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Biogeosciences Discussions

Interactive comment on "Variable metabolic responses of Skagerrak invertebrates to low O_2 and high CO_2 scenarios" *by* Aisling Fontanini et al.

Anonymous Referee #1

Received and published: 14 November 2017

Fontanini and co-workers investigated the respiratory responses of a range of invertebrates to short-term changes in oxygen and CO2. The paper gives some interesting insights which revealed the highly variable responses of invertebrates from a single area towards future scenarios. This reveals that e.g. general ecosystem models based on few species are not yet reliable. Therefore, this study highlights the need for more experimental work. Currently the manuscripts lack some important experimental de- tails which make it complicated to judge the quality of the measurements. For instance what was the rational behind measurements on day 3 and 6 and were any differences observed? Considering the strong physiological differences between the investigate phyla e.g. a crustacean and a tunicate or echinoderm it is not intuitive why the animals were grouped according to the habitat and not according to the lifestyle in figure 1. This could mask certain significances.

> Reply: The decision about the duration of the experiment was based on a meta-analysis by Vaquer-Sunyer & Duarte (2008) which found the median lethal time (LT_{50}) for over 400 studies to be just over 5 days. Therefore, we felt we should be able to detect a sub-lethal response such as respiratory changes within that time-frame. Moreover, as we wanted to measure the respiration rate, we had to make sure that the individuals were still alive. We have added a statement to the introduction to highlight this.
> The habitat background was the same for the species collected from the same habitat. Most of the tested species were echinoderms and gastropods. As for crustaceans, bivalves and tunicates, we just had 1 species each and thus didn't see the need of grouping per taxa.

I have a couple comments and the MS would benefit from some careful corrections thus I recommend major revision.

Specific comments

Page 1 Line 25 that the responses of respiration of the respiration – please change

Reply: Changes made as suggested.

P 2 line 6 please change to acidic water

Reply: We changed the phrase and it is now reading “leading to a decreased pH” as we try to avoid the term “acidic water”.

P2 Line 7 what do you mean by ‘Control’? more precisely speaking: the biochemical processes which change seawater pH?

Reply: Yes, we were referring to the biochemical processes and relationships that may cause pH to fluctuate over various temporal and spatial scales, drawing particular attention to the role of metabolism. This sentence has been altered to better reflect this.
The sentence now reads “The involvement of metabolic processes in the regulation of pH in coastal water is particularly evident when eutrophication stimulates algal CO_2 for marine organisms reach further than the highly documented impacts on calcification rates (Doney et al., 2009).”.

P2 Line 18 Internal is not a precise term, intra- and extracellular pH regulation are two completely different processes. I assume you refer to extracellular pH as intracellular pH is commonly well regulated? Please specify.

Reply: Yes, we agree and changed the phrase to “extracellular acid-base regulation”.

P2 Line 20 Hemoglobin is not common in invertebrates which this study is focused on.

Reply: We have removed the reference to haemoglobin as well as the definition of hypercapnia as we do not investigate the impact on blood

fluids within this study.

P3 Line 31 what is meant by ‘a history of North Sea upwelling’? commonly observed?

Reply: Salinity at the surface of the fjord can change by 10psu in the summer months as salty water originating from the North Sea comes to the surface. We have decided to remove this sentence as the water we used during experiments had a stable salinity for all animals.

P3 line 3 and following? Where the salinity similar at surface and bottom of the Fjords as all animals were exposed to the same high salinity during the acclimation phase?

Reply: The Sven Loven Centre has three water inflows from different depths in the Fjord. We chose to use North Sea (deep water) as animals from shallow environments are able to cope with the salinity due to the aforementioned upwelling events.
As we have removed the reference to summer upwelling, we have also removed the reference to North Sea water from this sentence. This will have minimal impact as we have stated the salinity over the course of the experiment in subsequent sentences.

P4 Line 3 and following Based on this paragraph, P. bernhardus was the only species which was fed during the experiment? Is there any specific reason for this decision?

Reply: This decision was based on advice from the lab technician in charge of looking after animals at the facility, which considerably experience in maintaining the organisms tested here. Given the short exposure periods, and the fact that most individuals are filter feeders, there was no need to add food to the aquaria. The crustacean *P. bernardus* on the other hand is a carnivore and was fed *ad libidum* before the experiment started. We added the sentence “No animals were fed during their experimental period” to clarify.

P4 Line 8 and following Several species and sometimes specimens were kept in the same aquaria for logistic purposes? However, can you exclude that a number of co-variable influenced respiration rates similar to the observed abnormal mortality in one tank?

Reply: Respiration rates of animals were measured individually in glass chambers, where no other organisms were present.
All aquaria had the same 'mixture' of organisms at the same time, so we would expect to see the impact of co-variables across all treatments. Moreover, we made sure no predator and preys were in the tanks at the same time to exclude stress due to their presence.

P4 Line 16 and following The animals were kept in closed systems without any waster exchange. Did you check the water quality in order to monitor potential accumulation of waste products due to metabolism and mortality?

Reply: Should that read Page 5 Line 16? As described on Page 5 Line 1, we replenished the water in the aquaria with a continuous flow of water. For the incubations to measure the respiration rate it was essential to keep the glass chambers/containers sealed as we measured oxygen at the beginning and end of the incubation. Any water-exchange would have influenced and changed those measurements.

P 5 Line 15 and following As respiration was only response variable measured in this study, more detailed information needs to be provided such as: Volume of the containers, did you control for a linear decline of oxygen concentrations? In particular, as this is the focus of the study, how much did oxygen decline during the incubation? Strong declines would severely affect the study concept.

Reply: Oxygen consumption has been updated to **mg L^{-1} O_2 min^{-1} L^{-1} g DW^{-1}** as the different size of glass chambers was accounted for when calculating the respiration rates.

P 4 line 34 Why did you use two different pH meters and what differences did you observe?

Reply: The Metrohm 827 pH meter was actually used to take daily point measurements and is shown in the information in the results section. The data logger was used as a reference for us to see what was happening overnight, but could only be placed in one tank and so has not contributed to any data reflected in this paper. We have removed the reference to the data logger.

P 6 line 7 Here you state a target of 1000 ppm whereas it is 1300 µatm in the M&M, even if target and measurement were not identical, the target should be uniform.

Reply: Changes made as suggested.

P6 Line 37 A non significant response may only called a 'trend towards'

Reply: Changes made as suggested.

P 7 line 7 the experiment did not last long enough to draw any reliable conclusion on survival rates

Reply: As shown by Vaquer-Sunyer & Duarte (2008)'s meta-analysis that 90% of 282 studies experienced LC_{50} at 4.6 mg O_2 L^{-1} with the mean LC_{50} for all organisms at 2.1 mg O_2 L^{-1}. The median LT_{50} (460 studies) was 117 hours or nearly 5 days. We therefore still believe that the high survivorship of organisms over 3-6 days in the low O_2 treatments indicates a tolerance (acclimation or adaption) to these conditions and is worthy of noting.
Nevertheless, we added the phrase "short-term" to the sentence and it reads now "The Baltic species tested were highly resistant to short-term hypoxia and high CO_2, alone or in combination, as they experienced very high survival rate across treatments in the relatively short-duration experiment reported here".

P7 line 14 To support this hypothesis you need to add a reference which documents higher mortality for populations from habitats with less abiotic stress

Reply: In this particular line (and following), we make a suggestion and already added references.

P7 line 17 and following Please consider that the RI hypothesis and in particular the definition of an exact threshold is still under debate: https://www.biogeosciences.net/10/2815/2013/bg-10-2815-2013.pdf

Reply: We have attempted to use Brewer and Peltzers RI's to test its ability to predict marine responses to O_2 and CO_2 and state in the discussion that we feel it did not hold predictive power in the context of

this experiment. We hope this may contribute to the ongoing discussion.

P8 Line 37 Even though calcified structures and the calcification process might be affected by undersaturation it is not clear why this should be detectable in the rates of aerobic metabolism

Reply: We agree and changed the phrasing. It now reads “Hence, the RI does not hold predictive power on the effects of hypoxia and/or pCO_2 on the species tested here, which seemed best predicted from consideration of the ranges of O_2 and CO_2 they experience in their habitat.”.

P9 line 6 Hypoxia is necessarily always coupled to elevated CO2

Reply: Changes made as suggested. It now reads “and is, therefore, coupled with elevated pCO_2”.

Table 3 please give the unit for respiration rates

Reply: Changes made as suggested and added the information requested.

Some references are either missing in the text or in the reference list e.g. Grans or Gräns? et al. is not in the list

Reply: We checked the reference list and made the changes as suggested.

Interactive comment on Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-321, 2017.

