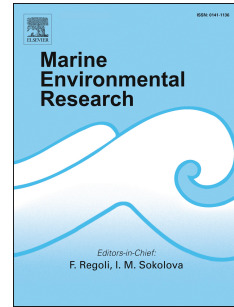# Accepted Manuscript

Condition of pteropod shells near a volcanic CO_2 vent region

Clara Manno, Paola Rumolo, Marco Barra, Sergio d'Albero, Gualtiero Basilone, Simona Genovese, Salvatore Mazzola, Angelo Bonanno

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Abstract

15 Natural gradients of pH in the ocean are useful analogues for studying the projected impacts

16 of Ocean Acidification (OA) on marine ecosystems. Here we document the *in situ* impact of

17 submarine CO$_2$ volcanic emissions (CO$_2$ vents) on live shelled-pteropods (planktonic

18 gastropods) species *Creseis conica* in the Gulf of Naples (Tyrrhenian Sea, Mediterranean).

19 Since the currents inside the Gulf will likely drive those pelagic calcifying organisms into and

20 out of the CO$_2$ vent zones, we assume that pteropods will be occasionally exposed to the

21 vents during their life cycle. Shell degradation and biomass were investigated in the stations

22 located within and nearby the CO$_2$ vent emission in relation to the variability of sea water

23 carbonate chemistry. A relative decrease in shell biomass (22%), increase in incidence of

24 shell fractures (38%) and extent of dissolution were observed in *Creseis conica* collected in

25 the Gulf of Naples compared to those from the Northern Tyrrhenian Sea (control stations).

26 These results suggest that discontinuous but recurrent exposure to highly variable carbonate

27 chemistry could consistently affect the characteristic of the pteropod shells.

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28 **Key words**
29 Ocean Acidification, Mediterranean, calcification, pteropods
30

1. Introduction

32 Marine ecosystems are increasingly influenced by decreasing seawater pH and carbonate

33 chemistry changes resulting from oceanic absorption of anthropogenic CO_2, a process now

34 well known as Ocean Acidification (OA) (Feely et al., 2004). Calcifying organisms are

35 particularly susceptible to OA because perturbations in the seawater carbonate system,

36 including changes in H^+ and $CO_{2\ (aq)}$, can reduce their ability to synthesize and/or maintain

37 calcium carbonate skeletons and shells. In efforts to understand the implications of these

38 changes on marine organisms, shallow submarine volcanic CO_2 vents have been identified as

39 useful analogues for studying the prospective impacts of Ocean Acidification on marine

40 ecosystems (Hall-Spenser et al., 2008) since the water surrounding the CO_2 vent naturally

41 lowers the pH of the water column (Williams et al., 1992).

42 Identifying the natural response of marine organisms to OA is a difficult task in laboratory

43 conditions since the behaviour of the organism is constrained and the feeding environment is

44 poorly simulated (Howes et al., 2015). However, the combination of laboratory experiments

45 with the assessment of naturally acidified environmental gradients (such as CO_2 vent

46 environments), can provide further insights into the threshold pH affecting the performance

47 of vulnerable marine species (Basso et al., 2015). Volcanic CO_2 vents have been widely used

48 as a proxy for future OA conditions by numerous authors showing the negative response of

49 the higher pCO_2 conditions to which benthic organisms have commonly been exposed for

50 their entire life span (i.e. Ricevuto et al., 2012; Milazzo et al, 2014; Langer et al., 2014).

51 Marine volcanic CO_2 vents are abundant in the Mediterranean Sea, especially around Italy

52 (Dando et al., 1999). Recent studies in the Gulf of Naples (Tyrrhenian Sea, Italy), on the

53 impact of CO_2 vents on marine benthic organisms inhabiting shallow coastal waters, showed

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54 a shift from benthic calcareous communities to communities lacking scleractinian coral (Hall-

55 Spenser et al., 2008). Furthermore, settlement and colonization by mollusks and microfauna

56 decreased at the acidified stations (Ricevuto et al., 2012; Milazzo et al., 2014). In the same

57 region, the natural pH gradient negatively affected the growth and survival in bivalves *Pinna*

58 *nobilis* (Ricevuto et al., 2012) while the patellogastropod limpet *Patella caerulea* was able to

59 counteract the low pH induced shell corrosion by the addition of aragonitic shell layers

60 (Langer et al., 2014).

61 With specific reference to the hydrological features, the Gulf of Naples is characterised by

62 the presence of two main water masses typical of the southern Tyrrhenian Sea: the Modified

63 Atlantic Water (MAW) and the Levantine Intermediate Water (LIW) (Uttieri et al., 2011).

64 Even if in the study area the water masses are essentially the same as for the southern

65 Tyrrhenian Sea, the presence of CO_2 submarine emissions alters the carbonate chemistry

66 nature of the water masses. The presence of natural submarine gas emissions was suggested

67 by (Sacchi et al., 2005). More recently (Passaro et al., 2014, 2016), detected and mapped the

68 gas discharge (dominated by CO_2) at the seafloor of the Gulf of Naples and suggested that the

69 occurrence of CO_2 vents in this area could be linked to the interaction between volcanic

70 related seafloor morphologies and the main, North East striking faults present in the area,

71 (i.e., Vesuvian fault).

72 However, all the CO_2 vent related studies have been mainly focused on the response of the

73 coastal benthic ecosystem, while the impact of these natural pH gradients on the planktonic

74 calcifying population has not been explored. Unlike sessile benthic organisms, pelagic

75 species can move in and out of waters surrounding the CO_2 vents and experience a

76 pronounced variability of pCO_2 conditions over time. This mobility makes it difficult to

77 quantify the exposure of pelagic organisms to high pCO_2 levels. However, a recent study on

3

78 corals found that repetitive exposure to high pCO_2 conditions may cause greater responses

79 within certain organisms than exposure to static OA (Roleda et al., 2015).

80 Euthecosome pteropods (planktonic shelled gastropods) have been identified as indicator for

81 OA (OSPAR/ICES advisor group, 2015); as their thin shells are made of aragonite, a

82 metastable form of biogenic $CaCO_3$ (Mucci, 1983), shelled pteropods are extremely sensitive

83 to changes in marine carbonate chemistry. These organisms have been widely studied for OA

84 effects, both in simulated OA conditions in the lab and in the field where high pCO_2 levels

85 already occur. Short-term lab experiments (up to a month), examining the impact of exposure

86 to high pCO_2, document pteropod shell dissolution, lowered shell calcification, altered

87 metabolism, behavior, gene expression and decreased survivorship (i.e. Manno et al., 2007;

88 Comeau et al., 2010; Lischka and Riebesell, 2012; Moya et al., 2016). In the field, changes in

89 pteropod species community composition, geographical distribution and presence of shell

90 dissolution have been observed as a result of co-variation of natural high CO_2 and low

91 dissolved oxygen across a frontal system in the Southern California Current (Bednaršek et al.,

92 2014; 2015) and within an upwelling region in the Scotia Sea (Bednaršek et al., 2012b). Maas

93 et al. (2016) suggested that natural environmental exposure to low pH and oxygen influences

94 pteropod metabolic sensitivity in the Oxygen Minimum Zone in the North Atlantic.

