



Effects of elevated CO₂ and temperature on an intertidal harpacticoid copepod community

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Manuscripts

Effects of elevated CO₂ and temperature on an intertidal harpacticoid copepod community

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Among the major consequences of global climate changes, warming and ocean acidification have been shown to have significant impacts on many marine organisms and ecosystems. However, few studies have addressed the impact of these two stressors on meiofaunal organisms and none on harpacticoid copepod community structure and diversity specifically. A mesocosm experiment was conducted to assess the potential interactive impact of different levels of elevated CO₂ and temperature on an intertidal harpacticoid copepod community. Artificial Substrate Units (ASUs) colonized by meiofauna from the extreme low intertidal zone were exposed to eight experimental treatments (four pH levels: 8.0, 7.7, 7.3 and 6.7, crossed with two temperature levels: 12 and 16 °C). After 60 days exposure communities were ~~seen to be~~ significantly affected by reduced pH and increased temperature. The dominant harpacticoid species were mainly affected at treatments held at pH 6.7, but with divergent biological response patterns. At pH 6.7 *Tisbe* sp and *Ectinosoma* sp2 exhibited important density reductions, while considerable density increases were observed for *Amphiascus longarticulatus* and *Amphiascoides golikovi*. Furthermore, these changes were also accompanied by differential responses to temperature treatments. This study has demonstrated that the combination of elevated levels of CO₂ and ocean warming may have substantial effects on the structure of benthic harpacticoid communities, and that a precautionary approach may be required when interpreting predictions from single ~~species-stressor, but also~~ single species studies. Importantly, the significant increase in malformations observed in the most severe treatment indicated that, even though copepod species may survive to high pCO₂ ocean acidification and warming stress, we need to consider sub-lethal effects that could have negative consequences for populations after long periods of exposure.

38
39 **Keywords:** *climate change, ocean acidification, warming, meiofauna, benthos.*
40

41 **Introduction**

42 The increasing concentration of atmospheric carbon dioxide (CO₂) is altering
43 the levels of co-occurring stressors, resulting in increasing sea surface temperatures and
44 seawater pCO₂, as well as decreasing the oceans' pH and its level of saturation of
45 carbonate minerals (Feely *et al.*, 2009). Since the beginning of the industrial revolution
46 in the mid-eighteenth century, the release of CO₂ from human activities has resulted in
47 an increase in atmospheric CO₂ concentrations by nearly 40% (Feely *et al.*, 2009). The
48 present Earth's atmospheric CO₂ levels are higher than at anytime in at least the last
49 800,000 years (Lüthi *et al.*, 2008), and is expected to continue to rise at an accelerating
50 rate (Feely *et al.*, 2009). Globally averaged combined land and ocean surface
51 temperature data show a warming of 0.85 [0.65 to 1.06] °C over the period 1880 to
52 2012 (IPCC, 2014).

53 Due to its large volume and the ability of seawater to buffer CO₂, the ocean has
54 absorbed nearly a third of all the anthropogenic carbon added to the atmosphere,
55 attenuating the overall effects (Sabine *et al.*, 2004). However, oceanic uptake of CO₂
56 has resulted in changes in seawater carbonate chemistry, a process known as 'ocean
57 acidification' and the pH of ocean surface water has decreased by 0.1 units since the
58 beginning of the industrial era, corresponding to a 26% increase in acidity (IPCC,
59 2014).

60 The rise in greenhouse gas (primarily CO₂) atmospheric concentration is
61 predicted to continue, with estimates for the year 2100 ranging from 475 to 1313 ppm
62 ([IPCC, 2013](#)). Best estimates of ocean warming in the top one hundred meters of the
63 water column are about 0.6 °C to 2.0 °C by the end of the 21st century (IPCC, 2013).

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6 64 However, an additional warming of global mean surface temperatures is forecasted to
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8 65 reach 2.6 °C to 4.8 °C by the end of 21st century (IPCC, 2014). As a consequence of
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10 66 ocean CO₂ uptake, an additional drop in ocean pH of 0.3 units by 2100 and 0.7 units by
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12 67 2250 is predicted (Caldeira and Wickett, 2003).

13
14 68 Climate change is causing alterations to marine ecosystems with impacts that
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16 69 are evident from polar to tropical regions (Harley *et al.*, 2006; IPCC, 2014).

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18 70 Temperature and pH are among the most important environmental factors controlling
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20 71 the distribution, physiological performance, morphology and behaviour of marine
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22 72 invertebrates (e.g. Pörtner, 2008; Widdicombe and Spicer, 2008; Doney *et al.*, 2009).

23
24 73 However, global warming and ocean acidification are the ~~Moreover, among all of the~~
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26 74 ~~expected impacts of the global climate changes in coastal areas, the~~ two major
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28 75 consequences of climate change that ~~have are been already are being~~ observed ~~are global~~
29
30 76 ~~warming and ocean acidification. Climate change is thus causing alterations to marine~~
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32 77 ~~ecosystems with impacts that are evident from polar to tropical regions (Harley *et al.*,~~
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34 78 ~~2006; IPCC, 2014).~~

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36 79 Environmental stressors can have simple additive effects (both significant, but
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38 80 no significant interaction) or have complex interactive effects where they have
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40 81 synergistic (increased stress) or antagonistic (decreased stress) effects on biological
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42 82 processes (Folt *et al.*, 1999). Despite the well-known controlling influence of
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44 83 temperature on metabolism and development, the interactive effects of ocean warming
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46 84 and CO₂-driven acidification on organisms at community level have been poorly
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48 85 studied and require use of factorial experimental designs.

49
50 86 Harpacticoid copepods are usually the most important meiofaunal group in
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52 87 terms of abundance in phytal areas with high diversity (Giere, 2009). Due to their high
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54 88 nutritional value, they are a predominant element in the diet of many fishes of both

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6 89 ecological and economic importance (Huys and Boxshall, 1991). Furthermore,
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8 90 copepods have been extensively used to show natural environmental changes (e.g.
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10 91 Sarmiento *et al.*, 2012; Kitahashi *et al.*, 2014) as well as to evaluate different types of
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12 92 human impacts (e.g. Costa *et al.*, 2016; Sarmiento and Santos 2012). ~~Due~~Owing to the
13
14 93 ~~higher sensibility~~greater sensitivity that harpacticoid copepods can exhibit in
15
16 94 comparison to other dominant meiofaunal groups such as nematodes (Hale *et al.*, 2011;
17
18 95 Sarmiento *et al.*, 2015), ~~these organisms~~they have been recently suggested as a valuable
19
20 96 group for predicting climate changes (Zeppilli *et al.*, 2015). However, no studies on
21
22 97 how the combination of elevated seawater CO₂ and temperature will impact intertidal
23
24 98 harpacticoid multi-species assemblages are available as yet.

25
26 99 The present study used a mesocosm experiment to assess the potential
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28 100 interactive impacts of different levels of elevated CO₂ and temperature on the fauna
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30 101 from an intertidal zone using the harpacticoid copepod community as a model system to
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32 102 evaluate changes in community structure and species responses.~~The present study used~~
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34 103 ~~a mesocosm experiment to assess the potential interactive impacts of different levels of~~
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36 104 ~~elevated CO₂ and temperature on the harpacticoid copepod community from the~~
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38 105 ~~intertidal zone collected using Artificial Substrate Units (ASUs).~~

106

107 **Materials and methods**

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109 The meiofauna samples used in this study are from a mesocosm experiment
110 carried out at Plymouth Marine Laboratory in 2009 (Hale *et al.*, 2011), where intertidal
111 benthic communities were exposed to elevated temperature crossed with different levels
112 of reduction in the pH of seawater. Sample collection and mesocosm experimental set-
113 up were described in detail by Hale *et al.*, (2011), and are summarized here.

