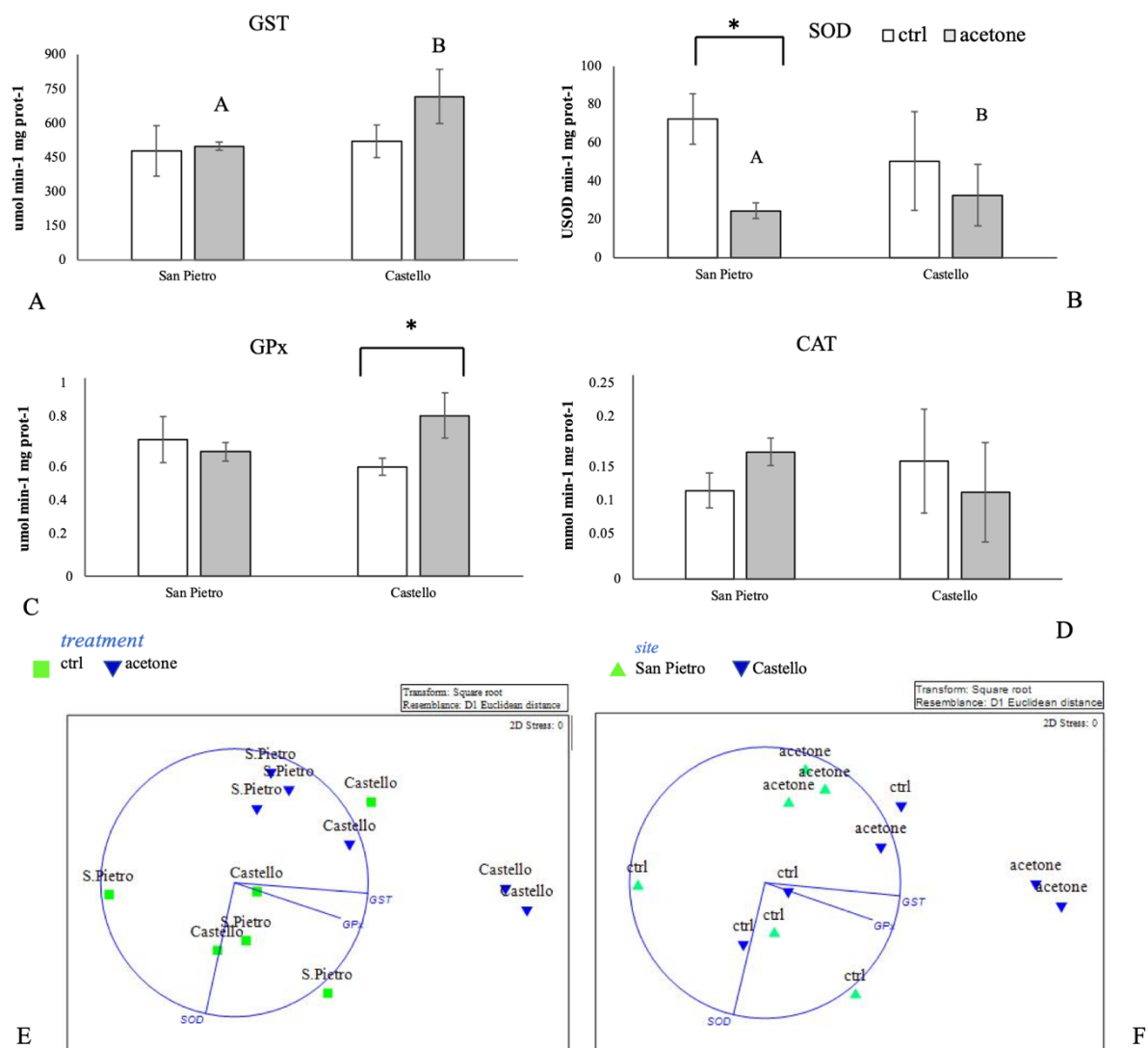


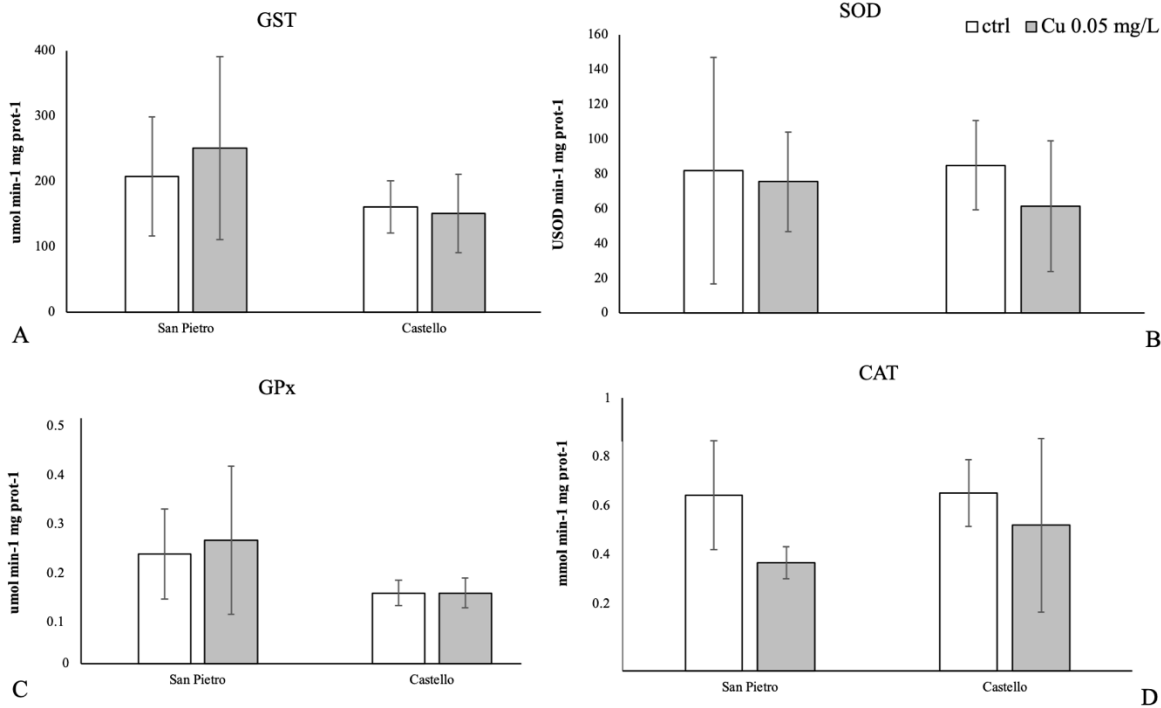
Fig. 4. Effects of acetone on antioxidant enzymes. Mean \pm s.d. of GST (A), SOD (B), GPx (C) and CAT (D) measured in *Syllis prolifera* from San Pietro and the vent system of Castello Aragonese treated with acetone 0.01% for five days. The asterisk (*) means statistically different activities due to 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).

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).

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3.2.1 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 (Figure 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).



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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.

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 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*

403 exposure experiments for a limited time and in controlled laboratory conditions, do not allow to differ between
404 plastic and adaptive mechanisms, the latter induced in organisms to cope with disturbances related to global
405 changes. This limits significantly our ability to realistically predict the impact of multiple stressors on marine
406 ecosystems (Rodriguez-Romero et al. 2021). In this study, a different experimental approach was used, since
407 for the first time, populations of species naturally exposed to OA during their entire life were employed.