Biogeosciences Discuss.,
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Biogeosciences Discussions

Interactive comment on "Variable metabolic responses of Skagerrak invertebrates to low O_2 and high CO_2 scenarios" *by* Aisling Fontanini et al.

Anonymous Referee #2

Received and published: 13 December 2017

General comments

The topic of the present work is interesting and the authors try to make a correlation between the obtained data and the environmental characteristics of habitats. However, my concern is the period of exposure to particular stressors, which may determine and the range of tolerance of marine invertebrates to environ- mental changes. For example, it is reported (page 8, line 43) that invertebrates from this ecosystem which showed little or no mortality in the presence of both stressors, reflects the range of conditions in the habitats these organisms occupy. However, mortality is depended on several factors including reproduction period, body size, etc. Moreover, it is depended on the period of species exposure to stressors. Also, a key point for the species to withstand stressful conditions for long term is their ability to keep a stable energy turnover since. Such metabolic responses and patterns determine and their thermal limits. Even more some species live at the edges of their range of thermal tolerance. Thus, long-term experiments might help further not only in estimating species' ability to withstand stressful conditions but to make a better correlation with the future climate projects. I consider that the authors should take into their consideration the above points and to reconsider the interpretation of some obtained data. I agree with author's statement about the complexity of stressors and the challenge of predicting how global stressors will affect marine ecosystems in the future.

Reply: We agree with the comments in regards to longer-term experiments, however it is beyond the scope of this work. Our research questions were targeted at being able to detect responses to

short-term and acute environmental changes that may occur suddenly as part of eutrophication events. We chose future climate change targets for O_2 and CO_2 levels as they are realistic representations of what will come in the future, or in some cases might already occur nowadays.

Specific comments

Page 2, line 8. This metabolic control. . .could be changed to The involvement of metabolic processes in the regulation of the pH in coastal water is... . ..

Reply: Changes made as suggested. It now reads “The involvement of metabolic processes in the regulation of pH in coastal waters is particularly evident when eutrophication stimulates algal blooms, leading to increased sedimentation of organic matter, subsequently degraded through microbial respiration, thereby consuming O_2 and releasing CO_2 (Conley et al., 2009).”.

Page 2, line 15. . . ...although the combined stress from depleted O2 and high CO2 is likely to provide a significant challenge to coastal invertebrates and less mobile species... could be changed to although the synergistic effect of O2 depletion and CO2 accumulation is likely to provide a significant challenge to coastal invertebrates and mostly to sessile species.

Reply: Changes made as suggested. It now reads “Whereas scientists have acknowledged this coupling over decades, the impacts of these two pressures have continued to be studied in isolation, although the synergistic effect of O_2 depletion and CO_2 accumulation is likely to provide a significant challenge to coastal invertebrates and mostly to sessile species.”.

Page 2, lines 24-26. There are many invertebrates tolerant to hypoxia (e.g. mussels). Thus, the authors should be focused on these species which rather are less tolerant (e.g. benthic invertebrates).

Reply: As the aim of the study was to test the combination of two stressors, we also used “tolerant” species to see how they react to the combination of the two stressors hypoxia and elevated pCO_2.
Moreover, it has been shown that responses are highly species specific

and not taxa-related (see Fabry, 2008; Malakoff, 2012; Calosi *et al.*, 2013).

Page 2, line 37. I would prefer synergistic instead joint

Reply: We made the change as suggested.

Page 2, line 38. ..future levels of what I consider that the two last paragraphs should be reorgasinized and rewritten in such a way so the firstly the authors to be reported at several hypotheses and secondly at their aims

Reply: We reorganized the paragraphs as suggested.

Methods and Materials

1. Merge the two first paragraphs

Reply: Changes made as suggested.

2. Make clear, when saying history, whether the reported environmental characteristics are long lasting. It is very important since species experiencing such environmental changes in their life cycle may have adapted to such environmental changes by developing the corresponding cellular and physiological mechanisms.

Reply: This has been re-worded to show that we are referring to natural and sustained seasonal events which occur in winter and can be exacerbated by nutrient enrichment.
It reads now “Both natural and anthropogenically enhanced hypoxia occur within the fjord when enrichment is high and seasonal water exchange over the sill is slow (Josefson and Widbom, 1988; Arneborg, 2004)”.

3. Report which of the examined rocky species are exposed or not to air because of tide. The latter characterizes sessile species tolerant to hypoxia.

Reply: There is no real tides in the fjord where we collected the speciements. The seawater level can change by a few dm (less than 1

meter) depending on atmospheric pressure, winds, etc. Among the tested species, only *Littorina sp.* and *Mytilus sp.* can be occasionally be exposed to air.

4. Change Metabolic response to Metabolic rate or Oxygen consumption. Metabolic responses usually is referred when we examine the metabolic patterns (e.g. enzyme activities, metabolites etc)

Reply: Changes made as suggested.

5. Page 5, line 8. For the readers describe briefly the physiological meaning of term respiration index.

Reply: We feel that this has been described in the following sentences. But if the editor wants us to describe it in a different way we will add a description.

6. Page 5, lines 15-21. The experimental procedure for determining the oxygen consumption should be written in details. For example, chamber volume, was it the same for all species examined?

Reply: We updated the formula in the manuscript to **mg L^{-1} O_2 min^{-1} L^{-1} g DW^{-1}** as the volume of the glass chamber was included in the calculation. Thus, we don't feel the need to report all the chamber sizes in the manuscript.
But if the editor is of the opinion that those data (mean ±SE for each treatment) are essential for the manuscript, we will off course add this information.

Also report the temperature, salinity and pH of water. It is very important to report the period (hours) of experimental procedure since under a particular level of PO2 metabolism sifts from aerobic to anaerobic and this point is species depended.

Reply: The water for incubation had the same values as the experimental aquaria and held in the same room so the temperature and salinity would be the same as reported in Table 2.

Incubations lasted a maximum of 5.5 hours, to make sure there is some oxygen left in the glass chamber. None of them reached 0.0 mg L^{-1} oxygen. We added the max. incubation time to the manuscript but don't see the need of reporting time (minutes) in detail. But if the editor is the opinion that those data (mean ±SE for each treatment) are essential for the manuscript, we will off course add this information.

7. Page 5, line 23. Ratio of what?

Reply: Changes made as suggested and it reads now "The response ratio of the respiration rate…".

Results

1. Respiration. Report the consumption of oxygen rate for each examined species and give possible differences between each other.

Reply: The data are provided in Table 3 (mean ± SE).

2. Give more information the differences or not for the oxygen consumption for each species at each treatment

Reply: The data and results of statistical tests and GLM are shown in Table 3. We don't feel the need to report all of them twice and mention them again in the text of the manuscript.
But if the editor is of the opinion that it is 100% necessary we will make the change as suggested.

3. In general the results should be rewritten in such a way so to be more clear what is happening in each species at tested treatments and whether differences were recorded from species to species.

Reply: Our research questions were more targeted towards the differences between treatments for each species and how their habitats may have played a role.

Table 3. In the column day it is marked 3/6, 3/5 etc. In the legend it is reported pooled

data where we had 3 and 6 days. Thus the number 4, 5 2 what do they mean.

Reply: These were days of measurement, that were pooled. We have altered the wording to reflect this (in Table 3 and methods).

Discussion

Page 7, line 29-30. It is unclear what the authors report.

Reply: Changes made for clarity. It now reads “The community in this area has already been sieved of species vulnerable to low O_2 concentrations due to a history of hypoxia and even complete anoxia within the last four decades (Nordberg et al., 2000; Polovodova et al., 2011).”.

Page 7, line 32-33. It is very important to report whether such changes in pH regard fluctuations or permanent changes. In the first case the organisms face waves of such changes and how long such waves last.

Reply: These just represent fluctuations. This sentence has been reworded to reflect this difference.

Page 7, line 36-37. Community of what? Rewrite the sentence (line 37-39), since it is unclear what it is meaning.

Reply: Changes made as suggested. It now reads “Exposed *A. filiformis* live in sediment burrows that experience much lower oxygen and higher pCO_2 than surrounding water which intensifies with depth (Hu et al., 2014). *A. filiformis* have been shown to withstand a pH of 7.0 and O_2 levels below 2.0 mg L-1 and experience no mortality (Hu et al., 2014). Hence, the species tested here already has O_2 and pH values comparable to those used as treatments here, particularly for infauna, such as *A. filiformis* and *B. lyrifera* which appear to be exposed to low O_2 and pH conditions on a regular basis.”.

Page 9, line 16. Responses . . .which responses?

> Reply: Changes made as suggested, it reads now “Respiratory responses”.