95 Here we present our observations of pteropods collected around the CO_2 vent region in the

96 Gulf of Naples (Tyrrhenian Sea). We aim to assess the condition of pteropod shells (in terms

97 of biomass and dissolution) to episodic exposure to high pCO_2 in the presence of volcanic

98 CO_2 vents. We focus on the species *Creseis conica (C. conica)* which are common and

99 distributed in tropical and subtropical water masses worldwide.

100 This study documents, for the first time, the impact of natural CO_2 volcanic emissions on live

101 pteropods extracted directly from the natural environment. In particular the present work adds

102 new insight to the in situ response of pteropods *C. conica* to recurrent exposure to critical

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103 carbonate chemistry environments. This study also highlights the importance of including

104 CO_2 vent regions within a long term monitoring program to investigate the potential ability of
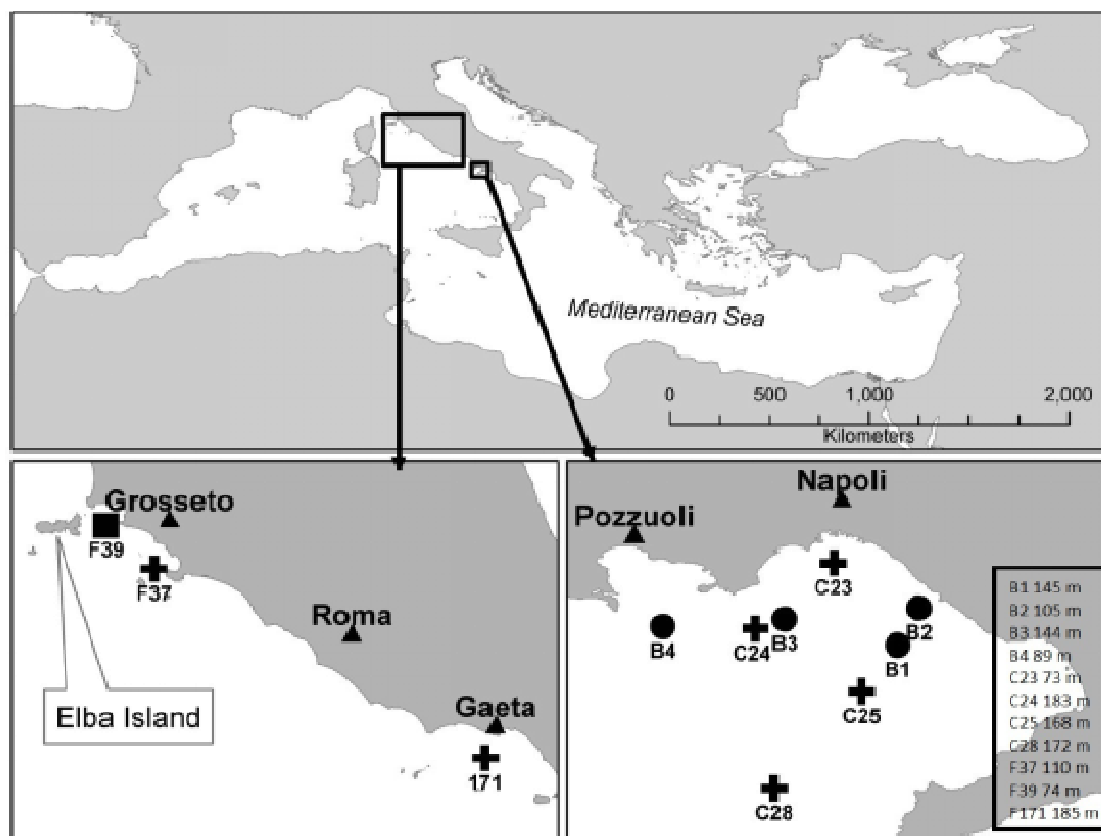
105 pteropods to persist in a high CO_2 ocean.

2. Methods

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2.1 Study region

109 This study was performed within the framework of the Medias (Mediterranean International

110 Acoustic Survey) project in the Tyrrhenian and Ligurian seas. All the samples and data were

111 collected on August 2015 during an oceanographic cruise in the Tyrrhenian Sea on board of

112 the R/V "G. Dallaporta". A total of 8 stations were sampled in the Gulf of Naples

113 characterized by on site (4 stations, group "B") and nearby (4 stations, group "C") presence

114 of natural submarine volcanic CO_2 emissions. Since currents inside the gulf will likely drive

115 the pteropods in (B stations) and out (C stations) the CO_2 vent zones, we assume that those

116 organisms will be periodically exposed to the vents during their life. In addition, more

117 stations (3 stations, group "F") were sampled outside of the Gulf of Naples, in the northern

118 Tyrrhenian Sea (Fig 1), where no CO_2 vents have been identified, to provide a control suite of

119 samples (Fig 1).

120 Stations characterized by natural gas emissions were identified during a previous

121 oceanographic survey in the same area (Passaro et al., 2014) by means of the Simrad EK60

122 Scientific Echosounder. Such instrumentation is typically used for estimating biomass and

123 distributions of small pelagic fish species in many areas of the Mediterranean Sea (Bonanno

124 et al., 2014) but also readily identify plumes of bubbles derived from CO_2 vents at the

125 seafloor.

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128 **Fig 1 Sampling station positions in the Northern Tyrrhenian Sea and in the Gulf of**

129 **Naples (Mediterranean Sea)**. Stations B1-B2-B3-B4 are characterized by the presence of

130 natural submarine volcanic CO_2 emissions. Each station depth is indicated. See Passaro et al.,

131 2014; 2016 for a detailed map of CO_2 vents emission points.

132 **2.2 Hydrology and Carbonate Chemistry measurements**

133 Full depth hydrological casts were acquired across all the stations using an SBE 9/11 Plus

134 CTD, with temperature, oxygen, conductivity and fluorometer sensors. The probes were

135 calibrated before the cruise at Sea-Bird Electronics in Kempten, Germany. The collected

136 downcast data were quality-checked and processed using the Seasoft-Win32 software. The

137 overall accuracies are within $0.001°C$ for temperature, 0.001 sm^{-1} for conductivity, and

138 0.015% of full scale for pressure. Raw fluorescence values were converted to Chl a biomass

139 $(\mu g*l^{-1})$ using the factory calibration.

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140 Discrete Total Alkalinity (TA) and Dissolved Inorganic Carbon (DIC) samples were

141 collected at different depths of the water column using a carousel equipped with Niskin

142 bottles and then poisoned with $HgCl_2$ (2% saturated solution) to prevent biological alteration.