114 Material collection

115 Fifty Artificial Substrate Units (ASU, each one made from 4 nylon mesh pan
116 scourers tied together, 9 cm \varnothing , 2.5 cm thick) were deployed in a sheltered area of rocky
117 intertidal at Mount Batten, Plymouth, UK (50.3567 N, 4.1277 W). The area is
118 characterized as a kelp habitat dominated by brown and red algae. The ASUs were
119 attached to the rock between 0.6 m and 1 m above lowest chart datum (LCD), during
120 the spring low tide on ~~the~~ 14 January 2009. They were left for a period of twelve weeks
121 to allow colonization and collected on 8 April 2009. The ASUs were retrieved and
122 transported in plastic bags to the mesocosm facility at the Plymouth Marine Laboratory
123 (PML) 1h after collection. Once at PML, five ASUs were randomly selected and
124 preserved in 10% formaldehyde solution, to represent the standard invertebrate
125 communities at the start of the exposure period.

127 Mesocosm experiment

128 Forty of the remaining ASUs were each placed individually in separate food
129 grade plastic buckets (vol. 6 L) containing seawater at ambient pH and temperature.
130 Each bucket was randomly allocated to one of eight treatments (four pH levels crossed
131 with two temperature levels), with five replicates for each combination. Control pH was
132 8.0 (the ambient seawater pH measured at the fauna collection site) ~~-, and the decreased~~
133 pH levels used were 0.3 units below ambient (the predicted drop in ocean pH by 2100),
134 0.7 units below ambient (the predicted drop in pH by 2250, Caldeira and Wickett, 2003)
135 and a pH of 6.7 (to simulate CO₂ storage site continuous point source leakage under
136 already acidified conditions, Blackford *et al.*, 2009). Seawater was acidified to pH levels
137 of 7.7, 7.3 and 6.7 by bubbling with 100% CO₂. The mesocosm was held at a control
138 temperature of 12 °C (the ambient temperature measured at the fauna collection site)

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6 139 and the elevated temperature treatment was 4 °C above the control ([simulating a rise in](#)
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8 140 [temperature midway within the range predicted, 2 to 6.4 °C, as a result of increased](#)
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10 141 [atmospheric CO₂ by 2099 \(Sokolov *et al.*, 2009\). Buckets containing the ASUs were](#)
11
12 142 [maintained in water baths \(5 buckets per water bath\) and the artificial manipulation of](#)
13
14 143 [temperature was achieved and regulated by heaters \(Hale *et al.*, 2011\). Artificial](#)
15
16 144 [manipulation of temperature was achieved by placing the treatment buckets in water](#)
17
18 145 [baths containing heaters.](#)

19
20 146 [Seawater was bubbled with CO₂ into the header tanks. ~~than Each e~~Each 6 L](#)
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22 147 bucket was continuously supplied with [high pCO₂ seawater \(8-10 ml min⁻¹ using](#)
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24 148 [peristaltic pumps\)](#) and oxygen was bubbled through the water held within the buckets to
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26 149 assist with maintenance of the correct pH and to increase water mixing and oxygen
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28 150 levels. [The monitoring system as described in Hale *et al.* \(2011\) maintained the](#)
29
30 151 [nominated pH and temperature treatments throughout the experimental period with little](#)
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32 152 [variation \(Table 1\) and was therefore considered a suitable method for the artificial](#)
33
34 153 [manipulation of seawater pH and temperature.](#)— The natural light regime was
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36 154 approximated using daylight simulation lights within the mesocosm with an average 8-h
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38 155 photoperiod per day. The experiment ran for 60 days, with little variation in the
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40 156 treatment levels (Table 1). During that time, each bucket received 1.68 ml of shellfish
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42 157 feed once a week to simulate the food availability at the Mount Batten collection site.
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44 158 No tidal simulation was applied during the experiment ([Hale *et al.*, 2011](#)).

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47 160 [Table 1]

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51 162 At the end of the exposure period, the ASUs were removed from the buckets
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53 163 and the resident fauna were extracted. The collected material was passed through two

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6 164 sieves (0.5 mm and 63 μm) to separate the macrofauna fraction from the meiofauna
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8 165 fraction (Somerfield *et al.*, 2007). Results from the macrofauna fraction are published in
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10 166 Hale *et al.* (2011) and ~~from the~~ meiofauna (major taxonomic groups) and Nematoda
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12 167 community ~~results have been are~~ published in Meadows *et al.* (2015). Due to the high
13
14 168 number of meiofauna organisms in each sample, thirty-six samples (the four first
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16 169 replicates per treatment + 4 initial samples; instead of 5) were ~~randomly~~ selected for
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18 170 copepod species identification. Under a stereo microscope, the first sixty copepod
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20 171 individuals were selected from each replicate, placed in Eppendorf tubes and preserved
21
22 172 in 75% Industrial Methylated Spirit (IMS). The identification of Copepoda
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24 173 Harpacticoida (copepodite V and adult stages) was done under a compound microscope
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26 174 (1000 \times magnification) and identified to species by the analyses of the entire animal
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28 175 following the taxonomic keys of Lang (1948, 1965), Huys *et al.* (1996) and Wells
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30 176 (2007) as well as publications with specific descriptions.
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33 34 178 Statistical analysis

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36 179 Permutational multivariate analysis of variance (PERMANOVA) (McArdle
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38 180 and Anderson, 2001), based on Bray-Curtis dissimilarities of copepod abundance
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40 181 $\log_{(x+1)}$ transformed data, was used to evaluate the impact of different temperatures
41
42 182 (factor Temperature) and pH levels (factor pH) on the community structure. For all
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44 183 analyses, 9,999 random permutations were used. Pair-wise *a posteriori* comparisons
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46 184 (the multivariate version of the *t* statistic) were made for calculation of significant
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48 185 differences. A similarity percentage (SIMPER) analysis was applied to determine which
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50 186 species were responsible for the dissimilarities among pH and temperatures. The
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52 187 Shannon–Wiener (H' , using \log_2), Pielou's evenness (J') and the number of species in
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54 188 each sample (S) were calculated. The population parameters malformed animal ratio,

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6 189 copepodite ratio, ovigerous female and female/male ratios were also calculated. In the
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8 190 present study, malformations were considered as external morphological abnormalities
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10 191 in important taxonomic characteristics, i.e., when parts of the body do not have the
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12 192 normal or expected shape for a given species (e.g. the number and shape of limbs or
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14 193 thorns). Minor variations in ornamentation were not considered malformations.

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16 194 Two-way analysis of variance (ANOVA) was used to examine the effects of
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18 195 the different pH and temperatures on the densities of harpacticoid's more abundant
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20 196 species (>2% of total), on the ecological descriptors (S, J' and H') and on population
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22 197 parameters (ratios of copepodites, female/male, ovigerous females and malformed
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24 198 animals).

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26 199 PERMANOVA, SIMPER and MDS were applied using the software Primer®
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28 200 6 with add-on PERMANOVA+ (Plymouth Routines in Multivariate Ecological
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30 201 Researches). The two-way ANOVAs were calculated using the software STATISTICA
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32 202 12. ~~Data normally distribution~~ Distribution normality and homogeneity of variance were
33
34 203 checked. The level of significance was set at P<0.05 for all analyses. Confidence
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36 204 intervals of 95% (CI) were used to express the variation of the calculated means.
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38 205 Parametric statistical analysis followed Zar (1996).

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207 **Results**

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44 208 A total of 2160 copepod individuals were analyzed, 60.09% of which were
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46 209 identified as harpacticoids at the species level, 0.79% were adult cyclopoids, 38.7%
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48 210 were copepodites (total) and 0.42% were broken animals which could not be identified
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50 211 to species level. Among the harpacticoids, 12 families, 33 genera and 51 species were
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52 212 recorded (Table 2).

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6 214 [Table 2]

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10 216 *Tisbe* sp (37.42%), *Harpacticus obscurus* (11.91%), *Ectinosoma* sp2 (5.45%),

11 217 *Ectinosoma* sp1 (4.8%), *Amphiascoides* sp1 (4.55%), *Paradactylopodia* sp (4.49%),

12 218 *Dactylopusia vulgaris dissimilis* (4.26%), *Ameiropsis mixta* (3.94%), *Amphiascus*

13 219 *longarticulatus* (3.25%), *Amphiascoides golikovi* (3.06%) and *Ameira* sp (1.67%),

14 220 accounted for ~85% of total.