Page 9, line 1-2. Do you know how long these events last? Is it an acute environmental change or long-term change?

> Reply: There is an overall trend towards decreasing oxygen over time in the Fjord (based on foraminifera populations) however hypoxic events can vary in duration. There is also a seasonal trend of decreasing oxygen over winter months before new water comes into the fjord.

Page 9, line 10. It could be nice if the authors could support such adaptive responses, genetically determined, by reporting differences from individuals of the same species but from different populations habiting environments differing in the tested abiotic factors. The observed responses in the present work may regard phenotypic plasticity which may be observed and in individuals from populations living in other environments when treated similarly.

> Reply: We were limited in time and logistics, thus there was no option for us to test different populations of the same species. But we agree that this should be taken in consideration for future experiments to compare if there are differences in responses depending on populations, water quality and conditions the individuals experienced previously.

Interactive comment on Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-321, 2017.

Variable metabolic responses of Skagerrak invertebrates to low O_2 and high CO_2 scenarios

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Abstract

Coastal hypoxia is a problem that is predicted to increase rapidly in the future. At the same time we are facing rising atmospheric CO_2 concentrations, which are increasing the pCO_2 and acidity of coastal waters. These two drivers are well studied in isolation however; the coupling of low O_2 and pH is likely to provide a more significant respiratory challenge for slow moving and sessile invertebrates than is currently predicted. The Gullmar Fjord in Sweden is home to a range of habitats such as sand and mud flats, seagrass beds, exposed and protected shorelines, and rocky bottoms. Moreover, it has a history of both natural and anthropogenically enhanced hypoxia as well as North Sea upwelling, where salty water reaches the surface towards the end of summer and early autumn. A total of 11 species (Crustacean, Chordate, Echinoderm and Mollusc) of these ecosystems were exposed to four different treatments (high/low oxygen and low/high CO_2; varying pCO_2 of 450 and 1300 µatm and O_2 concentrations of 2-3.5 and 9-10 mg L^{-1}) and respiration measured after 3 and 6 days, respectively. This allows us to evaluate respiration responses of species of contrasting habitats to single and multiple stressors. Results show that respiratory responses were highly species specific as we observed both synergetic as well as antagonistic responses, and neither phylum nor habitat explained trends in respiratory responses. Management plans should avoid the generalized assumption that combined stressors will results in multiplicative effects and focus attention on alleviating hypoxia in the region.

KEYWORDS: Hypoxia, acidification, low O_2, elevated pCO_2, high CO_2, low pH, respiration rate, invertebrates, Gullmar Fjord.

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1 Introduction

Resolving the responses of marine organisms to the multiple pressures associated with global change is a major challenge for marine scientists (Duarte et al., 2014). This challenge is particularly pressing for coastal ecosystems, where human populations and impacts often concentrate. Among these pressures, decreasing O_2 concentrations (hypoxia) and ocean acidification (OA) are receiving particular attention (Diaz and Rosenberg, 2008; Doney et al., 2009; Vaquer-Sunyer and Duarte, 2008; Kroeker et al., 2013). Whereas uptake of anthropogenic CO_2, is leading to decreased pH in the open ocean (Doney et al., 2009; Caldeira and Wickett, 2003), explaining fluctuations of pH in coastal waters is more complex, often involving metabolic processes (Duarte et al., 2013). The involvement of metabolic processes in the regulation of pH in coastal waters is particularly evident when eutrophication stimulates algal blooms, leading to increased sedimentation of organic matter, subsequently degraded through microbial respiration, thereby consuming O_2 and releasing CO_2 (Conley et al., 2009). Hence, elevated CO_2 through excess respiration is associated with reduced O_2 in coastal ecosystems, as these two gases are linked through metabolic processes. Indeed hypoxia is affecting a growing number of coastal ecosystems (Diaz and Rosenberg, 2008; Vaquer-Sunyer and Duarte, 2008), suggesting that eutrophication-driven acidification (Borges and Gypens, 2010; Cai et al., 2010) should be spreading as well. Whereas scientists have acknowledged this coupling over decades, the impacts of these two pressures have continued to be studied in isolation, although the synergistic effect of O_2 depletion and CO_2 accumulation is likely to provide a significant challenge to coastal invertebrates and mostly to sessile species.

The consequences of elevated pCO_2 for marine organisms reach further than the highly documented impacts on calcification rates (Doney et al., 2009). For example, extracellular acid-base regulation is a challenge as some organisms are unable to compensate for increased environmental acidity (e.g. Pane and Barry, 2007), which may lead to elevated CO_2 and low pH in their blood fluids, referred to as hypercapnia, reducing the affinity of haemoglobin for O_2 and further interfering with respiratory processes. Depending on the severity of the pH change, organisms can experience mortality and a range of sub-lethal effects such as increased respiration, reduced growth, behavioural changes such as compromised ability to avoid predators (see summary by Kroeker et al., 2013), and increased susceptibility to parasites (Burgents et al., 2005). Similarly, hypoxia has been shown to cause mortality, reduced growth and reproduction, decrease respiration rates, and induce behavioural changes such as forced migrations, which may make organisms more susceptible to predation (Vaquer-Sunyer and Duarte, 2008). In a review of 460 studies, half showed that 50% population mortality in response to low O_2 occurred within just five days (Vaquer-Sunyer and Duarte, 2008). There are growing concerns that the combined impacts of elevated pCO_2 and hypoxia may prove to be a more significant challenge for marine life that the predictions from isolated effects (Burnett, 1997; Brewer and Peltzer, 2009; Mayol et al., 2012; Melzner et al., 2013). While other studies have considered combined stressors such as low O_2 and high/low temperature (reviewed by Vaquer-Sunyer and Duarte, 2011), low O_2 and increased hydrogen sulphide (reviewed by Vaquer-Sunyer and Duarte, 2010), increased pCO_2 and temperature (Doney et al., 2009; Lischka et al., 2010), research focusing on how elevated pCO_2 and low O_2 will impact marine organisms has been of recent attention (e.g. Gobler et al., 2014; Steckbauer et al., 2015; Sui et al., 2016ab). For example, recent reports have shown that low O_2 and elevated pCO_2 can cause additive impacts for the growth and survivorship of bivalve larvae and later stage clams (Gobler et al., 2014), however similar research indicates some early life stage bivalves are largely tolerant of these combined effects (Frieder et al., 2014). Moreover, invertebrates along the coastline of Chile show rather additive than synergetic affects to the combination of low O_2 and elevated pCO_2 (Steckbauer et al., 2015).

Here we examine the independent and synergistic impacts of short-term elevated pCO_2 and low O_2 scenarios on the respiration of Skagerrak marine invertebrates at projected future levels, as down-regulation of metabolic rates has been proposed as a short-term evolutionary strategy to balance energy supply and demand when physiological processes are

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impaired by environmental stresses (Calosi et al., 2013). Elevated pCO_2 and low O_2 imposes a significant strain on aerobic animals as the available energy acquired from oxic respiration can be reduced in the presence of increased pCO_2 (Brewer and Peltzer, 2009). This energy could otherwise be directed towards growth, reproduction, and other biologically and ecologically important activities. Reduced respiration is known to occur during hypoxia and both increases and decreases have been observed when pH is reduced (e.g. Whiteley, 2011; Wei and Gao, 2012). However, responses are often highly species-specific (Fabry, 2008; Malakoff, 2012; Calosi et al., 2013). Reduced metabolism is a beneficial response for organisms in the short-term, however could become problematic over extended periods (Melzner et al., 2009; Rosa et al., 2013), as they may be unable to produce the energy required to support key processes.

We evaluated the respiratory responses of 11 species of Skagerrak marine invertebrates representing four phyla and contrasting habitats, such as shallow rocky shores, typically growing in Baltic waters, and deeper (about 30 m) muddy sediments in Atlantic waters, as well as behavioural strategies, including sessile (e.g. blue mussels) and mobile (e.g. hermit crab, sea starts and sea snails, Table 1). We used a two-way full factorial design enabling us to resolve additive and interactive effects. We hypothesize that responses could be driven by phyla and/or the habitat or niche the species occupy (Table 1). In particular, we expect all species to be able to cope with elevated pCO_2, as they experience broad fluctuations of pCO_2 in their habitat (Table 1), but should be vulnerable to hypoxia, as they experience high O_2 levels in their habitat, except for those with an infaunal growth habitat, which are expected to be resistant to low O_2 and elevated pCO_2, as in their habitat (Table 1). We also expect calcifiers to be particularly vulnerable to elevated pCO_2 as additional energy to support calcification is required to cope with the reduced saturation state of carbonate minerals associated with elevated pCO_2 (Hendriks et al., 2015).