143 Seawater TA and DIC were measured by potentiometer titration, employing the open-cell

144 procedure. The precision for TA was ±2.0 mmol kg^{-1} and 4 mmol kg^1 for DIC. Data accuracy

145 was confirmed by regular analyses of Certified Reference Materials (Scripps Institution of

146 Oceanography). Carbonate saturation states of aragonite (Ω_{ar}) were indirectly calculated

147 from TA and DIC data using the CO2SYS software (Lewis and Wallace, 1998), with

148 carbonate dissociation constants by (Mehrbach et al., 1973) refitted by (Dickson et al., 1997)

149 and sulfate dissociation constants by (Dickson et al., 1990). Note that for logistical reasons no

150 chemistry samples, were collected at station F39.

151 **2.3 Pteropod collection and investigation**

152 On board, living pteropods were collected from near bottom depth (ranging between 65 m

153 and 170 m) to the surface by a Bongo-40 zooplankton net (200 µm mesh size). Sampling took

154 place over one time at each station during the day time. The volume of sea water sampled was

155 measured by General Oceanics mechanical flow-meters attached to the ring net. Samples

156 were stored for 3 weeks within buffered formalin solution and kept at 4°C. pH was measure

157 in all the samples, at the beginning and the end of the storing period to ensure that the state of

158 the shells were not affected by the preservation technique. After three weeks from the

159 collection, pteropod species were identified and counted using a light microscope Olympus

160 SZX16. Pteropod abundance within the water column was calculated as individuals per cubic

161 meter (Ind*m^{-3}).

162 Investigation of shell morphology and shell biomass was determined only for the dominant

163 pteropod species *C. conica*. Shell morphology was performed using a Scanning Electron

164 Microscope (SEM). The number of individuals analysed for SEM ranged between 10 or 20

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165 for each station (except for station B2 where we analysed only 5 organisms) depending on the

166 availability of specimens. Only individuals with similar shell size (juveniles ranging between

167 280 µm-320 µm) were selected to facilitate comparison between different groups assuming

168 same life stage has similar susceptibility to high pCO_2 level. Before SEM imaging,

169 individuals were carefully washed with DI water to remove salt on the shell and then air-

170 dried for 24h. The shells outer organic layer (periostracum) was not removed. We

171 acknowledge that the exclusion (Bednaršek et al. 2016) or inclusion (Peck et al., 2016b) of

172 periostracum for evaluate shell dissolution is still in debate. However our rationale for not

173 removing the periostracum prior to imaging shells follows previous studies (Peck et al., 2015)

174 showing that the removal of the organic outer layer, which also has an intra-crystalline matrix

175 (Marin et al., 1996), can expose crystals in a way which could be mis-interpreted as shell

176 dissolution.

177 Shell degradation was evaluated by applying a semi-quantitative index of dissolution

178 (Gerhardt et al., 2001; Lischka et al., 2011; Manno et al., 2012). This Dissolution Index is

179 represented by six preservation stages (from 0 = best preservation to 5 = highest degree of

180 dissolution), determined by: shell surface lustre (whether lustrous or dull); shell damage

181 (surface with shell corrosion and/or perforation of at least one layer of aragonite). For each

182 station, we calculated the % of shell falling in four dissolution levels: no corrosion

183 (transparency, preservation stage 0); low corrosion (opacity with small sign of dissolution,

184 preservation stage 1-2); high corrosion (periostracum and the first aragonite prismatic are

185 partially missing, preservation stage 3-4); damage (presence of perforation, preservation stage

186 5).

187 Shell surface was inspected for the presence or absence of fracture zone (i.e., resulting from

188 in situ mechanical damage) and represented as % shells presenting fractures to the total

189 shells. To discriminate between "natural fractures" and fractures due to mechanical damage

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190 from the net and collection processing, we only considered the "historical fractures" where it

191 appears that the animal has built up shell material to weld the shell back together (Peck et al.

192 2018).

193 For the measurement of shell biomass (carbonate content expressed as µg $CaCO_3$),

194 individuals were heated to 550°C for 5 h to eliminate organic matter content and the ashes

195 (representing the remains of the shells) weighed using a Toledo microbalance. The ash

196 weight can be considered an indirect estimate of $CaCO_3$ content. As for SEM investigation, to

197 allow us to estimate shell biomass difference between groups, we only used individuals with

198 similar shell diameter (juveniles, 302µm ±11, for a total of 76 specimens) and presenting the

199 best shell condition within each group (31, F; 30 C; 15, B specimens).

2.4 Data analysis

201 For each station, the average values of carbonate chemistry parameters (pH, TA, Ω_{ar}, DIC,

202 pCO_2) were computed together with total abundance, shell biomass, shell dissolution level,

203 and percentage of fractured shells. Temperature, salinity, Chl a and oxygen values recorded at

204 the same depth of carbonate chemistry measurements were extracted from CTD profiles to

205 obtain the average hydrological conditions at each station. Obtained data matrix was then

206 used in the statistical analysis. The pairwise correlation between all above-mentioned factors

207 was computed by using Spearman correlation coefficient. PCA was used to investigate the

208 presence of pattern of variables (that could be interpreted as "processes") as well as to best

209 explain the variation observed among stations. The differences among the identified groups

210 of stations were assessed with parametric statistical tests (namely ANOVA and t-test

211 according to the nubers of groups). If serious violations in the assumption required by

212 parametric tests were identified, the non-parametric alternative were used (Kruskal-Wallis
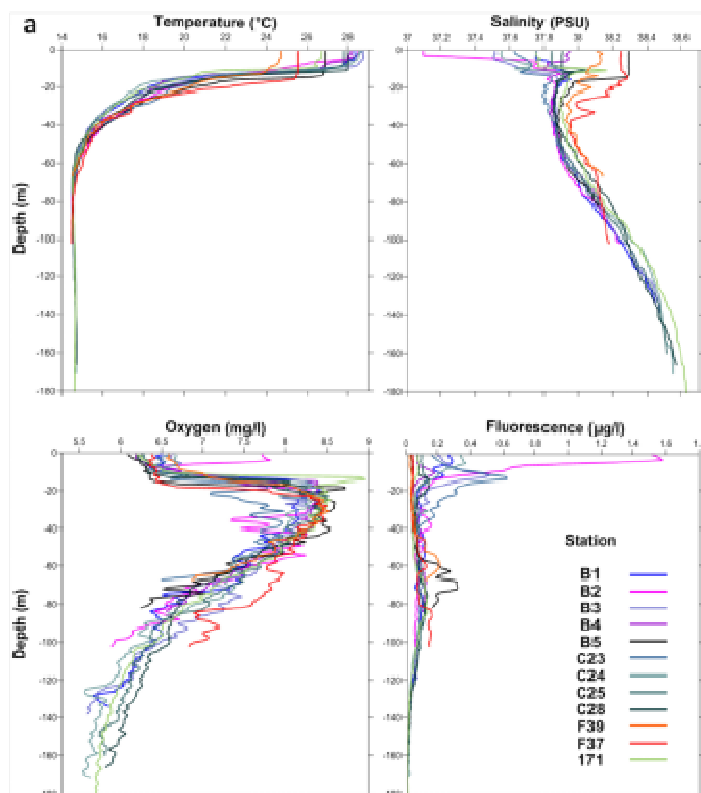
213 ANOVA and Wilcoxon rank sum test).