15
16 221 MDS ordination analyses indicated marked differences in the structure of

17 222 copepod community among field and treatment samples. Among treatments the most

18 223 important difference was observed between samples maintained at pH 6.7 and those

19 224 from the other pH treatments (Figure 1).

20 225

21 226 [Figure 1]

22 227

23 228 The pattern illustrated in the MDS ordination (Figure 1) was confirmed by

24 229 PERMANOVA. Significant differences in the copepod community structures were

25 230 detected for the factor pH and Temperature, but not for the interaction between the two

26 231 factors (Table 3).

27 232

28 233 [Table 3]

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30 235 The pattern of response of copepod community structure to the different pH

31 236 levels was mainly caused by differences among samples kept at pH 6.7, while no

32 237 differences were detected among control and the other treatments (Table 4).

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6 239 [Table 4]

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10 241 SIMPER analyses showed that decreases in the density of *Ectinosoma* sp2 and
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12 242 *Tisbe* sp in samples at pH 6.7, were important to dissimilarities among treatments
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14 243 (Table 5).

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18 245 [Table 5]

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23 247 SIMPER analyses showed that many species were important contributors to the
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25 248 dissimilarity between communities kept at the two different temperature levels (Table
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27 249 6).

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31 251 [Table 6]

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35 253 ANOVA results for species richness, evenness and diversity showed no
36
37 254 significant differences for the factors pH and temperature nor for interaction between
38
39 255 the two factors ($p > 0.1$ for all) (Figure 2).

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43 257 [Figure 2]

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47 259 ANOVA results for copepod population parameters showed no differences for
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49 260 female/male ratio ($p > 0.6$ for all comparisons) nor for the percentage of ovigerous
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51 261 females ($p > 0.15$ for all comparisons) for both pH and temperature or interaction
52
53 262 between factors. The copepodite ratio showed significant differences for the factor pH
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55 263 ($F_{(3,24)} = 3.12$; $p = 0.045$) and for the factor Temperature ($F_{(1,24)} = 5.41$; $p = 0.029$), but not

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6 264 for their interaction ~~between factors~~ ($p > 0.18$). The *a posteriori* Fisher test indicated that
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8 265 copepodite ratio at pH 6.7 was lower than at pH 7.7 ($p = 0.007$) and higher at 16 °C
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10 266 compared to 12 °C. Malformed animals ratio showed significant differences for factor
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12 267 pH ($F_{(3,24)} = 3.24$; $p = 0.039$). ~~The~~ Fisher test indicated that the ratio of malformed
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14 268 animals at pH 6.7 was significantly higher when compared to pH 8.0 ($p = 0.014$), 7.7
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16 269 ($p = 0.013$) and 7.3 ($p = 0.041$) (Figure 3).

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18 270
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20 271 [Figure 3]
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24 273 Only few harpacticoid species showed significant differences among
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26 274 treatments, and the majority were sensitive only to pH 6.7 (Figure 4). The two-way
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28 275 ANOVA indicated that *Tisbe* sp showed significant interaction between the factors pH
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30 276 and Temperature ($F_{(3,24)} = 5.22$, $p < 0.01$). At pH 6.7, *Tisbe* sp showed higher densities at
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32 277 12 °C than at 16 °C ($p < 0.001$). Moreover, the density of *Tisbe* sp in samples held at pH
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34 278 6.7 and 16 °C was lower than in the all other treatments ($p < 0.001$). *Ectinosoma* sp2 was
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36 279 sensitive ~~for to~~ the ~~factor~~ pH ($F_{(3,24)} = 6.99$, $p < 0.01$) and Temperature ($F_{(1,24)} = 5.33$,
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38 280 $p = 0.03$) ~~changes~~, but ~~not for the~~ interaction ~~was not significant~~ ($F_{(3,24)} = 0.44$,
39
40 281 $p = 0.72$). Results of the *a posteriori* test showed that the density of *Ectinosoma* sp2 was
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42 282 lower at pH 6.7 when compared to all other pH levels ($p < 0.01$ for all comparisons).
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44 283 Considering the factor temperature, *Ectinosoma* sp2 density was higher at 16 °C than at
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46 284 12 °C ($p = 0.03$). The species *Amphiascoides* sp1 showed significant differences only for
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48 285 the factor Temperature ($F_{(1,24)} = 5.11$, $p = 0.033$), with higher densities found at 16 °C.

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51 287 [Figure 4]
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Discussion

The results presented here indicate the potential impacts to intertidal copepods that are likely to occur across a range of predicted pH (in the context of global change at highly variable coastal areas [and CO₂ storage leakage](#)) and temperature levels (Caldeira and Wickett, 2003; IPCC, 2014). [Since ocean acidification and warming are the two major consequences that ~~are have~~ already been observed in response to increased atmospheric CO₂](#)~~Since ocean acidification and warming are both caused by increased atmospheric CO₂~~, organisms are being exposed to these ~~two~~ stressors simultaneously [in natural ecosystems](#) (Byrne, 2011; Hale *et al.*, 2011; Melatunan *et al.*, 2013). In the present study, harpacticoid community structure was affected by pH and temperature separately, with samples exposed to pH 6.7 showing the strongest effect. ~~Following~~ [Using the same ~~pattern~~ experimental procedures](#), Meadows *et al.* (2015) found that the total density of copepods was significantly affected by pH and temperature separately, and ~~that~~ copepod abundance at pH 6.7 was significantly lower compared to other pH levels (Meadows *et al.*, 2015). However, the [here](#) presented detailed analysis at lower taxonomic level showed that different harpacticoid species exhibited divergent response patterns highlighting the species-specific nature of responding to stressors.

[The direct effects on taxa and the indirect effects through ecological interactions are both important factors influencing the response of a multispecies intertidal community to increasing pCO₂ and warming \(Hale *et al.*, 2011; Melatunan *et al.*, 2013\).](#)~~The response of a multispecies intertidal community to ocean warming and acidification is influenced by direct effects on taxa and indirect effects through ecological interactions (Hale *et al.*, 2011; Melatunan *et al.*, 2013).~~ Species interactions may attenuate or amplify the direct effects on individual species (Kroeker *et al.*, 2012). In the present study, harpacticoid species responded mainly to treatments held at pH

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6 314 6.7. Differences between pH 6.7 and the other pH treatments were ~~due-caused to~~ by
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8 315 important reductions in the densities of *Tisbe* sp and *Ectinosoma* sp2, followed by a
9
10 316 considerable increase in the densities of *A. longarticulatus* and *A. golikovi* at ~~this~~-pH
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12 317 6.7. These changes were also accompanied by differential species response to
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14 318 temperature at this pH, where the densities of *A. longarticulatus* and of *Amphiascoides*
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16 319 sp1 were higher at 16 °C and the density of *Tisbe* sp was higher at 12 °C. These results
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18 320 show that in multispecies communities it is likely that certain trade-offs between species
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20 321 will occur under environmental change, with both species that suffer and benefit from
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22 322 the changing conditions altering the resulting communities.

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24 323 Most of the studies investigating the effects of increased pCO₂ ocean
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26 324 acidification on copepods have found that acidification associated ~~to-with~~ a wide range
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28 325 of CO₂ concentrations predicted for this century cause no significant effects on
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30 326 mortality, development, metabolism or reproductive parameters (Mayor *et al.*, 2012;
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32 327 McConville *et al.*, 2013; Vehmaa *et al.*, 2013; Hildebrandt *et al.*, 2014; Li *et al.*, 2015).
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34 328 However, when copepods are exposed to a range of pCO₂ predicted for a future ocean in
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36 329 combination with increasing temperatures they become more sensitive to high
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38 330 pCO₂ ocean acidification with impacts on offspring viability, hatching success, egg
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40 331 production and metabolic rates (Mayor *et al.*, 2012; Vehmaa *et al.*, 2013; Hildebrandt *et*
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42 332 *al.*, 2014; Zervoudaki *et al.*, 2014).