408

409 4.1 Basal characterization of antioxidant system and GLY content

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

420 Our results seem to confirm that there is a limited influence of acidified conditions on the functionality of the
421 antioxidant system also in *S. prolifera*. In fact, only the activity of the GPx was significantly different between
422 individuals from the two sites. This observation is in contrast with the findings obtained in laboratory studies
423 carried out on polychaetes. In fact, short-term *in vivo* exposure to low pH levels, similar to the ones observed
424 in the CO₂ vent of Castello Aragonese, triggered an alteration of antioxidant enzymes activities in *Diopatra*
425 *neapolitana* and *Hediste diversicolor*. In particular, an increase of CAT, SOD and GST activities and lipid
426 peroxidation was detected in organisms subjected to acidified conditions (Freitas et al. 2015; 2016). The
427 modulation of antioxidant enzymes has been observed also in another study in which an *in situ* transplant
428 experiment of *Sabella spallanzanii* was carried out in the CO₂ vent of Castello Aragonese (Ricevuto et al.
429 2016). In this case, a reduction of CAT and GPx has been observed in organisms translocated from the control
430 site to the vents for 30 days.

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

435 These discrepancies highlight how the responses to OA might be very different in the short and long term since
436 the induction of the antioxidant system could represent an acclimatization response to a sudden increase in
437 acidification levels, while this system does not appear to be induced in organisms living under low and
438 fluctuating pH throughout their entire life. This seems also supported by the results of the GLY content, which

439 suggest that in individuals from the vent an adaptation mechanism might be in place, which allows them to
 440 preserve a sufficient amount of metabolic reserves.

441 The nMDS analysis without showing a clear differentiation between individuals from the Castello vent
 442 compared to the San Pietro control site, highlighted on the contrary a distinct clustering between the two
 443 sampling periods. This indicates that the activity of the antioxidant system of the species is subject to seasonal
 444 variations with higher activities (except for SOD) in summer than in autumn, regardless of the pH conditions.
 445 Seasonal fluctuations of antioxidant enzymes have been described in several marine invertebrates (Chainy et
 446 al. 2016). Indeed, factors such as the intensity of solar radiation, temperature, salinity and oxygen
 447 concentration, might trigger prooxidant conditions for the organisms, which therefore increase the activity of
 448 antioxidant enzymes to maintain their oxidative status (Bocchetti and Regoli, 2006). Notwithstanding, the
 449 seasonal effect was different in the two sampling sites, with CAT increasing in individuals from San Pietro,
 450 while for vent polychaetes it was GST that increased, and thus suggesting that in organisms tolerant to OA the
 451 mechanisms induced to face the response to environmental fluctuations are different to those of individuals
 452 which are not subjected to stressful conditions.

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

459

460 *4.2 Combined effects of OA and pollutants*

461 Although living inside the vents does not seem to affect dramatically the efficiency of the antioxidant system,
 462 the response to harmful chemicals may be altered. To test this hypothesis, the organisms from the two sites
 463 were exposed *in vivo* to acetone. The results showed the absence of lethal effects for individuals from the
 464 control site, in line with what was found in aquatic species in previous studies (Hutchinson et al. 2006; Leoni
 465 et al. 2008). On the contrary, a high mortality rate was observed in individuals from the vent exposed to acetone
 466 (0.05% v/v). Also in the further experiment, with lower acetone concentrations (0.01% v/v), a greater lethality
 467 for individuals from the vent compared to those from San Pietro control was observed, thus suggesting that
 468 this substance has a higher toxic effect on the vent population.

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

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

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

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

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

501 The results of the two exposure experiments suggest that the combined effects of OA and environmental
502 pollutants are dependent on their specific mechanisms of actions and also on the metabolic and physiological
503 performances of the species. These observations highlight the need to increase our understanding of the
504 molecular, cellular and physiological mechanisms that underlie the species tolerance towards OA, which is
505 currently overlooked. This is essential for predicting the vulnerability of organisms such as *S. prolifera* to
506 further environmental disturbances.

507

508 **5. Conclusions**

509 Our study provided the first information on the antioxidant system of the polychaete *Syllis prolifera* living
510 inside the CO₂ vents of Castello Aragonese, helping to broaden the current knowledge on the mechanisms that
511 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.

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