214 Shannon diversity index was used to characterize the pteropods biodiversity in the stations.

215 All statistical analysis were carried out in R statistical environment (R Core Team, 2018).
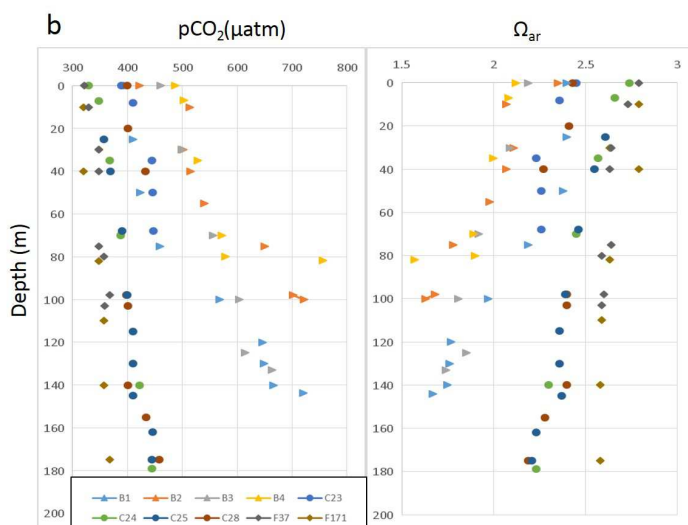
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3. RESULTS

3.1 Hydrology profiles

At all stations mean surface and bottom temperature ranged between 25.6 °C and 28.2 °C and between 14.45 °C and 14.67 °C respectively (Fig 2a). Surface salinity was strongly influenced by the river outflow with values ranging from 37.09 to 38.25. The salinity minimum due to the presence of the Modified Atlantic Water (MAW) was typically positioned between 30 and 45 m (Fig 2a). Oxygen concentration exhibited a similar profile at all stations except B2 (Fig 2a) where higher surface oxygen values were mainly influenced by the Sarno river outflow. Fluorescence values ranged between 0.01 and 1.58 $\mu g*l^{-1}$. The higher values were recorded in the Gulf of Naples and in particular in the B2, B3 and C23 stations (Fig. 2a). Dataset of the hydrological parameters is available in Table 1 in S1_Table.

227

228

Fig 2 Vertical hydrological and carbonate chemistry profiles. a) Temperature, salinity,

oxygen (mg*l^{-1}), Chl a (µg*l-1) and b) pCO$_2$ (µatm) and Ωar (aragonite saturation state) at

the sampling stations (F control, C nearby vents and B vents station).

3.2 Carbonate chemistry

Significant differences were recorded among the three groups of stations for both Ω_{ar}

(ANOVA, $F(2,7)=101.4$, $p<0.001$) and pCO$_2$ (ANOVA, $F_{(2,7)}=240.7$, $p<0.05$). In particular,

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235   although seawater was not under-saturated with respect to the aragonite at any of the stations

236   (i.e., $\Omega_{ar}>1$ at all stations), Ω_{ar} and pCO_2 values in the Gulf of Naples (stations B and C) were

237   respectively significantly lower and higher than at control site (stations F). Differences in

238   carbonate chemistry were also evaluated by grouping the B and C stations (Gulf of Naples)

239   and comparing such group with the stations outside the gulf (control). Obtained results

240   showed that stations outside the gulf were significantly different from the B+C group

241   ($\Omega ar; t_{(7)}=5.78$; $p<0.05$ and pCO_2; $t_{(7)}=4.7$, $p<0.05$).

242   Dataset of carbonate chemistry is available in Table 2 in S1_Table.

243 3.3 Difference in pteropod abundance and "shell fitness" between

244 stations

245   Pteropod abundance was significantly different between the three groups of stations (K-W

246   ANOVA, $H_{(2)}= 7.13$, $p<0.05$). In particular, pteropod abundance was significantly lower at

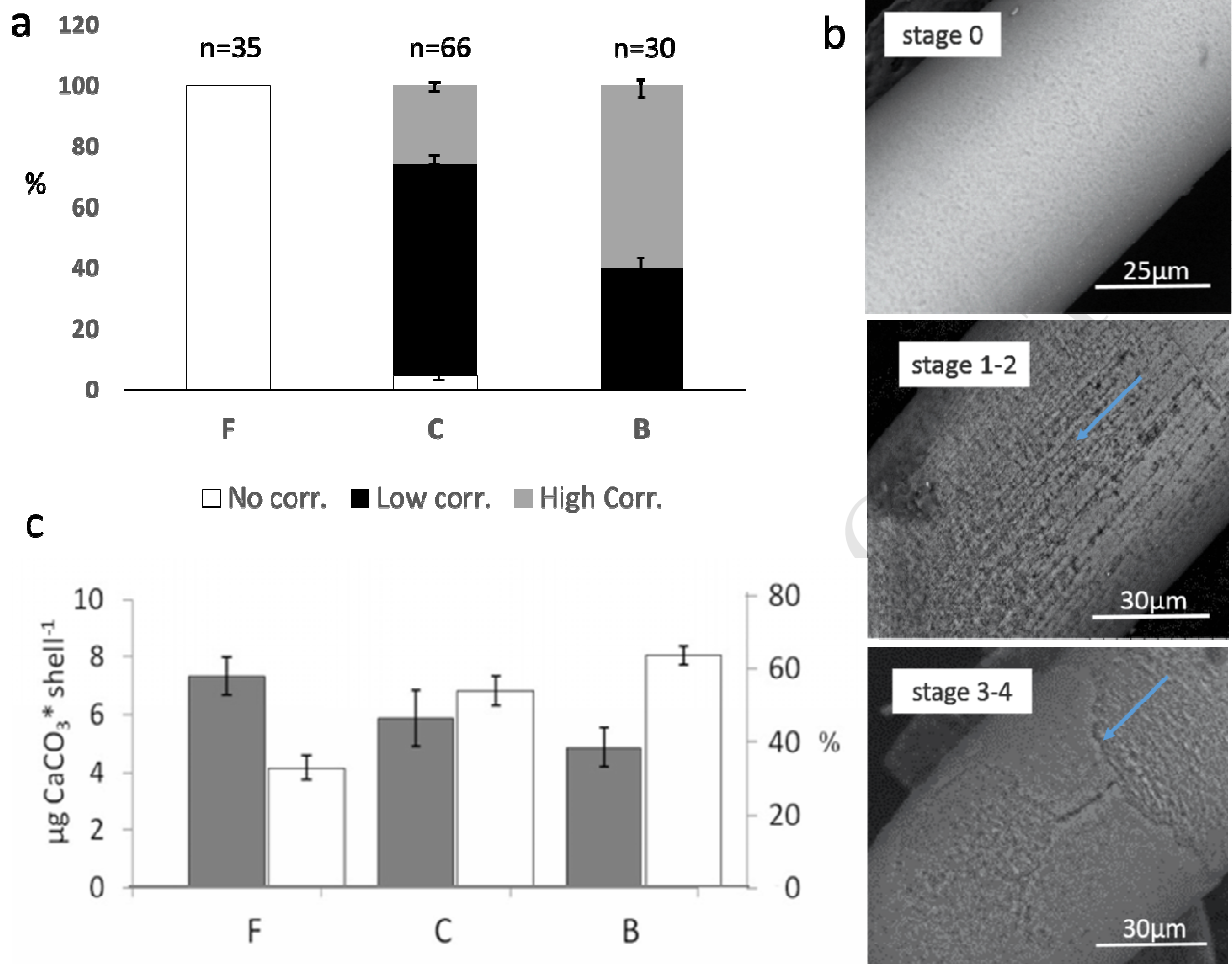247   group C (40%) and B (82%) stations than the control stations, group F.