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44 333 Some studies have applied CO₂ concentrations far beyond those expected for
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46 334 the next 100 years by-as reported by the IPCC reports. However, such predictions are
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48 335 for open ocean conditions and coastal environments already experiencing pCO₂ levels
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50 336 much higher than those predicted under future open ocean scenarios (Blackford *et al.*,
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52 337 2009; Hofmann *et al.*, 2011). Consequently, these higher treatment levels can still be
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54 338 considered as relevant to future ocean acidification impacts in highly variable inshore

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6 339 systems. At these levels (5000–10,000 ppm CO₂, pH 7.02 – 6.7), copepods were
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8 340 negatively affected in terms of reproduction but not in terms of mortality, with
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10 341 reductions in hatching success and egg production and with increases in hatching and
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12 342 nauplius mortality (Kurihara *et al.*, 2004a,b; Mayor *et al.*, 2007; McConville *et al.*,
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14 343 2013).

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16 344 Contrarily to what was observed for single-species experiments, in the present
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18 345 study, the copepod communities were negatively affected in terms of mortality at these
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20 346 high pCO₂ levels. Under these conditions, the ~~patterns of response~~ response patterns
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22 347 were complex. Despite the sensitivity of the dominant species *Tisbe* sp, the densities of
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24 348 other species increased, suggesting that the impact of increased pCO₂ ocean acidification
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26 349 in combination with warming can cause responses that are not predictably
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28 350 unidirectional. Our results confirm the increasing consensus in literature that
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30 351 experiments on whole assemblages should be prioritized performed alongside species
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32 352 and population specific studies. The former can reveal ~~since they comprise complex~~
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34 353 changes in ecological and biological interactions and present an ecosystem-level view
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36 354 of changes, whilst the latter may provide the necessary information on physiology and
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38 355 ecology of species and population to interpret a more complex system with many
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40 356 ecological interactions. ~~Thus~~ This way, results and predictions from single species
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42 357 studies ~~could~~ can be interpreted in the light of multi-specific assemblages (e.g. Kroeker
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44 358 *et al.*, 2013; Gaylord *et al.*, 2015). ~~The present results confirm the increasing consensus~~
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46 359 in literature that experiments on whole assemblages should be prioritized since they
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48 360 ~~comprise complex changes in ecological and biological interactions.~~ The present results
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50 361 confirm the increasing consensus in literature that experiments on whole assemblages
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52 362 can be more informative than single species ones, revealing complex changes in
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54 363 ecological and biological interactions. ~~Thus, predictions from single species studies~~

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6 364 ~~could be interpreted in the light of multi specific assemblages. Thus, a precautionary~~
7 ~~approach may be required when interpreting predictions from single species studies,~~
8 365 ~~once potential changes due to species interactions are not considered (e.g. Kroeker et~~
9 ~~al., 2013; Gaylord et al., 2015).~~
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12 368 In the present study, strong impacts were observed only at the most severe pH
13
14 369 treatment, and copepods were not affected at less severe pH reductions. This apparent
15
16 370 high tolerance is to be expected considering that communities from temperate intertidal
17
18 371 environments experience high variability of abiotic factors in their natural environment
19
20 372 and thus, most intertidal animals would have developed effective physiological
21
22 373 adaptations for surviving such a highly variable and ~~harsh-testing~~ environment. In fact,
23
24 374 there have been studies that found no effects of ~~high pCO₂ ocean acidification~~ (pH
25
26 375 between 7.78 to 7.2) on copepods from the Arctic or temperate environments and/or
27
28 376 from laboratory cultures (Mayor *et al.*, 2012; McConville *et al.*, 2013; Vehmaa *et al.*,
29
30 377 2013; Hildebrant *et al.*, 2014; Li *et al.*, 2015). It is expected that fauna from habitats
31
32 378 characterized by strong abiotic variability (e.g. areas with volcanic emissions in the sea,
33
34 379 areas with excessive respiration in confined areas filled with plant and animal life, like
35
36 380 rockpools of the intertidal zone, marine sediments or hypoxic bottom waters) would
37
38 381 exhibit ~~higher-greater~~ tolerance to climate change predictions expected for this century
39
40 382 (Pörtner *et al.*, 2004). Pascal *et al.* (2010) suggested that among two harpacticoid
41
42 383 species, the species (*Shizopera knabeni*) that came from environments that are more
43
44 384 prone to hypercapnia (e.g. mudflats) was less sensitive to future acidification than
45
46 385 *Amphiascoides stopus*, which is found on large grained beaches. Li *et al.* (2015) found
47
48 386 that the combination of heat shock and ~~high pCO₂ ocean acidification~~ did not affect the
49
50 387 mortality of *Tigriopus japonicus*, a ~~harpacticoid~~ copepod which inhabits a highly
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52 388 variable intertidal environment. However, when exposed to very high CO₂ levels,
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6 389 reduction in egg production rate and hatching success was observed for *Centropages*
7
8 390 *typicus*, but not for *Temora longicornis* ([both calanoid copepods](#)). Since both species
9
10 391 were collected from the western English Channel the results suggest that even species
11
12 392 from the same locality and with similar life histories could present different tolerances
13
14 393 to ocean acidification (McConville *et al.*, 2013). Additionally, alternative theories
15
16 394 suggest that for some of ~~the~~ organisms, particularly if they are from populations at the
17
18 395 edges of their geographical distribution, individuals may already be close to their upper
19
20 396 tolerance capacity and even a slight increase in pCO₂ could have significant impacts on
21
22 397 community structure ([Findlay *et al.*, 2010](#)).

23
24 398 ~~[Tisbe sp was the dominant species and the general community pattern of](#)~~
25
26 399 ~~[response to increased pCO₂ and warming observed was influenced by this species.](#)~~
27
28 400 ~~[Species of this genera are characterized by having high fecundity and a short generation](#)~~
29
30 401 ~~[time \(7 – 16 days, Williams and Jones, 1994; Pinto *et al.*, 2001\), a wide range of body](#)~~
31
32 402 ~~[sizes, tolerance to a wide range of environmental changes and by having the ability to](#)~~
33
34 403 ~~[grow on different food sources and attaining high population densities \(e.g. 205 ind.](#)~~
35
36 404 ~~[mL⁻¹\) \(Souza-Santos *et al.*, 2006\), characteristics that allows *Tisbe* species to be easily](#)~~
37
38 405 ~~[reared in the laboratory \(e.g. Williams and Jones, 1999; Souza-Santos *et al.*, 2015\).](#)~~
39
40 406 ~~[However, despite the absence of significant effects on *Tisbe* sp density at pH 7.7 and](#)~~
41
42 407 ~~[7.3, it is possible – if not likely - that sub-lethal impacts could occur \(Fitzer *et al.*,](#)~~
43
44 408 ~~[2012\).](#)~~ ~~[Tisbe sp was the dominant species in all treatment samples and general](#)~~
45
46 409 ~~[community pattern of response to ocean acidification and warming observed was](#)~~
47
48 410 ~~[influenced by this species. Species of the genera *Tisbe* are characterized by having high](#)~~
49
50 411 ~~[fecundity and a short generation time, a wide range of body sizes, tolerance to a wide](#)~~
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52 412 ~~[range of environmental changes and by having the ability to grow on different food](#)~~
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54 413 ~~[sources and attaining high population densities. *Tisbe* species are easily reared in the](#)~~

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6 414 ~~laboratory and have been extensively cultured for tests as live food for fish and~~
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8 415 ~~crustacean larvae as well as for ecotoxicological bioassays (e.g. Williams and Jones,~~
9
10 416 ~~1999; Souza Santos *et al.*, 2006; Souza Santos *et al.*, 2015). However, despite the~~
11
12 417 ~~absence of significant effects on *Tisbe* sp density at pH 7.7 and 7.3, it is possible if not~~
13
14 418 ~~likely that sub-lethal impacts could occur (Fitzer *et al.*, 2012).~~

15
16 419 In the present study, the positive increase of malformed adult animals with the
17
18 420 increased level of warming and pCO₂ ocean acidification is presented for the first time
19
20 421 for copepods. This kind of approach has been conducted only for large representatives
21
22 422 of macrobenthic species at early development stages. For those animals, an increase in
23
24 423 abnormal development in larval and juveniles stages of some coral, molluscs and
25
26 424 echinoderms has been correlated to increases in pCO₂ ocean acidification and warming
27
28 425 (Byrne, 2011). Since the time difference between the nauplii stage and the last
29
30 426 copepodite stage is very short (10 and 18 days) for most of harpacticoid species (Giere,
31
32 427 2009), the evaluation of abnormality at these stages would be a very difficult task, and
33
34 428 for studies at community level almost impossible. On the other hand, assessing the
35
36 429 presence of malformed appendices in adults during microscope identification does not
37
38 430 increase time significantly. The analysis of this parameter indicates that, species that do
39
40 431 not suffer mortality in response to high pCO₂ ocean acidification and warming entering
41
42 432 the adult stage (like *Tisbe* sp) are not free from sub-lethal symptoms that could have
43
44 433 negative consequences for populations after long periods of ~~exposition~~exposure.