2 Methods and Materials

2.1 Site and Location

The experiments were conducted during August 2013 at the Sven Lovén Centre for Marine Sciences in Kristineberg, of the University of Gothenburg, Sweden (58° 14' 58" N and 11° 26' 44" E). The centre provided access to a diversity of marine life as it is located at the mouth of the Gullmar Fjord. This fjord is home to a mix of habitats with varying complexity and a salinity gradient of three distinct water masses: 1) the surface layer from the Kattegat Sea (salinity 24 – 27); 2) the more saline mid-waters (32 - 33) from the Skagerrak; and 3) the salty (34.4) North Sea water mass in the deeper sections of the fjord (Polovodova et al., 2011). The fjord is home to a range of habitats such as sand and mud flats, seagrass beds, exposed and protected shorelines, and rocky bottoms, which together with the diversity of water masses results in high biodiversity (University of Gothenburg, 2011). Both natural and anthropogenically enhanced hypoxia occur within the fjord when enrichment is high and seasonal water exchange over the sill is slow (Josefson and Widbom, 1988; Arneborg, 2004).

2.2 Species, collection and maintenance

Specimens from 11 invertebrate species (Table 1) were collected from either surface or deep water within the Gullmar Fjord. *Ciona intestinalis* and *Littorina littorea* were collected by hand from mooring ropes and rocky shores,

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respectively, in the Grunsund boat harbour. *Asterias rubens* was also collected by hand from the rocky shore at the research station. All other specimens were retrieved with an Agassiz trawl aboard the research vessel *Oscar von Sydow* at up to 30 m depth over both rocky bottom and muddy sediment. *Amphuira filiformis* were collected with a 0.5 m sediment grab at 20 m depth. Only the top 10 cm of sediment from each grab was retained, as this was the oxygenated layer where organisms could be found. All organisms were maintained in flow-through tanks for at least two days before being placed into experimental aquaria. Water conditions followed the natural fluctuations occurring in the fjord (average pH ~ 8.0, salinity = 32.1 ± 0.02 ranging from 31.5 to 32.7, and temperature = 16˚C ± 0.06 ranging from 14.1 to 17.3˚C, data from http://www.weather.loven.gu.se/en/data).

Based on earlier experience in holding these species for experimental purposes, *Pagrus bernhardus* was fed, by allowing them to feed *ad libidum* on blue mussel meat, while being held in the tank prior to the experiment. No animals were fed during the experimental period. *C. intestinalis* and *L. littorea* were placed in plastic mesh cages (~ 0.5 cm^2) so that they were not lost through the outflow or escaped the aquarium. All gastropods, bivalves, and hermit crabs were cleaned with a toothbrush prior to use in order to remove any algae that could alter O_2 concentrations during measurements.

Invertebrates were exposed to one of the four treatments for a maximum of six days. Mortality events were rare across species (7 individuals died out of 168 used in the experiments) and insufficient to allow robust calculations of mortality rates. Of these seven, three specimens (one each of *P. bernhardus*, *P. miliaris,* and *A. rubens*) died at the same time in the same aquarium indicating that there was likely an anomaly in the tank, although we could not determine its nature. The other four specimens that experienced mortality were *A. rubens* under $L_{O2}L_{CO2}$, and *P. bernhardus, M. edulis*, and *A. filiformis* under $L_{O2}H_{CO2}$. Survivorship in the control was 100%, 97.6% in $L_{O2}L_{CO2}$, and 92.9% in the $H_{O2}H_{CO2}$ and $L_{O2}H_{CO2}$ treatment.

2.3 Treatment protocol

Four treatments (3 replica aquaria each) with two levels of dissolved oxygen (DO) and pCO_2 concentration were used: a) $H_{O2}L_{CO2}$ – ambient pCO_2 (400 µatm) and high O_2 (100% saturation or 9 - 10 mg L^{-1}); b) $L_{O2}L_{CO2}$ – ambient pCO_2 and low O_2 (20 - 35% saturation or 2 - 3.5 mg L^{-1}); c) $H_{O2}H_{CO2}$ – elevated pCO_2 (~1300 µatm) and high O_2; and d) $L_{O2}H_{CO2}$ – elevated pCO_2 and low O_2.

The high O_2 aquaria were bubbled with ambient air, whereas the low O_2 aquaria were bubbled with a mixture of air and N_2 using an Aalborg GFC17 Mass Flow Controller (MFC) and a jar filled with glass marbles (allowing even mixing of gases) to create a mixture with reduced O_2 content. This was then bubbled through the six low O_2 treatments maintaining the DO between 2.0 - 3.5 mg L^{-1}, which was chosen after Vaquer-Sunyer and Duarte (2008)'s meta-analysis to be a bit higher than the traditional definition of hypoxia by Diaz and Rosenberg (1995, 2008). The DO content of each aquaria was measured daily with PresSens oxygen micro-optodes (OXY 4 v2.11 Micro) that were calibrated in O_2 saturated deep-sea water (~10 mg DO L^{-1} for 100% DO) and a 1 g ml^{-1} sodium sulphite solution (0 mg DO L^{-1} for 0% DO).

To increase the pCO_2, pure CO_2 was bubbled through elevated pCO_2 aquaria. The low O_2 treatments also received CO_2 gas to maintain pCO_2 at an ambient level due to the displacement of CO_2 in the presence of N_2. A reduction of 0.4 pH units (equivalent to 1,300 µatm for elevated pCO_2) from the ambient waters (at ~450 µatm in low CO_2) was chosen. These values correspond to the annual average atmospheric pCO_2 level for the high-end projected level for 2100 (IPCC, 2007) and for 2005, respectively. pH was controlled with Aqua Medic pH computers and 2.5W M-ventil valves. Each pH controller had a sensor attached to the aquarium, which opened the valve to release a burst of CO_2 when the pH was increasing beyond the

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set level (i.e. 7.6 or 8.0). The pH_{NBS} (NBS scale) was measured daily of each aquaria with a Metrohm 827 pH meter, calibrated at 15˚C (with pH solutions of 3.99, 7.04, and 9.08).

Aquaria were continuously replenished by allowing water to flow through the tanks (filtered through a 20µm mesh) in a flow-through system with aquaria volume maintained at 17 L. Each replica aquaria held one individual from each species with the exception of *C. intestinalis* and *L. littorea*, which had two individuals per replica tank.

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2.4 Carbonate chemistry

Gran titration method was used to measure total alkalinity (TA) every third day. Two 25 ml water samples were collected from each aquarium and filtered through a 45 µm filter. TA was measured at room temperature with a SI Analytics TitroLine alpha plus machine and TitriSoft 2.6 software. Spectrophotometry was used to measure the pH_{TOT} (total scale) at 25 °C of treatments with a Perkin Elmer Lambda 25 UV/VIS spectrometer and Perkin Elmer UV WinLab software to confirm the values of the daily pH_{NBS} measurements (after Dickson, 2009). TA, pH_{NBS}, with temperature and salinity, were used to calculate the pCO_2, and aragonite and calcite saturation states (Ω_{arag} and Ω_{cal} respectively) in CO2SYS (Pierrot and Wallace, 2006) with K_1 and K_2 constants from Mehrbach et al. (1973; refit by Dickson and Millero, 1987) and KSO_4 from Dickson (1990).

Respiration Index (*RI*) was calculated after Brewer and Peltzer (2009) as

$$RI = \log_{10} (pO_2 / pCO_2) \quad \text{Eq. 1}$$

where $RI \leq 0$ corresponds to the thermodynamic aerobic limit, a formal dead zone; at $RI = 0$ to 0.4 aerobic respiration does not occur; the range $RI = 0.4$ to 0.7 represents the practical limit for aerobic respiration, and the range $RI = 0.7$ to 1.0 delimits the aerobic stress zone (Brewer and Peltzer 2009). Therefore, an *RI* less than 1.0 represents conditions in which organisms experience a physiological constraint on the free energy available to them to do work, with increasing severity of this constraint as the *RI* declines.

2.5 Metabolic rate

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Respiration was measured on day three and six of exposure (with the exception of *O. nigra* and *C. intestinalis* which were recorded on days two and four and three and five respectively) to detect any change in metabolic rate in response to short-term hypoxia, elevated pCO_2 water or both. Invertebrates were placed in pre-treated water for approximately five hours (depending on their size) in hermetically sealed containers. Oxygen was measured at the beginning and end of the incubation (max. 5.5 hours) using the PresSens micro-optodes. A blank sample was measured to see if there was any natural 'drift'. Initial and final measurements were used to calculate the consumption rate standardized to dry weight (DW) as mg L^{-1} O_2 min^{-1} L^{-1} g DW^{-1}. DW was measured after placing the individuals in the dry oven at 60˚C for at least 24 hours to remove any moisture. All weight measurements were recorded with a Mettler Toledo AT261 Delta Range analytical balance (readability 0.01 mg).