248   Pteropod diversity was significantly different (Shannon diversity index, (K-W ANOVA, H

249   (2)= 6.76, $p<0.05$) between the three groups of stations also. In particular, diversity was

250   significantly higher at the control stations outside the Gulf of Naples (group F, 100% of

251   identified species) than in the stations of the groups B and C. Dataset of pteropod relative

252   abundance is available in Table 3 in S1_Table.

253   The state of *C. conica* shell condition is presented in (Fig 3a). ANOVA test showed that the

254   percentage of shells presenting no signs of corrosion, low corrosion and high corrosion were

255   significant different among the considered groups (No Corr: $F(2,8)=9284$, $p<0.05$; Low Corr:

256   K-w: $H(2)=9.07$, $p<0.05$; High Corr: $F(2,8)=102.6$, $p<0.05$). Pteropods collected from the

257   CO_2 vent stations (group B) presented a significantly higher degree of dissolution than

258   pteropods collected from stations C and F. In particular all pteropods collected in group F had

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259 a well preserved and transparent shells (stage 0). Conversely, within group B, 60% of shells

260 showed stage 4 levels of dissolution. Shell dissolution (even if moderate) was also observed

261 in the group C, with 70% of shells exhibiting opacity and dullness (stage 3). SEM pictures in

262 the Fig 3b are representative of the different *C. conica* shell dissolution stages observed. We

263 did not observe evidence of shell perforation (stage 5) in any specimens.

264 Significant differences among the three groups were evidenced also in terms of incidence of

265 shell fractures (F $(2, 8)$=51, $p<0.001$) and biomass. In particular, at stations within group B

266 the highest incidence of shell fractures and the lowest biomass was recorded. Significant

267 differences were also recorded between C and F stations, the latter presenting the lowest

268 incidence of shell fractures and the highest biomass. Dataset of pteropod shell biomass,

269 fractures and dissolution are available in Table 4, 5 and 6 in S1_Table.

270 Comparing the B+C stations against the F group, the presence of significant differences

271 between the Gulf of Naples (B+C) and the Control station (F) were confirmed (No Corr:

272 $t_{(7)}$=103.97, $p<0.05$; Low Corr: $t_{(7)}$=9.26, $p<0.05$; High Corr: $t_{(7)}$=6.32, $p<0.05$; incidence of

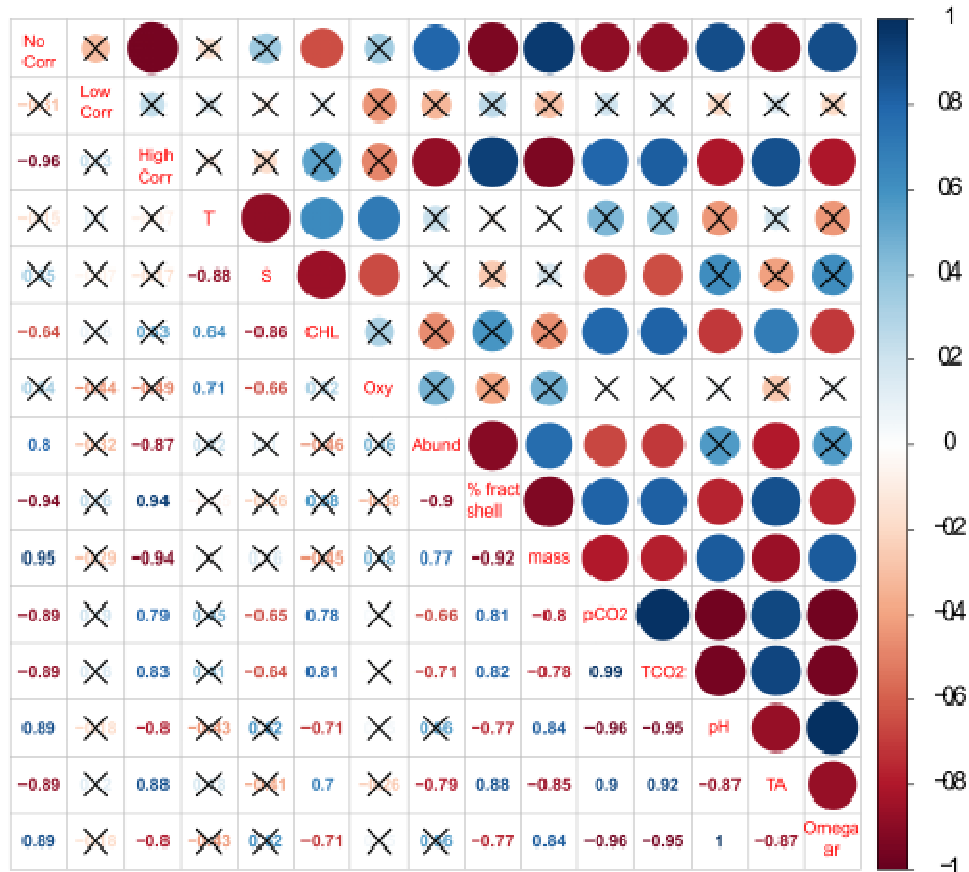273 shell fractures: $t_{(7)}$=7.8, $p<0.05$; biomass: $t_{(7)}$=7.35, $p<0.05$;).