45 434 The exposure period of 60 days could be a brief time scale to detect subtle
46
47 435 effects for other communities such as macrofauna (Hale *et al.*, 2011). However, due to
48
49 436 the rapid life cycles of harpacticoid copepods, the present results were most probably
50
51 437 the response of a natural community exposed to elevated pCO₂ and warming over 6-8
52
53 438 generations (Giere, 2009). A limitation of the present study is the exposure of intertidal

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6 439 [communities to artificially constant low pH. Consequently, high priorities for future](#)
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8 440 [research should consider the natural variability of pH and temperature that organisms](#)
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10 441 [are subjected in field in order to investigate climate change effects on species and](#)
11
12 442 [community responses through natural and manipulative experiments.](#)

13
14 443 The results presented in this study demonstrated that the combination of
15
16 444 elevated levels of CO₂ and ocean warming may have substantial effects on harpacticoid
17
18 445 communities from intertidal environments. Moreover, they showed that ecological
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20 446 interactions may lead to complex community responses to pH and temperature changes
21
22 447 that cannot be encompassed by single species and/or single stressor experiments.

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20 646 Zervoudaki, S., Frangoulis, C., Giannoudi, L., and Krasakopoulou, E. 2014. Effects of
21 647 low pH and raised temperature on egg production, hatching and metabolic rates of a
22 648 Mediterranean copepod species (*Acartia clausi*) under oligotrophic conditions.
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25 649 *Mediterranean Marine Science*, 15: 74–83.
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Figure legends

651
652 Figure 1. MDS ordination plots for the Bray–Curtis similarity for Copepod community
653 structure. + (cross) Field samples, ● (circle) 8.0, ▲ (triangle) 7.7, ■ (square) 7.3, ◆
654 (diamond) 6.7 (12 °C closed symbols, 16 °C open symbols).

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656 Figure 2. Shannon diversity ($H' \log_2$), Pielou's evenness (J') and Species richness (S)
657 for copepod community at different pH and temperatures. Values: mean ± 95 %
658 confidence intervals.

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660 Figure 3. Mean (± 95 % confidence intervals) of ovigerous female, female/male ratios,
661 copepodite and malformed animals ratios at different pH and temperatures.

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663 Figure 4. The effects of pH and temperature on the mean abundance and relative
664 abundance (± 95 % confidence intervals) of the main harpacticoids species.

Table 1. Seawater chemistry within a) buckets and b) reservoir tanks during the experimental exposure period (Hale *et al.*, 2011). (Sal – salinity, TCO₂ – total water carbon dioxide concentration, TA – total alkalinity, pCO₂ – partial pressure of carbon dioxide, Ω_{Ca} – calcite saturation state, Ω_{Ar} – aragonite saturation state, HCO₃⁻ – bicarbonate concentration, CO₃²⁻ – carbonate concentration). Values: mean, ±SD, 95% CI.

	Nominal	Temp					pCO ₂					
	pH	(°C)	pH	Sal	TCO ₂	TA	(µatm)	Ω _{Ca}	Ω _{Ar}	HCO ₃ ⁻	CO ₃ ²⁻	
Buckets												
	12°C	8	11.78	7.86	34.88	1858.80	1956.61	729.23	1.59	1.01	1784.99	66.59
			0.35	0.09	0.19	314.04	293.36	160.4	0.3	0.19	275.4	12.5
			0.07	0.02	0.05	72.54	74.23	40.59	0.08	0.05	69.68	3.16
		7.7	11.93	7.66	34.89	2084.49	2155.52	1295.53	1.17	0.75	2031.97	49.03
			0.35	0.08	0.24	331.50	303.58	244.51	0.24	0.15	286.88	10.09
			0.07	0.01	0.06	76.57	76.81	61.87	0.06	0.04	72.59	2.55
		7.3	11.66	7.35	34.94	2181.94	2098.44	2729.23	0.55	0.35	2039.57	23.22
			0.41	0.07	0.13	227.68	228.21	499.36	0.11	0.07	222.75	4.5
			0.09	0.01	0.03	52.59	57.74	126.35	0.03	0.02	56.36	1.14
		6.7	11.53	6.81	34.82	2409.95	1942.01	2268.73	0.16	0.1	1925.14	6.6
			0.38	0.23	0.14	313.33	221.04	3127.42	0.11	0.07	220.99	4.56
			0.08	0.04	0.04	72.37	55.93	791.33	0.03	0.02	55.92	1.15
	16°C	8	16.04	7.85	35.31	1915.97	1984.25	822.18	1.91	1.23	1779.26	80.3
			0.40	0.13	0.26	216.12	226.77	743.4	0.38	0.27	204.34	15.83
			0.08	0.02	0.07	49.92	57.38	188.1	0.1	0.06	51.7	4.01
		7.7	16.01	7.61	35.13	2046.30	2072.27	1422.98	1.21	0.78	1943.53	50.94
			0.63	0.15	0.21	241.83	246.16	388.9	0.29	0.19	233.63	12.18
			0.13	0.03	0.05	55.86	62.29	98.4	0.07	0.05	59.12	3.08
		7.3	15.76	7.37	35.06	2105.32	2051.74	2611.66	0.67	0.43	1980.85	28.01
			0.31	0.1	0.15	254.15	232.74	547.02	0.15	0.1	225.48	6.43
			0.07	0.02	0.04	58.70	58.89	138.41	0.04	0.02	57.05	1.63
		6.7	15.48	6.66	34.99	2423.61	1957.02	3010.36	0.15	0.1	1940.63	6.5
			1.52	0.19	0.18	284.04	25.08	4141.2	0.08	0.05	212.54	3.41
			0.32	0.04	0.04	65.60	54.42	1047.85	0.02	0.01	53.78	0.86
Reservoir tanks												
		8	14.08	7.89	34.95	1930	2018.97	680.76	1.93	1.24	1811.93	81.15
			0.44	0.14	0.10	273.13	307.01	156.68	0.31	0.2	287.81	12.89
			0.19	0.06	0.05	138.22	166.89	85.17	0.17	0.11	156.45	7.01
			15.56	7.98	34.84	1860	1970.58	527.15	2.38	1.53	1715.41	99.82
			0.41	0.14	0.13	257.68	290.3	96.68	0.39	0.25	231.67	16.49
			0.18	0.06	0.07	130.40	157.81	52.56	0.21	0.14	142.08	8.97
		7.7	15.46	7.68	34.82	2086.67	2116.32	1211.65	1.38	0.89	1970.45	57.92