The response ratio of the respiration rate was calculated as the average metabolism in the experimental treatment (X_E), divided by the average metabolism in the control (X_C). The effect size for each treatment was the ln-transformed Respiration Rate (Kroeker et al., 2010):

$$\text{Ln Effect Size} = \text{LnRR} = \ln (X_E) - \ln (X_C), \quad \text{Eq. 2}$$

where X_E and X_C are the mean values of the response variable in the experimental and control treatments, respectively, where the control treatment was represented by the $H_{O2}L_{CO2}$ treatment. Bias-corrected bootstrapped 95% confidence interval was calculated after Hedges et al. (1999) and Gurevitch and Hedges (1999). The zero line indicates no effect, and significance of mean effects is determined when the 95% confidence interval does not overlap zero.

2.6 Data analyses

One-way ANOVA's were conducted to test for differences in the respiration rate between treatments for each species. As there was no significant difference between time (i.e. difference between day 3 and 6) all data from days three and six were pooled together. Where the respiration showed significant differences between treatments, a Student's t-test and post-hoc Tukey HSD test were conducted to resolve which treatments resulted in different respiration rates. A regression comparison was done to test the overall differences between the treatments. Moreover, a General Linear Model (GLM) was used to quantify species response to changes in pCO_2, oxygen and their interaction. A significant, positive interaction term indicates synergistic effects between the stressors, while a significant, but negative interaction term implies antagonistic effects, using the statistical software JMP (version 10.0; https://www.jmp.com) with the level for significance set at 0.05.

3 Results

3.1 Water conditions

The average measurements and calculated carbonate chemistry data for the experimental period are shown in Table 2. On average (±SE) the targeted pH levels of 8.04±0.07 in low pCO_2 treatments and 7.59±0.02 in the elevated pCO_2 treatments were achieved, respectively, and significantly different from each other ($p < 0.0001$, ANOVA). The corresponding atmospheric CO_2 levels were higher than our expected targets of 380 µatm ($H_{O2}L_{CO2}$ and $L_{O2}L_{CO2}$) and 1,000 ppm ($H_{O2}H_{CO2}$ and $L_{O2}H_{CO2}$).

The desired average (±SE) oxygen content of 9.51±0.05 mg L^{-1} for high oxygen treatments, and 2.98±0.15 mg L^{-1} for low oxygen treatments were also attained (Table 1; $p < 0.0001$, ANOVA). O_2 concentrations remained relatively stable for the $H_{O2}L_{CO2}$ and $H_{O2}H_{CO2}$ treatments (SE = 0.06 for both) where 100% saturation was targeted. DO concentrations in the $L_{O2}L_{CO2}$ and $L_{O2}H_{CO2}$ treatments were more variable ranging from 1.81 mg L^{-1} up to 3.88 mg L^{-1} over the course of the experiment. The pH was also most variable where manipulation was required in the $H_{O2}H_{CO2}$ (SD = 0.08 units) and $L_{O2}H_{CO2}$ (SD = 0.09 units) treatments, however there was also natural variation in the seawater as seen in the $H_{O2}L_{CO2}$ and $L_{O2}L_{CO2}$ treatments (SD = 0.28). Overall pH and O_2 level were quite well maintained around the targeted averages.

The *RI* averaged 1.60 ± 0.02 for the $H_{O2}L_{CO2}$, 1.15 ± 0.03 for the $L_{O2}L_{CO2}$, 1.14 ± 0.03 for the $H_{O2}H_{CO2}$ and 0.69 ± 0.04 for the $L_{O2}H_{CO2}$ treatment (Table 2). The *RI* values for the hypoxic and elevated pCO_2 treatment were similar as the differences in pO_2 and pCO_2 had a similar affect on *RI*. All treatments matched the target values and were held to an acceptable level and variability within each treatment (Table 2).

3.2 Respiration

Although the overall response was not significant for any experimental treatment ($p = 0.357$; ANOVA), when plotting the mean respiration rate of each species of the $H_{O2}L_{CO2}$ treatment versus the different experimental treatments (Fig. 1), results of regression analysis show that there is a significant difference between the 1:1 line in the $H_{O2}H_{CO2}$ treatment ($p < 0.05$; Regression comparison), whereas the other two treatments didn't differ significantly ($L_{O2}L_{CO2}$: $p = 0.701$; $L_{O2}H_{CO2}$: $p = 0.070$; regression comparison). When comparing results of the different habitats a significant difference between treatments and habitats was observed ($p < 0.01$; two-way ANOVA), as the result of the mooring was different from the other three habitats throughout treatments (Student's t).

The general trend for the $L_{O2}L_{CO2}$ and $L_{O2}H_{CO2}$ treatment was for organisms to reduce their metabolism, as metabolic rates for most species fell below the 1:1 line (Fig. 1). The metabolic rate for *C. intestinalis* under ambient conditions was over 2.5 times greater than that for any other species. Echinoderms generally displayed lower respiration rates, with the exception of *A. filiformis* who had comparatively high metabolism (Fig. 2). The three species of molluscs had similar metabolic rates, which differed amongst treatments.

When looking at the Ln Effect Size of each species separately, six of the 11 species tested experienced reduced respiration in response to the $L_{O2}L_{CO2}$ treatment compared to the $H_{O2}L_{CO2}$ treatment, with a further three species experiencing a trend towards reduced respiration (Fig. 2; Table 3). The species *A. filiformis* and *A. rubens* responded with increased respiration, although not significantly (Fig. 2). As for the $H_{O2}H_{CO2}$, six species increased respiration, with a significant difference in *O. fragilis* and *M. edulis*. The other five species responded with decreased metabolic rates (Fig. 2). The $L_{O2}H_{CO2}$

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treatment also had quite variable results with seven species experiencing lower respiration rates than the control, with significant differences in the species *A. rubens*, *A. filiformis*, *L. littorea* and *T. granifera* (Fig. 2). The majority of species exposed to $L_{O2}L_{CO2}$, $H_{O2}H_{CO2}$, and $L_{O2}H_{CO2}$ did not experience changes in respiration that differ significantly from those observed under $H_{O2}L_{CO2}$ conditions. This is confirmed by the results of the GLM (Table 3), which showed that the responses to oxygen and CO_2 are highly species specific, as we observed synergetic effects in only four out of 11 species (*O. fragilis*, *O. nigra*, *A. rubens* and *T. granifera*; Table 3).

4 Discussion

The Baltic species tested were highly resistant to short-term hypoxia and elevated pCO_2, alone or in combination, as they experienced very high survival rate across treatments in the relatively short-duration experiment reported here. Whereas lethal responses to elevated pCO_2 are seldom observed (Kroecker et al., 2013), the level of hypoxia imposed is sufficient to cause mortality of half of the populations of most marine species (Vaquer-Sunyer et al., 2008), with elevated pCO_2 expected to enhance respiratory stresses (Brewer and Peltzer, 2009). This suggests that the species tested have adapted to hypoxia and elevated pCO_2, which are experienced regularly in the ecosystem (Table 1), as more vulnerable species would have been already removed from the community.

The resistance of all species tested to short-term (3 to 6 day exposure) hypoxia, elevated pCO_2, and their combined effects, reflected in negligible mortality rates and modest metabolic responses, suggest that the community in the Gullmar Fjord have already been sieved to contain species and lineages resistant to these stressors, to which they have been exposed, at least for short periods of time, for generations (Josefson and Widbom, 1988; Arneborg, 2004). Whereas physiological limits to low O_2 concentrations seem unavoidable (Brewer and Peltzer, 2009), the existence of thresholds for elevated pCO_2 are less evident. Moreover, the fact that no or negligible mortality was experienced in our experiments at *RI*'s of 0.69, where Brewer and Peltzer (2009) predict the organisms to be severely compromised, in the thermodynamic limit of aerobic respiration, supports the idea that organisms have acclimatized to reoccurring events of low O_2 (and low pH), which are well documented within the Gullmar Fjord (Rosenberg, 1985; Johannessen and Einar, 1996; Nordberg et al., 2000; Polovodova and Nordberg, 2013). While there is a relatively long history of monitoring in the Gullmar Fjord, one of the longest-studied ecosystems in the world (seawater temperature records exist since the 1700's), pH data collection has been erratic and often only recorded at the surface (SMHI, 2011). However available data shows pH has fluctuated between 7.6 and 8.7 over the last six decades (Dorey et al., 2013).