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Fig 3 Difference in pteropod abundance and "shell fitness" between stations a) Shell dissolution level (%) of *C. conica* collected at the group B (vent stations), C and station F; b) SEM images showing different levels of dissolution for *C. conica* shells. The top image shows a detail of *C. conica* shell in perfect condition (stage 0, mainly found in the group F); in the middle *C. conica* shell lustreless with sign of dissolution (stage 2-3, mainly found in group C and B); on the botttom *C. conica* shell with high dissolution where the periostracum and the first aragonite prismatic are partially missing (stage 4, mainly found in the group B); c) shell biomass (grey histogram, µg CaCO3* shell^{-1}) and shell presenting fractures (white histogram, %) of *C. conica*
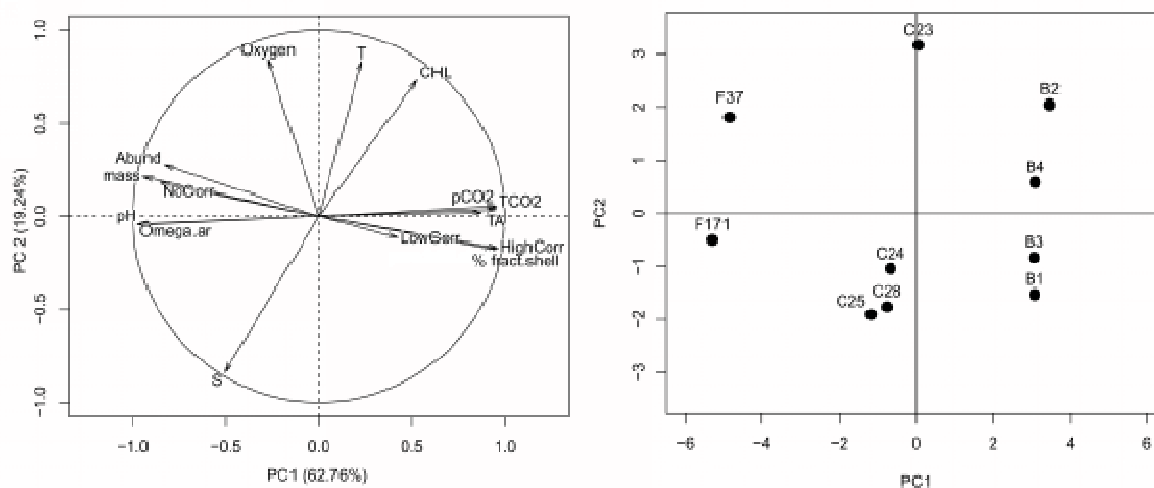
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3.4 Relationships with carbonate chemistry and hydrology

The pairwise correlation analysis (Fig 4a) showed the presence of strong correlations between carbonate chemistry and some parameters related to the condition of pteropods (such as the lowest and the highest dissolution levels, the biomass and the percentage of shell fractures). In particular, the absence of dissolution was positively related to high Ω_{ar} values. Conversely, the highest level of corrosion was negatively related to Ω_{ar}. The abundance and the shell biomass were found positively correlated with Ω_{ar} while the opposite was true for the percentage of shell presenting fractures. No significant differences were found among the three groups of stations in terms of temperature, salinity and oxygen, evidencing the presence of comparable hydrological conditions..

PCA analysis further confirm the relationships observed in correlation analysis, providing a more clear picture of the factors driving differences among the three groups of stations (Fig. 4b,c). The first two PCA axis explained 82% of the total variance. In particular, the 1st PC axis was significantly (see Table 7 in S1_Table) related to Ω_{ar}, and pCO_2, absence of corrosion (No Corr) and higher corrosion, as well as to abundance, percentage of shell with fractures and biomass. Such patterns evidenced that stations having lower values on the first PCA axis were characterized by higher abundance, biomass, Ω_{ar} and lower shell dissolution (No Corr.) as well as by lower pCO_2, percentage of shell with fractures and lower proportion of pteropods shell characterized by higher degree of corrosion. As the 1st axis accounts for 62.76% of the total variance it is clear that most of the variability among stations is related to pteropods and carbonate chemistry parameters. In this context all the B stations were clustered on the right side of the 1st PC axis, while the F stations showed the lowest values with respect to such axis. C stations were mainly found in intermediate position along the 1st PC axis evidencing the presence of intermediate conditions between B and F stations in terms of the parameters related to the 1st PC. Regarding the 2nd PC axis, it was found strongly related only to the hydrological parameters. Also, as it accounts for a much lower proportion

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310   of the total variance, the weak effect of hydrological condition in driving the differences

311   among the stations was confirmed. Dispersion of stations along the 2nd PC axis is much lower

312   than the one along the 1st PC, and the observed differences along the 2nd PC are mainly due to

313   local factors, such as distance from the coast (leading to higher Chl a) or the presence of

314   freshwaters input (it is the case of C23 and F37 stations).

315

316   **Fig. 4a: Pairwise Spearman correlation plot among the considered variables.** Correlation

317   values are reported in the lower triangular matrix. In the upper triangular matrix a graphical

318   representation of correlation values is reported (higher the correlation, bigger the circle; blue

319   and red colours indicate positive and negative correlations respectively). The "X" symbol is

320   used to mark the non-significant (p>0.05) correlations.. Note: no corrosion=% shell

321   presenting preservation stage 0; low corrosion=% shell presenting preservation stage 1-2;

322   high corrosion = % shell presenting preservation stage 3-4; DIC =TCO$_2$.

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Fig 4b: PCA of the considered variables. Visual representation of the correlation among environmental, chemistry, biological factors variables and PCs (left panel) and distribution of the stations along the 1th and 2nd PCs space (right panel). Note corr = corrosion; TCO_2=DIC

4. DISCUSSION

4.1 Pteropod shell fitness around CO_2 vents

This study documents, for the first time, the impact of natural CO_2 volcanic emissions on live pteropods extracted directly from the natural environment. We illustrated that the decrease of Ω_{ar}, associated with the presence of CO_2 vents, can alter the chemical environment for planktonic calcifying organisms in the vicinity. In particular, *in situ* shell dissolution and change in shell biomass were the predominant features observed in the live pteropods collected in the Gulf of Naples (in the station located within and nearby the CO_2 vent discharge) compare to pteropods collected in the control stations. Unfortunately, so far there are no studies on seasonal variability of the carbonate chemistry in this region, however, the pH difference between the CO_2 vent stations and the controls is higher than the natural seasonal variability of the Liguria coastal site (Howes et al., 2015), located on the border with the Tyrrhenian Sea.

17

341 The difficulty in investigating pelagic organisms along a "natural gradient" is determining the

342 residence-times of populations within the CO_2 vent stations, so as to parameterise the

343 duration of their exposure to the stressor. However, pteropods may perform diel and/or

344 seasonal vertical migration, spending part of their time under low Ω_{ar} (nearby the CO_2 vent

345 source at the bottom) and part in the more saturated waters at the surface (Bednaršek et al.,

346 2012; Manno et al., 2016). In particular, Creseidae (such as *C. conica*) seems to perform diel

347 vertical migration (Be and Gilmer, 1977; Hsueh, 1995) with a vertical distribution >100 m

348 (van der Spoel, 1967; Be and Gilmer, 1977). Consequently, despite we do not have

349 information on the vertical distribution of pteropods at the time of collection, we can assume

350 that the organisms collected at the CO_2 vent stations (group B, emission depth ranging from

351 89m to 145m) will have been daily exposed to pCO_2 fluctuation.