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2											
3		0.43	0.19	0.12	206.13	225.18	243.27	0.23	0.15	216.55	9.6
4		0.19	0.08	0.06	104.31	122.41	132.24	0.12	0.08	117.71	5.22
5		14.33	7.63	34.75	2033.33	2084.55	1308.57	1.21	0.77	1957.12	50.53
6		0.56	0.18	0.15	287.90	323.71	230.09	0.37	0.23	297.01	15.36
7		0.24	0.08	0.08	145.70	175.97	125.07	0.2	0.13	161.46	8.35
8											
9	7.3	15.39	7.26	34.86	2156.67	2066.85	3279.5	0.54	0.35	2010.12	22.48
10		0.55	0.19	0.13	130.21	141.82	584.35	0.12	0.08	135.28	5.21
11		0.24	0.08	0.07	65.90	77.09	317.65	0.07	0.04	73.54	2.83
12		14.26	7.35	34.82	2106.67	2043.55	2905.94	0.55	0.35	1984.88	23.14
13		0.43	0.2	0.14	264.49	286.94	484.55	0.12	0.08	278.31	5.16
14		0.19	0.08	0.07	133.85	155.98	263.4	0.07	0.04	151.29	2.8
15											
16	6.7	13.86	6.33	34.76	2686.67	1770.66	243.69	0.05	0.03	1765.47	2.16
17		0.44	0.14	0.14	311.7	247	42.29	0.02	0.01	245.84	0.64
18		0.19	0.06	0.08	157.74	134.27	22.99	0.01	0.01	133.64	0.35
19		15.3	6.34	34.76	2693.33	1763.27	238.83	0.06	0.04	1757.66	2.31
20		0.7	0.2	0.16	358.17	220.64	41.23	0.02	0.01	219.47	0.63
21		0.3	0.08	0.09	181.26	119.94	22.41	0.01	0.01	119.31	0.34
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Table 2. List of Copepoda Harpacticoida species from collected at the rocky shore at Mount Batten, Plymouth, UK.

Order Harpacticoida Sars, 1903	
Suborder Oligarthra Lang, 1944	
Family Laophontidae T. Scott, 1905	Family Canthocamptidae Brady, 1880
<i>Laophonte cornuta</i> Philippi, 1840	<i>Mesochra pygmaea</i> (Claus, 1863)
<i>Laophonte setosa</i> Boeck, 1865	<i>Nannomesochra arupinensis</i> (Brian, 1925)
<i>Laophonte sima</i> Gurney, 1927	Family Dactylopusiidae Lang, 1936
<i>Laophonte</i> sp	<i>Dactylopusia vulgaris dissimilis</i> Brian, 1921
<i>Laophontinae</i> sp1	<i>Diarthrodes</i> sp
<i>Laophontinae</i> sp2	<i>Paradactylopodia</i> sp
<i>Paralaophonte brevirostris</i> (Claus, 1863)	Family Ectinosomatidae Sars, 1903
Family Miraciidae Dana, 1846	Ectinosomatidae sp 1
<i>Amonardia normani</i> (Brady, 1872)	<i>Ectinosoma</i> sp1
<i>Amphiascoides golikovi</i> Chislenko, 1977	<i>Ectinosoma</i> sp2
<i>Amphiascoides</i> sp1	<i>Halectinosoma</i> sp1
<i>Amphiascopsis</i> sp	<i>Halectinosoma</i> sp2
<i>Amphiascus minutus</i> (Claus, 1863)	<i>Halectinosoma</i> sp3
<i>Amphiascus longarticulatus</i> Marcus, 1974	<i>Pseudobradya</i> sp1
<i>Amphiascus</i> (varians) sp	<i>Pseudobradya</i> sp2
<i>Amphiascus parvus</i> Sars, 1906	<i>Pseudobradya</i> sp3
<i>Amphiascus angustipes</i> Gurney, 1927	<i>Sigmatidium</i> sp
<i>Bulbamphiascus</i> sp	Family Longipediidae Boeck, 1865
<i>Delavalia</i> sp	<i>Longipedia</i> sp
<i>Haloshizopera lima</i> Becker, 1974	Family Normanellidae Lang, 1944
<i>Paramphiascella</i> sp	<i>Normanella</i> sp
<i>Robertgurneya</i> sp	Family Harpacticidae Dana, 1846
<i>Robersonia</i> sp	<i>Harpacticus obscurus</i> T. Scott, 1895
Family Ameiridae Boeck, 1865	Family Pseudotachidiidae Lang, 1936
<i>Ameira</i> sp	<i>Idomene purpurocincta</i> (Norman & T. Scott, 1905)
<i>Ameiropsis mixta</i> Sars, 1907	Family Peltidiidae Claus, 1860
<i>Nitocra</i> sp	<i>Alteutha depressa</i> (Baird, 1837)
<i>Proameira hiddensoensis</i> (Schäfer, 1936)	<i>Eupelte</i> sp
<i>Proameira thetiensis</i> Pallares, 1982	Family Tisbidae Stebbing, 1910
<i>Psyllocamptus</i> (L) <i>triarticulatus</i> Lang, 1965	<i>Tisbe</i> sp
	Harpacticoida sp

Table 3. PERMANOVA results for the Copepod community exposed to different pH and temperatures. Significant values are highlighted in bold.

Source	<i>df</i>	MS	<i>F</i>	<i>P</i>
Temperature (T)	1	3584.5	2.52	0.0044
pH	3	3147.1	2.22	0.0004
pH x T	3	1467.1	1.03	0.422
Residual	24	1420.3		

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Table 4. Pair-wise *a posteriori* comparisons for pH. Significant values are highlighted in bold.

pH Comparisons	<i>t</i>	<i>P</i>
8.0 x 7.7	0.821	0.759
7.7 x 7.3	1.000	0.466
7.7 x 6.7	1.951	0.0001
8.0 x 7.3	0.911	0.6551
8.0 x 6.7	2.066	0.0001
7.3 x 6.7	1.839	0.0002

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For Review Only

Table 5. Percent contribution (Contrib. %) of Cyclopoida and species of Harpacticoida to average dissimilarity (Diss.) among different pH (Cut off for low contributions: 70%).

8.0 vs 6.7		7.7 vs 6.7		7.3 vs 6.7	
Diss.= 63.88	Contrib. %	Diss.= 60.36	Contrib. %	Diss.= 58.61	Contrib. %
<i>Amphiascus longarticulatus</i>	5.84	<i>Ectinosoma</i> sp2	6.63	<i>Ectinosoma</i> sp2	6.18
<i>Tisbe</i> sp	5.80	<i>Tisbe</i> sp	6.45	<i>Tisbe</i> sp	6.02
<i>Ectinosoma</i> sp2	5.62	<i>Amphiascoides golikovi</i>	5.40	<i>Paradactylopodia</i> sp	5.51
<i>Dactylopusia vulgaris dissimilis</i>	5.04	<i>Amphiascoides</i> sp1	4.89	<i>Amphiascus longarticulatus</i>	5.31
<i>Amphiascoides golikovi</i>	4.81	<i>Delavalia</i> sp	4.85	<i>Amphiascoides</i> sp1	4.66
<i>Laophonte cornuta</i>	4.65	<i>Amphiascus longarticulatus</i>	4.82	<i>Laophonte cornuta</i>	4.44
<i>Amphiascoides</i> sp1	4.65	<i>Laophonte cornuta</i>	4.70	<i>Robertgurneya</i> sp	4.01
<i>Paradactylopodia</i> sp	4.54	<i>Ameiropsis mixta</i>	4.44	<i>Ameiropsis mixta</i>	3.68
<i>Normanella</i> sp	4.09	<i>Paradactylopodia</i> sp	4.32	<i>Pseudobradya</i> sp2	3.67
<i>Ectinosoma</i> sp1	4.07	Cyclopoida	4.23	<i>Ectinosoma</i> sp1	3.58
<i>Ameira</i> sp	4.01	<i>Dactylopusia vulgaris dissimilis</i>	4.14	<i>Dactylopusia vulgaris dissimilis</i>	3.54
Cyclopoida	3.76	<i>Ectinosoma</i> sp1	4.01	<i>Amphiascoides golikovi</i>	3.52
<i>Ameiropsis mixta</i>	3.22	<i>Normanella</i> sp	3.69	<i>Pseudobradya</i> sp1	3.25
<i>Pseudobradya</i> sp1	2.88	<i>Idomene purpurocincta</i>	3.39	<i>Idomene purpurocincta</i>	3.18
<i>Pseudobradya</i> sp2	2.86	<i>Pseudobradya</i> sp1	3.35	<i>Ameira</i> sp	3.17
<i>Laophonte sima</i>	2.84			<i>Normanella</i> sp	2.82
				Laophontinae sp2	2.53

Table 6. Percent contribution (Contrib. %) of Cyclopoida and species of Harpacticoida to average dissimilarity (Diss.) between temperatures (Cut off for low contributions: 70%).