Our experimental treatments explored a more limited range of O_2 and CO_2 than present across Gullmar Fjord. The community in this area has already been sieved of species vulnerable to low O_2 concentrations due to a history of hypoxia and even complete anoxia within the last four decades (Nordberg et al., 2000; Polovodova et al., 2011). Indeed, our $H_{O2}L_{CO2}$ values, involving saturating O_2 concentrations, are unlikely to be experienced at the fjords depths (Fig. 3). It is, therefore, possible that the low O_2 conditions better represented the environment in which the organisms were growing prior to the experiments. The experimental CO_2 values tested need also be compared with ambient levels. Dorey et al. (2013) found that pH in the Gullmar Fjord has varied between 8.7 and 7.6 over the last 66 years (average monthly fluctuation was 0.34 to 0.89 units). Therefore the minimum pH level conducive to a rise in pCO_2 to 1,000 µatm would be closer to 7.2. Dorey et al. (2013) conducted lab experiments with pH values as low as 6.5 for urchin larvae, which are generally more sensitive to pH change than adults (Dupont and Thorndyke, 2009). Exposed *A. filiformis* live in sediment burrows that experience much

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lower oxygen and higher pCO_2 than surrounding water which intensifies with depth (Hu et al., 2014). *A. filiformis* have been shown to withstand a pH of 7.0 and O_2 levels below 2.0 mg L^{-1} and experience no mortality (Hu et al., 2014). Hence, the species tested here already has O_2 and pH values comparable to those used as treatments here, particularly for infauna, such as *A. filiformis* and *B. lyrifera* which appear to be exposed to low O_2 and pH conditions on a regular basis.

Sublethal responses, in terms of metabolic depression or enhancement, were observed in response to hypoxia and elevated pCO_2, alone or in combination. We expected that $L_{O2}H_{CO2}$ would be the most significant respiratory stress for organisms, as it would affect all species except those with an infaunal growth habit, and thus would result in a reduced metabolism. However, only one species (*A. filiformis*) with an infaunal growth habit (Table 1) experienced a significantly reduced metabolism due the coupled impacts of H_{CO2} and L_{O2}. Two of the three species with an infernal growth habit showed no metabolic response to hypoxia, whereas all except two of the species growing in other habitats, generally experiencing high oxygen levels, experienced a metabolic depression under hypoxia. Whereas there were no consistent patterns in the responses across phyla, they showed consistency among habitats, reflecting the conditions the species were adapted to in their natural habitat.

There is growing interest in understanding the response of marine organisms to multiple stressors such as rising temperature, OA, increased UVB radiation, and reduced O_2 (e.g. Pörtner et al., 2005; Fredersdorf et al., 2009; Vaquer-Sunyer and Duarte, 2010; 2011, Duarte, 2014). For example, Mayol et al. (2012) examined the co-occurrence of low O_2 with elevated pCO_2 in the Pacific Ocean off the Chilean coast, identifying layers where both stressors co-occur. Yet, most experimental evidence of the response of marine invertebrates to stressors focus on individual effects, where their combined effects may differ from those derived (or calculated) from combinations of individual effects (cf. in Kroeker et al., 2013). Indeed, multiplicative, rather than additive, effects of the impacts of the individual stresses are expected (Pörtner et al., 2005; Clapham and Payne, 2011; Ateweberhan et al., 2013). However, our results demonstrate that there is a broad range of possible impacts within species from the Gullmar Fjord ecosystem including species that show an amplification of the responses beyond that expected under an additive model and those that show a buffering or compensation of responses when multiple stressors co-occur. The *A. rubens* exhibited a synergistic response to hypoxia and elevated pCO_2 as it showed a significant metabolic depression under both stressors, but no significant response to either one alone. The echinoderm *O. fragilis* experienced enhanced metabolic rates when exposed to elevated pCO_2, consistent with the sensitivity to elevated pCO_2 reported for their larvae, which experienced 100 % mortality when pH was reduced by just 0.2 units (Dupont and Thorndykee, 2008). In contrast, *M. edulis* experienced depressed metabolism when exposed to hypoxia. As a result, these effects operated into an antagonistic mode, resulting in no significant change in metabolic rates when the organisms were exposed to both hypoxia and elevated pCO_2. However, there was no general trend for responses to be either additive or synergistic across species. Indeed, our result suggests that responses are mostly dependent on the fluctuations in the stressors in their habitats, so that the prior selective and adaptive history of the species plays an important role in determining their vulnerability to different stressors.

Whereas a theoretical framework to predict the response of marine organisms to multiple stressors is generally lacking, Brewer and Pelzer (2009) derived a theoretical expectation of the expected responses in the particular case of combined hypoxia and high CO_2, the organisms tested show a *RI* decrease with intensity of alterations in our treatments as expected. Although we reached the 0.7 threshold value (0.69 under $L_{O2}H_{CO2}$), which represents the thermodynamic limit for aerobic respiration, the organisms are expected to be severely compromised. Yet, we observed little or no mortality and the organisms exposed to $L_{O2}H_{CO2}$ should have experienced aerobic stress, yet, our results showed that they were more likely to reduce respiration under hypoxia. Hence, the *RI* does not appear to hold predictive power as to the response of marine

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invertebrates to the interactions between O_2 and CO_2. All but one of the tested species were calcifiers, and were expected to be impacted by elevated pCO_2. Indeed, the treatment with elevated pCO_2 reached an undersaturated concentration of aragonite ($\Omega_{arag} < 1$), where calcifiers are expected to be stressed (Doney et al., 2009). Molluscs rely chiefly on aragonite to construct their shells (Porter, 2007), while echinoderms and crustaceans use calcite (Raup, 1959; Raabe et al., 2005). Yet, the impacts of elevated pCO_2 were not greater in molluscs than for echinoderms and crustaceans in our experiments. Hence, neither the RI_{crit} does not hold predictive power on the effects of hypoxia and/or pCO_2 on the species tested here, which seemed best predicted from consideration of the ranges of O_2 and CO_2 they experience in their habitat.

Responses to low O_2 and elevated pCO_2 were variable amongst phyla and species in the community tested here, ranging from antagonistic to synergistic responses. The very limited impacts of low O_2 and elevated pCO_2 of the invertebrates from this ecosystem, which showed little or no mortality in the presence of both stressors, reflects the range of conditions in the habitats these organisms occupy. This ecosystem has been reported to experience recurrent seasonal hypoxic events characterised by low pH values and elevated pCO_2 (Nordberg et al., 2000; Dorey et al., 2013). Hence, the organisms tested were resistant to both stressors within the levels used in this experiment, which, while ranging within values reported to negatively impact on marine invertebrates for both O_2 (Vaquer-Sunyer and Duarte, 2008) and CO_2 (Kroeker et al., 2013), were within the range present in their ecosystem. Eutrophication-driven hypoxia, such as experienced in Baltic fjords, derives from excess metabolic O_2 consumption and is, therefore, coupled with elevated pCO_2 (e.g. Duarte et al., 2013, Melzner et al., 2013, Wallace et al., 2014). Hence, low O_2 and elevated pCO_2 often co-occur in areas affected by hypoxic events, such as Gullmar Fjord. Haselmair et al. (2010) observed that pH declined by up to 0.7 units during an induced anoxic event in the Adriatic Sea and Melzner et al. (2013) predict that pCO_2 can reach up to 3,200 µatm during anoxic events in brackish waters (salinity of 20), with those values decreasing as salinity increases. Hence, adaptive responses of organisms in the Gullmar Fjord should be coupled for low O_2 and elevated pCO_2, thereby accounting for the limited effects to the experimentally imposed stressors used here.

5 Conclusions

Respiratory responses to low O_2 and elevated pCO_2 were variable amongst phyla and species in the community tested here, ranging from buffered to amplified metabolic responses. The very limited impacts of low O_2 and elevated pCO_2 of the invertebrates from this ecosystem, which showed little or no mortality in the presence of both stressors, reflects the past history of this ecosystem, which has been reported to experience recurrent hypoxic events characterised by low pH values and elevated pCO_2 (Nordberg et al., 2000; Dorey et al., 2013). Hence, the organisms trialled were resistant to both stresses within the levels used in this experiment, which were within values reported to negatively impact on marine invertebrates for both O_2 (Vaquer- Sunyer and Duarte, 2008) and CO_2 (Kroeker et al., 2013). Hypoxia impacted the greatest number of organisms and represents, therefore, the most concerning stress in the region. Management plans addressing hypoxia should also avoid the generalized assumption that synergistic stressors will result in multiplicative effects and focus research into understanding the mechanisms calcifiers and other invertebrates employ to cope with these changes. Our results also highlight the idiosyncratic nature of responses, which were strongly species-specific, suggesting that extrapolations from experiments conducted on a few species to the phylum level may be strongly misleading. This adds complexity to the challenge of predicting how global stressors will affect marine ecosystems in the future.