352 Another challenge is that pteropods are also not static spatially and will likely move around

353 and outside the Gulf of Naples transported by currents. Two different water inflow and

354 outflow regimes are present in the Gulf of Naples, with a tendency towards stagnation inside

355 the basin during spring and summer and a more effective water renewal mechanism in fall

356 and winter under NE winds (Cianelli et al., 2015). In particular, Mazzocchi et al. (2012)

357 outlined that the only few species representative of the coastal area dominate the zooplankton

358 assemblage in summer owing to coastal retention (Cianelli et al., 2015). Thus, given a growth

359 rate of about 0.33 mm per month (Bednaršek et al., 2012; Well, 1976) and a mean diameter

360 of pteropod shell investigated in this study of 302µm, it is likely that specimens were retained

361 in the Gulf of Naples from the very beginning of their life cycle and within the same water

362 mass condition. As Bednaršek et al., 2012 and Well, 1976 reported similar values of shell

363 growth rates on pteropods collected from very different regions (i.e. Scotia Sea and West

364 India respectively), we assume that the used growth rate can be representative for pteropods

365 collected in the present study.

18

366 Uttieri et al., 2011 using a model simulations of particle transport (in the summer period)

367 demonstrate the presence of a scarce renewal of coastal waters, both over short (i.e., 48 h)

368 and long (i.e., 1 month) periods. The authors found that the residence times was very high,

369 with particles remaining in the deployment area on average for more than 15 days. This

370 simulation confirms that pteropods will spend a relevant amount of time in the station of

371 collection before to be moved back and foward around the Gulf. Thus, although we do not

372 have information about the resident time of pteropods in the Gulf of Naples, we can assume

373 that pteropods collected in August (this study) have likely been trapped in the Gulf and have

374 experienced intermittent CO_2 vent impact for months. The presence of impacted pteropod

375 shell in the stations (group C) not directly located on the CO_2 vent discharge, confirm the role

376 of currents within the Gulf of Naples, driving the pelagic calcifiers inside and outside the CO_2

377 vent emissions. Conversely, sessile benthic calcifiers (as for result of their sedentary

378 behaviour) experience shell degradation only when directly located around the CO_2 vents

379 (i.e., Hall-Spenser et al., 2014, Basso et al., 2015; Milazzo et al. 2014).

380 The variability nature of the CO_2 vent system over the time is a key factor in the

381 interpretation of the observed negative impact on pteropods shell and in part explains the high

382 level of shell dissolution despite the presence of oversaturated seawater ($\Omega_{ar}>1$). We are

383 aware that our data are not representative of the carbonate chemistry condition over time and

384 a detailed survey throughout the year will be an important next step. However, Passaro et al.

385 (2016) found that in the Gulf of Naples bubble plumes generated at the CO_2 vent are

386 highly variable: from a continuous, dense bubble-flux to short-lived phenomena. In

387 particular, the authors found the pH values above a shallow CO_2 vent emission (75 m

388 depth) decrease from 8.4 (at 70 m depth) to 7.8 (at the bottom). Unfortunately, the authors

389 did not provide Ω_{ar} values but the pH values at the bottom are lower than the pH we

390 observed near the CO_2 vent emissions and it could likely correspond to lower Ω_{ar} than the

19

391   values observed in our study. Therefore, periodical exposition to critical low Ω_{ar} values may

392   drive the dissolution state of pteropod shells.

393   Overall, we suggest that pteropods around the CO_2 vents in the Gulf of Naples, are

394   negatively impacted when periodically exposed to high spatial and temporal variability in

395   Ω_{ar}. Evidence of impact on pteropod shell dissolution has already been reported in the field.

396   Within an upwelling system, Bednaršek et al. (2014), observed higher levels of shell

397   dissolution (up to preservation stage 5) than the present study. This can reflect either the

398   higher magnitude as well as time of exposure of pteropods in this region compared to the

399   Gulf of Naples. Further, the absence of additive environmental stressors in the Gulf of Naples

400   such as variability in oxygen and nutrient concentration could also partially explain the lower

401   impact on the shell compared to the upwelling system.

402   In the future targeted research, focused on the investigation of vertical distribution and

403   migration of pteropods in the CO_2 vent regions, will be crucial to improve our understanding

404   on the potential ability of these organisms to avoid water depths with critical carbonate

405   saturation state. It will be important to use Lagrangian modelling studies (to track pteropods

406   across temporal and spatial scales) since in addition to intensity and duration of exposure

407   (Manno et al., 2012; 2016), the impact of CO_2 vents on pteropods is likely to be also a

408   function of the recovery time between the exposures itself. Lagrangian particle tracking

409   models coupled with hydrodynamic models are particularly efficient tools to examine the role

410   played by various physical processes and to study transport processes over an entire basin to

411   simulate zooplankton dispersion and distribution at different scales (e.g. Speirs et al., 2006;

412   Lett et al., 2007).

4.2 Impact on the pteropod shell biomass

414   We found that shell biomass was significantly lower in pteropods living within the Gulf of

415   Naples compared to those in the Northern Tyrrhenian Sea. Only individuals of the similar

20

416 length (juveniles, 302 µm ± 11) were used to measure shell biomass in order to compare the

417 different groups. The decrease in shell biomass suggests that calcification was lower than

418 dissolution and in turn the shell biomass decrease. This was more the case of the individuals

419 presenting high level of shell dissolution (group B) where likely the dissolution exceed the

420 calcification. However, shell biomass of individuals in the group C, which present manly

421 shells with opacity and/or low level of dissolution was still significantly lower, suggesting

422 that the lower shell biomass was a common features of pteropods in the Gulf of Naples

423 (compared to the shell biomass values of the control stations outside the Gulf). We are aware

424 that other environmental factors can play a role in pteropods shell growth (e.g. temperature

425 and salinity) (Lalli and Gilmer, 1989), however differences in salinity and temperature

426 between the stations during the summer were within the natural seasonal variability of the

427 Tyrrhenian Sea and well within the pteropods' tolerance window (Lalli and Gilmer, 1989).

428 Further, incubation experiment of pteropods under a range of salinity (Manno et al., 2012)

429 and temperature (Lischka and Riebsell, 2012) show that those parameters have to change

430 quite considerably before a negative effect is detectable (i.e., shell growth, behaviour,

431 survival). Similarly, the potential role of temperature on shell dissolution was excluded

432 because previous works found that under manipulate water condition increasing in

433 temperature not leads to dissolution on pteropods (Lischka et al. 2011, Gardner et al. 2018).

434 Food availability may also play a critical role in determining the shell growth because food

435 supply is required to support the metabolic processes facilitating bio-calcification as well as

436 the resistance of calcifiers to adverse condition such as OA (Ramajo et al., 2016). Particulate

437 food availability to pteropods, as inferred indirectly from average Chl a fluorescence in each

438 station was not significantly different between the three groups of stations, suggesting

439 pteropods were not limited by food availability in the region around the CO_2 vents.