12 °C vs 16 °C	
Diss.= 57.39	Contrib. %
<i>Amphiascoides</i> sp1	5.18
<i>Paradactylopodia</i> sp	4.98
<i>Amphiascus longarticulatus</i>	4.81
<i>Ectinosoma</i> sp	4.68
<i>Amphiascoides golikovi</i>	4.38
<i>Dactylopusia vulgaris dissimilis</i>	4.25
Cyclopoida	4.11
<i>Tisbe</i> sp	4.11
<i>Ameiropsis mixta</i>	4.06
<i>Ectinosoma</i> sp1	3.83
<i>Laophonte cornuta</i>	3.81
<i>Normanella</i> sp	3.72
<i>Ameira</i> sp	3.68
<i>Pseudobradya</i> sp1	3.42
<i>Delavalia</i> sp	3.38
<i>Robertgurneya</i> sp	3.15
<i>Idomene purpurocincta</i>	2.93

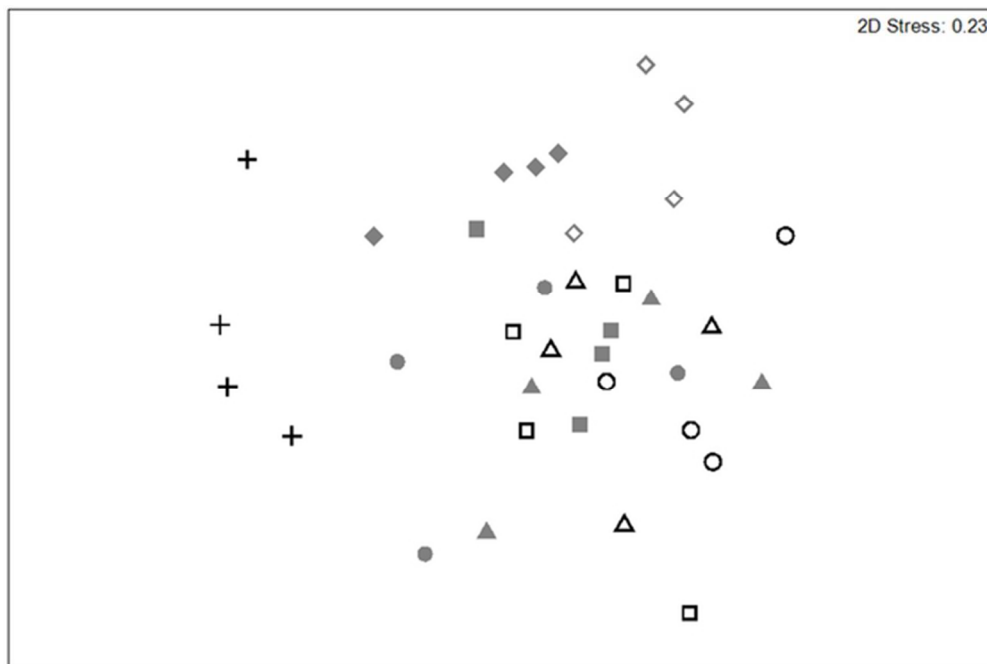


Figure 1. MDS ordination plots for the Bray–Curtis similarity for Copepod community structure. + (cross) Field samples, ● (circle) 8.0, ▲ (triangle) 7.7, ■ (square) 7.3, ◆ (diamond) 6.7 (12 °C closed symbols, 16 °C open symbols).

56x38mm (300 x 300 DPI)

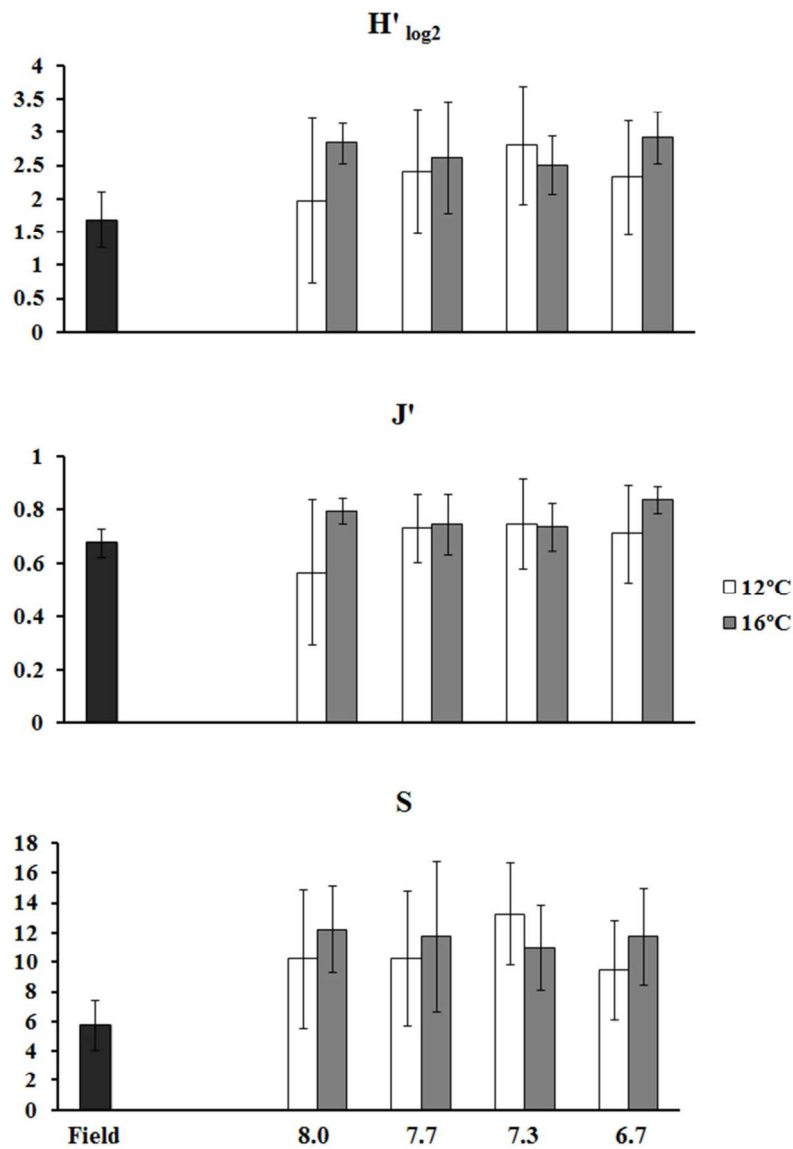


Figure 2. Shannon diversity ($H' \log_2$), Pielou's evenness (J') and Species richness (S) for copepod community at different pH and temperatures. Values: mean \pm 95 % confidence intervals.

126x187mm (300 x 300 DPI)

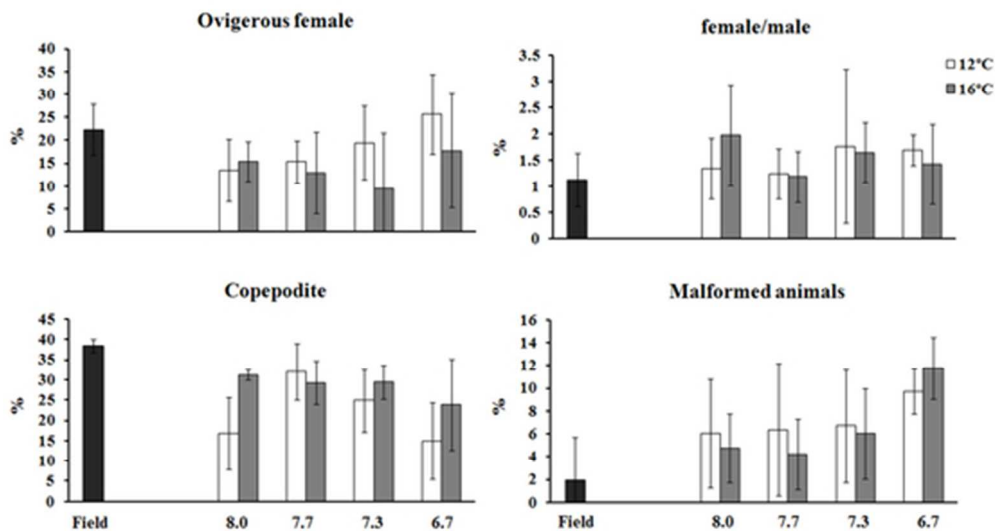


Figure 3. Mean ($\pm 95\%$ confidence intervals) of ovigerous female, female/male ratios, copepodite and malformed animals ratios at different pH and temperatures.