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Author contribution

design of the experiment: AF, AS, CMD

experimental part: AF, AS, SD

analysis: AF, AS, SD, CMD

writing: AF, AS, SD, CMD

Competing interests

The authors declare that they have no conflict of interest.

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References

Arneborg, L.: Turnover times for the water above sill level in Gullmar Fjord, Cont Shelf Res, 24, 443 - 460, 10.1016/j.csr.2003.12.005, 2004.

Ateweberhan, M., Feary, D. A., Keshavmurthy, S., Chen, A., Schleyer, M. H., and Sheppard, C. R. C.: Climate change impacts on coral reefs: Synergies with local effects, possibilities for acclimation, and management implications, Mar Pollut Bull, 74, 526 - 539, 10.1016/j.marpolbul.2013.06.011, 2013.

Borges, A. V., & Gypens, N.: Carbonate chemistry in the coastal zone responds more strongly to eutrophication than to ocean acidification. Limnol Oceanogr, 55, 346-353. 10.4319/lo.2010.55.1.0346, 2010.

Brewer, P., and Peltzer, E.: Limits to Marine Life, Science, 324, 347 - 348, 10.1126/science.1170756, 2009.

Burgents, J. E., Burnett, K. G., and Burnett, L. E.: Effects of hypoxia and hypercapnic hypoxia on the localization and the elimination of *Vibrio campbellii* in *Litopenaeus vannamei*, the Pacific white shrimp, Biol Bull, 208, 159 - 168, 2005.

Burnett, L.: The challenges of living in hypoxic and hypercapnic aquatic environments, Amer Zool, 37, 633 - 640, 1997.

Cai, W., Hu, X., Huang, W., Murrell, M. C., Lehrter, J. C., Lohrenz, S. E., and Gong, G.: Acidification of subsurface coastal waters enhanced by eutrophication. Nat Geosci, 4, 766-770. 10.1038/ngeo1297, 2011.

Caldeira, K., and Wickett, M.: Anthropogenic carbon and ocean pH, Nature, 425, 365, 2003.

Calosi, P., Rastrick, S., Lombardi, C., H.J., d. G., Davidson, L., Jahnke, M., Giangrande, A., Hardege, J. D., Schulze, A., Spicer, J. I., and Gambi, M. C.: Adaptation and acclimatization to ocean acidification in marine ectotherms: an in situ transplant experiment with polychaetes at a shallow CO_2 vent system, Phil Trans R Soc B, 368, 1 - 15, 10.1098/rstb.2012.0444, 2013.

Clapham, M. E., and Payne, J. L.: Acidification, anoxia, and extinction: A multiple logistic regression analysis of extinction selectivity during the Middle and Late Permian, Geology, 39, 1059 - 1062, 10.1130/G32230.1, 2011.

Conley, D.: Save the Baltic Sea, Nature, 486, 463 - 464, 10.1038/486463a, 2012.

Diaz, R., and Rosenberg, R.: Marine Benthic Hypoxia: A review of its ecological effects and the behavioural responses of benthic macrofauna, Oceanogr Mar Biol, 33, 245 - 303, 1995.

Diaz, R. J., and Rosenberg, R.: Spreading dead zones and consequences for marine ecosystems, Science, 321, 926 - 929, 10.1126/science.1156401, 2008.

Dickson, A. G., and Millero, F. J.: A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media Deep-Sea Res Pt I, 34, 1733 - 1743, 1987.

Dickson, A. G.: Standard potential of the reaction: $AgCl_{(s)} + {}^1/_2H_{2(g)} = Ag_{(s)} + HCl_{(aq)}$, and the standard acidity constant of the ion HSO_4^- in synthetic sea water from 273.15 to 318.15 K, J Chem Themodyn, 22, 113 - 127, 1990.

Doney, S., Fabry, V., Feely, R., and Kleypas, J.: Ocean Acidification: The Other CO_2 Problem, Annu Rev Mar Sci, 1, 169 - 192, 10.1146/annurev.marine.010908.163834, 2009.

Dorey, N., Lancon, P., Thorndyke, M., and Dupont, S.: Assessing physiological tipping point of sea urchin larvae exposed to a broad range of pH, Glob Change Biol, 19, 3355 - 3367, 10.1111/gcb.12276, 2013.

Duarte, C.M. Global Change and the Future Ocean: A Grand Challenge for Marine Sciences. Frontiers in Marine Sciences, doi: 10.3389/fmars.2014.00063, 2014.

Field Code Changed

Deleted: Alldredge, A. L., and Madin, L. P.: Pelagic Tunicates: Unique Herbivores in the Marine Plankton Bioscience, 32, 655 - 663, 1982.¶
Anderson, P., Håkansson, B., Håkansson, J., Sahlsten, E., Havenhand, J., Thorndyke, M., and Dupont, S.: Marine Acidification - On effects and monitoring of marine acidification in the seas surrounding Sweden, Swedish Meteorological and Hydrological Institute, Gothenburg, Sweden, 2008.¶

Deleted: Berteaux, D., Reale, D., McAdam, A., and Boutin, S.: Keeping pace with fast climate change: can arctic life count on evolution?, Integr Comp Biol, 44, 140 - 151, 2004.¶

Deleted: Canadell, P., Lequre, C., Raupach, M. R., Ciais, P., Conway, T., Field, C. B., Houghton, S., and Marland, G.: Global carbon sources and sinks: 2007 update, OP Conference Series: Earth Environ Sci, 10.1088/1755-1307/6/8/082001, 2009.¶
Carpenter, J.: New Measurements of Oxygen Solubility in Pure and Natural Water, Limnol Oceanogr, 11, 264 - 277, 1966.¶

Duarte, C., Hendriks, I., Moore, T., Olsen, Y., Steckbauer, A., Ramajo, L., Carstensen, J., Trotter, J., and McCulloch, M.: Is Ocean Acidification an Open-Ocean Syndrome? Understanding Anthropogenic Impacts on Seawater pH, Estuar Coast, 36, 221 - 236, 10.1007/s12237-013-9594-3, 2013.

Dupont, S., and Thorndyke, M. C.: Ocean acidification and its impact on the early life-history stages of marine animals, CIESM, Impacts of acidification on biological, chemical and physical systems in the Mediterranean and Black Seas. N° 36 in *CIESM Workshop Monographs* [F. Briand Ed.], Monaco, 89 - 97, 2008.

Fabry, V. J.: Marine Calcifiers in a High-CO_2 Ocean, Science, 320, 1020 - 1022, 10.1126/science.1157130, 2008.

Fredersdorf, J., Müller, R., Becker, S., Wiencke, C., and Bischof, K.: Interactive effects of radiation, temperature and salinity on different life history stages of the Arctic kelp *Alaria esculenta* (Phaeophyceae), Oecologia, 160, 483 - 492, 10.1007/s00442-009-1326-9, 2009.

Frieder, C., Gonzalez, J., Bockmon, E., Navarro, M., and Levin, L.: Can variable pH and low oxygen moderate ocean acidification outcomes for mussel larvae?, Glob Change Biol, 20, 754 - 764, 10.1111/gcb.12485, 2014.

Gobler, C., DePasquale, E., Griffith, A., and Baumann, H.: Hypoxia and Acidification Have Additive and Synergistic Negative Effects on the Growth, Survival, and Metamorphosis of Early Life Stage Bivalves, PLoS ONE, 9, 1 - 10, 10.1371/journal.pone.0083648, 2014.

Gurevitch, J., and Hedges, L. V. 1999. Statistical issues in ecological meta-analyses. Ecology, 80: 1142–1149.

Haselmair, A., Stackowitsch, M., Zuschin, M., and Riedel, B.: Behaviour and mortality of benthic crustaceans in response to experimentally induced hypoxia and anoxia *in situ*, Mar Ecol Prog Ser, 414, 195 - 208, 10.3354/meps08657, 2010.

Hendriks, I. E., Duarte, C. M., Olsen, Y. S., Steckbauer, A., Ramajo, L., Moore, T. S., McCulloch, M. Biological mechanisms supporting adaptation to ocean acidification in coastal ecosystems. Estuar Coast Shelf S, 152, A1-A8. http://dx.doi.org/10.1016/j.ecss.2014.07.019, 2015.

Hedges, L. V, Gurevitch, J., Curtis, P. S., and Jun, N. 1999. The Meta-Analysis of Response Ratios in Experimental Ecology. Ecology, 80: 1150–1156.