21

440 Evidence of change in shell morphology in response to change in carbonate chemistry

441 associated with shallow-water CO_2 vents has already been observed in benthic molluscs (i.e.,

442 Langer et al., 2014, Garilli et al., 2015). Garilli et al. (2015) show that benthic gastropod

443 species (*Cyclope neritea* and *Nassarius corniculus*) adapted to acidified seawater (Ω_{ar}=0.68)

444 were smaller than those found in normal pH conditions (8.1) while Langer et al., (2014)

445 found that the patellogastropod limpet *Patella caerulea* counteracted the induced shell

446 dissolution in the CO_2 vent waters (Ω_{ar}=3.01) by enhanced production of internal aragonite

447 shell layers. Incubation experiments on the Mediterranean pteropod, *Creseis acicula*, reported

448 a 30% decrease in calcification with a decrease in Ω_{ar} from 3.3 to 2.0 (Comeau et al., 2012).

449 Moya et al. (2016) show that pteropod *Heliconoides inflatus* exhibited a 50% decrease in

450 gross calcification when exposed to waters of $\Omega_{ar} = 2$ (compare to control condition $\Omega_{ar} =$

451 2.9).

452 Our results provide *in situ* evidence that shifts away from an organisms optimum Ω_{ar} values

453 can significantly affect calcification despite waters remaining oversaturated. In support our

454 observation, pteropod shells collected within sediment traps became significantly lighter over

455 recent decades as Ω_{ar} decreased (Robert et al., 2011). A decrease in the shell thickness of

456 modern (2000+, Ω_{ar}=4.0) tropical pteropod *D. longirostris* compared to 1960s (Ω_{ar}=3.5)

457 samples has been observed (Roger et al., 2012). Further, Howes et al. (2017) compared the

458 difference in shell thickness of pteropod samples (*Cavolinia inflexa* and *Styliola subula*)

459 collected in the Tyrrhenian Sea with archived samples from 1910's. The authors observed

460 that shell thickness from modern pteropods (Ω_{ar}=3.4) was significantly less than from

461 individuals collected on 1910's (Ω_{ar}=3.88) (despite they state those decrease in shell

462 thickness should be treated with caution). Comparison with the present study and Howes et

463 al. (2017), both made in the Tyrrhenian Sea, highlights the relevance of using natural

464 environmental gradients to forecast the impact of high pCO_2 on marine organisms as spatial

22

465   change (natural variability of the carbonate chemistry, associated to CO_2 vents) can be a

466   substitute for time (100's older vs. modern samples, Howes et al., 2017). Further short time

467   experimental studies (up 29 days), where pteropods were incubated at undersaturated Ω_{ar}

468   levels, found a decrease of calcium carbonate precipitation and shell diameter, respectively

469   up 28% (Comeau et al., 2010) and 12 % (Lischka et al., 2012) confirming the relevance of

470   short episodic exposure in natural environments.

471   We observe an inverse relationship between shell biomass and the incidence of shells

472   presenting fractures, indicating that fractures are most commonly found in shells with low

473   biomass i.e., thinner/low density shells. Assuming predation pressure is comparable across all

474   sites, we consider that thin shells found at station B are more fragile and therefore more prone

475   to fracture than the more robust, high biomass shells. Although the effectiveness of the

476   periostracum for pteropods is a matter of debate (Peck et al., 2016b; Bednaršek et al. 2016).

477   Peck et al. (2016a) indicated that the shells of healthy, living pteropods are only susceptible

478   to dissolution of the shell where the periostracum has been breached and the aragonite

479   beneath is exposed to undersaturated waters. The susceptibility of the thin, fragile shells of

480   pteropods at the CO_2 vent stations to fracturing increases the incidence of aragonite being

481   exposed beneath the damaged periostracum. The consequence of increased incidence of

482   mechanical damage to the shell and exposure to undersaturated waters is consistent with our

483   observation of heightened incidence of shell dissolution.

484   In this study, *C. conica* (as well as the total pteropod assemblage) in the Gulf of Naples were

485   lower in abundance compared to those collected in the control stations (Northern Tyrrhenian

486   Sea). Due to the highly patchy distribution of pteropod abundance and sampling collection

487   being limited to one time, the impact of CO_2 vents on pteropod survivorship can only be

488   speculative and any interpretation have to be evaluated with extremely caution. However, it is

489   likely that the observed increasing in shell degradation and decreasing in shell biomass could

23

490 contribute to increasing in pteropod mortality (because affecting shell buoyancy, defence

491 against predator etc.). Bednaršek et al. (2014) also observed a relationship between shell

492 dissolution and decrease in pteropod abundance within the upwelling system and suggested

493 that increased dissolution combined with increased shell fragility could potentially induce

494 pteropod population decline.

495 Marine organisms   have the potential to adapt to changes in ocean pH and adaptation

496 potential can be inferred from existing genetic diversity related to patterns of local adaptation

497 across present gradients in environmental pH (Stilmann and Paganini, 2015). Even if not

498 explored in this study, the decrease in shell biomass of pteropods as potential local adaptation

499 to natural low saturation state of $CaCO_3$, is an interesting matter for future investigation. At

500 the high latitudes, for example, due to the natural lower saturation state of $CaCO_3$, shell-

501 building materials are more difficult to extract from seawater and calcifying organisms

502 present thinner shells than individuals living at medium and low latitudes (Grauss et al.

503 1974). Understanding the persistence of populations of marine organisms in future altered

504 environments requires first an understanding of extant phenotypic plasticity under realistic

505 environmental conditions and the potential for adaptation (Silmann and Paganini 2015). CO_2

506 vent regions might help to improve our understanding to predict if pteropod populations

507 possess adequate genetic variation to adapt to forecasted environmental change. Future long

508 term monitoring of the in situ population dynamics as well as study on phenotypic plasticity

509 and genetic variation across natural small scale gradients (such as CO_2 vent) will be crucial to

510 understanding the plasticity- adaptive-defence of this organism to persist in a more acidified

511 ocean over short (< 10 year) to medium (10–100 year) temporal scales.

512

513 **Appendix A. Supplementary data**

24

514　**S1 Table Hydrology and carbonate chemistry variables and pteropod dataset of all the**

515　**stations.** 1) Temperature, salinity, oxygen and fluorescence; 2) pH, Ω_{ar}, Total Alkalinity

516　(TA), Dissolved Inorganic ; 3) pteropods abundance and species contribution; 4) shell

517　biomass and length ; 5) shell fractures; 6) shell dissolution; 7) Correlation statistics

518　# Acknowledgements

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Highlights

1- *in situ* shell dissolution and change in shell biomass were the predominant features observed in the live pteropods collected within and nearby CO_2 vent regions.
2- Low pteropod biomass shells (collected nearby the CO2 vents) were more fragile and therefore more prone to fracture than the more robust, high biomass shells (collected in the control stations).
3- In the Gulf of Naples, intermittent shifts away from optimum Ω_{ar} values can significantly affect pteropod calcification despite waters remaining oversaturated.