47x26mm (300 x 300 DPI)

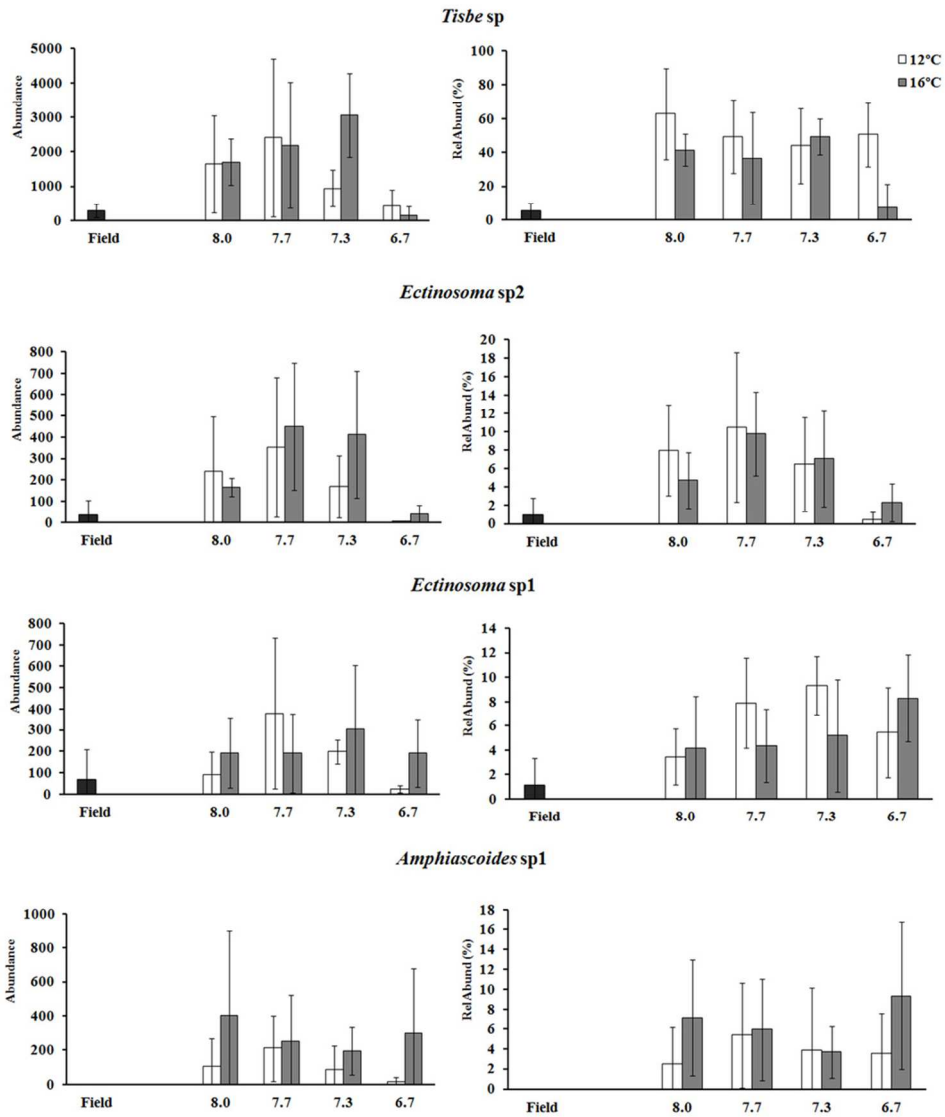


Figure 4. The effects of pH and temperature on the mean abundance and relative abundance ($\pm 95\%$ confidence intervals) of the main harpacticoids species.

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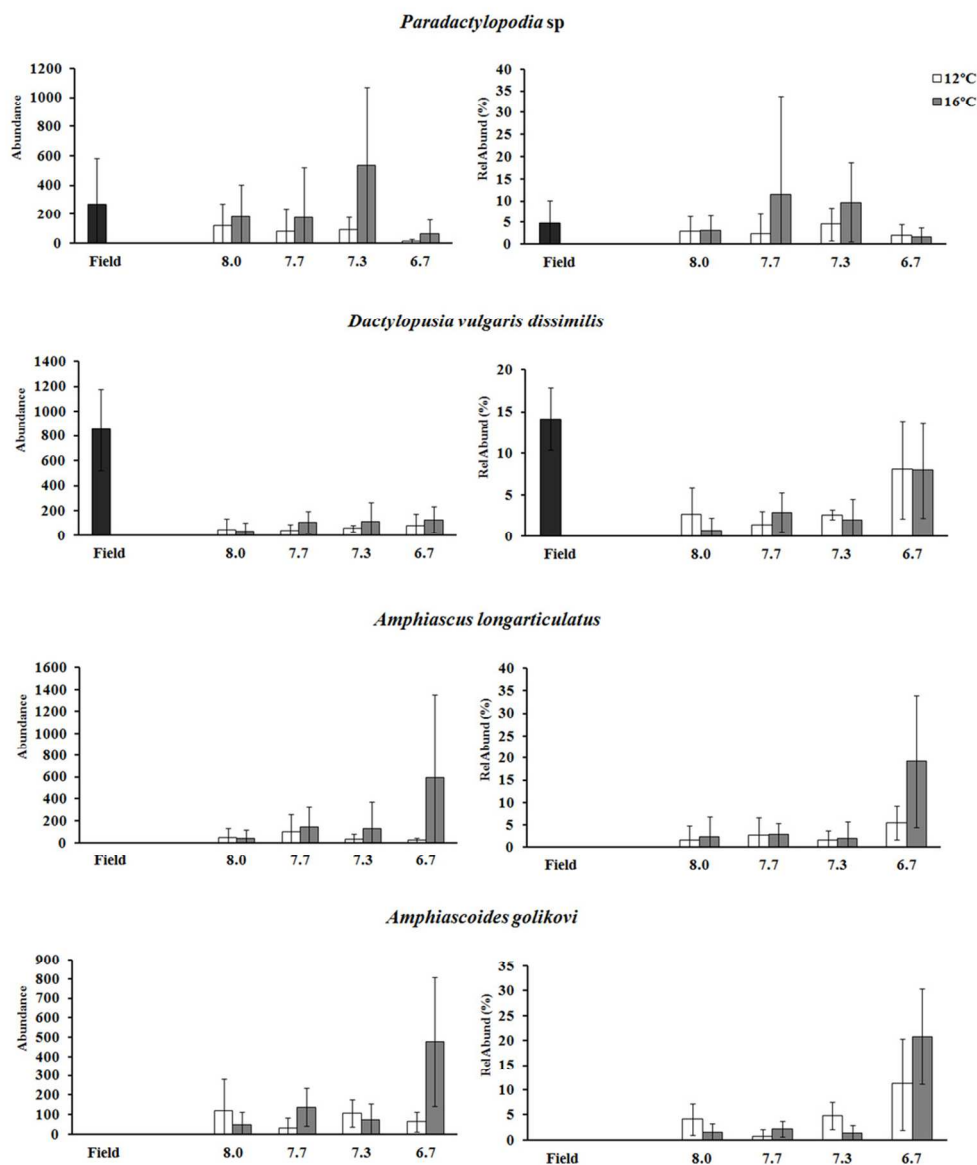


Figure 4. Continued

100x117mm (300 x 300 DPI)