Hu, M., Casties, I., Stumpp, M., Ortega-Martinez, O., and Dupont, S.: Energy metabolism and regeneration impaired by seawater acidification in the infaunal brittlestar, *Amphiura filiformis*, J Exp Biol, 10.1242/jeb.100024, 2014.

IPCC: Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change Cambridge University Press, Cambridge, 2007.

Johannessen, T., and Einar, D.: Declines in oxygen concentrations along the Norwegian Skagerrak coast, 1927-1993: A signal of ecosystem changes due to eutrophication, Limnol Oceanogr, 41, 766 - 788, 1996.

Josefson, A., and Widbom, B.: Differential response of benthic macrofauna and meiofauna to hypoxia in the Gullmar Fjord basin, Mar Biol, 100, 31 - 40, 10.1007/BF00392952, 1988.

Kroeker, K., Kordas, R., Crim, R., and Singh, G.: Meta-analysis reveals negative yet variable effect of ocean acidification on marine organisms, Ecol Lett, 13, 1419 - 1434, 10.1111/j.1461-0248.2010.01518.x, 2010.

Kroeker, K. J., Kordas, R. L., Crim, R. N., Hendriks, I. E., Ramajo, L., Singh, G. S., Duarte, C. M., and Gattuso, J.-P.: Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming, Glob Change Biol, 19, 1884 - 1896, 10.1111/gcb.12179, 2013.

Lindahl, O., Lundve, B., and Johansen, M.: Toxicity of *Dinophysis* spp. in relation to population density and environmental conditions on the Swedish west coast, Harmful Algae, 6, 218 - 231, 10.1016/j.hal.2006.08.007, 2007.

Deleted: HELCOM: Baltic Sea Action Plan, HELCOM Ministerial Meeting, Krakow, Poland, 2007, ¶
Hu, M., Casties, I., Stumpp, M., Ortega-Martinez, O., and Dupont, S.: Energy metabolism and regeneration impaired by seawater

Deleted: a

Deleted: idification in the infaunal brittlestar, *Amphiura filiformis*, J Exp Biol, 10.1242/jeb.100024, 2014.¶
IPCC: Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change Cambridge University Press, Cambridge, 2007.¶
IPCC: Climate Change 2013: The Physical Science Basis: Working Group I Contribution to the IPCC Fifth Assessment Report of the Intergovernmental Panel on Climate Change, UNEP and WHO, *Online*, 2013.¶

Deleted: Kurihara, H.: Effects of CO_2-driven ocean acidification on the early developmental stages of invertebrates, Mar Ecol Prog Ser, 373, 275 - 284, doi: 10.3354/meps07802, 2008.¶

Lischka, S., Budenbender, J., Boxhammer, T., and Riebesell, U.: Impact of ocean acidification and elevated temperatures on early juveniles of the polar shelled oteropod *Limacina helicina* mortality, shell degradation, and shell growth, Biogeosciences Discuss, 7, 8177 - 8214, 10.5194/bgd-7-8177-2010, 2010.

Malakoff, D.: Researchers Struggle to Assess Responses to Ocean Acidification Science, 338, 27 - 28, 10.1126/science.338.6103.27, 2012.

Mayol, E., Ruiz-Halpern, S., Duarte, C., Castilla, J., and Pelegri, J.: Coupled CO_2 and O_2-driven compromises to marine life in summer along the Chilean sector of the Humboldt Current System, Biogeosciences, 9, 1183 - 1194, 10.5194/bg-9-1183-2012, 2012.

Mehrbach, C., Culberson, C. H., Hawley, J. E., and Pytkowicz, R. M.: Measurement of the Apparent Dissociation Constant of Carbonic Acid in Seawater at Atmospheric Pressure, Limnol Oceanogr, 18, 897 - 907, 1973.

Melzner, F., Gutowska, M. A., Langenbuch, M. D., S., Lucassen, M., Thorndyke, M. C., Bleich, M., and Pörtner, H. O.: Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny?, Biogeosciences, 6, 2313 - 2331, 10.5194/bgd-6-4693-2009, 2009.

Melzner, F., Thomsen, J., Koeve, W., Oschlies, A., Gutowska, M. A., Bange, H. W., Hansen, H. P., and Kortzinger, A.: Future ocean acidification will be amplified by hypoxia in coastal habitats, Mar Biol, 160, 1875 - 1888, 10.1007/s00227-012-1954-1, 2013.

Nordberg, K., Gustafsson, M., and Krantz, A.-L.: Decreasing oxygen concentrations in the Gullmar Fjord, Sweden, as confirmed by benthic foraminifera, and the possible association with NAO, J Marine Syst, 23, 303 - 316, 2000.

Pane, E. F., and Barry, J. P.: Extracellular acid–base regulation during short-term hypercapnia is effective in a shallow-water crab, but ineffective in a deep-sea crab, Mar Ecol Prog Ser, 334, 1 - 9, 10.3354/meps334001, 2007.

Pierrot, D. L. E., and Wallace, D. W. R.: MS Excel Program Developed for CO_2 System Calculations. ORNL/CDIAC-105a. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, Tennessee, 2006.

Polovodova, A., Nordberg, K., and Filipsson, H.: The benthic foraminiferal record of the Medieval Warm Period and the recent warming in the Gullmar Fjord, Swedish west coast, Mar Micropaleontol, 81, 95 - 106, 10.1016/j.marmicro.2011.09.002, 2011.

Polovodova, A., and Nordberg, K.: Foraminiferal fauna from a deep basin in Gullmar Fjord: The influence of seasonal hypoxia and North Atlantic Oscillation, J Sea Res 79, 40 - 49, 10.1016/j.seares.2013.02.001, 2013.

Porter, S. M.: Seawater Chemistry and Early Carbonate Biomineralization, Science, 316, 1302, 10.1126/science.1137284, 2007.

Pörtner, H. O., Langenbuch, M., and Michaelidis, B.: Synergistic effects of temperature extremes, hypoxia, and increases in CO_2 on marine animals: From Earth history to global change, J Geophys Res-Oceans, 110, 1 - 15, 10.1029/2004JC002561, 2005.

Raabe, D., Sachs, C., and Romano, P.: The crustacean exoskeleton as an example of a structurally and mechanically graded biological nanocomposite material, Acta Mater, 53, 4281 - 4292, 10.1016/j.actamat.2005.05.027, 2005.

Raup, D. M.: Crystallography of Echinoid Calcite J Geol, 67, 661 - 674, 10.1086/626624 1959.

Rosa, R., Trübenbach, K., Repolho, T., Pimentel, M., Faleiro, F., Boavida-Portugal, J., Baptista, M., Lopes, V., Dionisio, G., Leal, M. C., Calado, R., and Pörner, H. O.: Lower hypoxia thresholds of cuttlefish early life stages living in a warm acidified ocean, Proc. R. Soc. B, 280, 1 - 7, 10.1098/rspb.2013.1695 2013.

Rosenberg, R.: Eutrophication - The future marine coastal nuisance?, Mar Pollut Bull, 16, 227 - 231, 1985.

Deleted: Melatunan, S., Calosi, P., Rundle, S. D., Moody, A. J., and Widdicombe, S.: Exposure to Elevated Temperature and pCO_2 Reduced Respiration Rate and Energy Status in the Periwinkle *Littorina littorea*, Physiol Biochem Zoo, 84, 583 - 594, 10.1086/662680, 2011.¶

Deleted: Michaelidis, B., Ouzounis, C., Palera, A., and Pörtner, H. O.: Effects of long-term hypercapnia on acid-base balance and growth rate in marine mussels *Mytilus galloprovincialis*, Mar Ecol Prog Ser, 293, 109 - 118, 10.3354/meps293109, 2005.¶

Deleted: Pespeni, M. H., Sanford, E., Gaylord, B., Hill, T. M., Hosfelt, J. D., Jaris, H. K., LaVigne, M., Lenz, E. A., Russel, A. D., Young, M. K., and Palumbi, S. R.: Evolutionary change during experimental ocean acidification, PNAS, 110, 6937 - 6942, 10.5061/dryad.6j51n., 2013.¶

SMHI: Swedish Oceanographic Data Centre, Swedish Meteorological and Hydrological Institute, Sweden, 2011.

Steckbauer, A., Ramajo, R., Hendriks, I. E., Fernandez, M., Lagos, N., Prado, L., and Duarte, C. M.: Synergetic effects of hypoxia and increasing CO_2 on benthic invertebrates of the central Chilean coast, Front Mar Sci, 2, 10.3389/fmars.2015.00049, 2015.

Sui, Y., Kong, H., Huang, X., Dupont, S., Hu, M., Storch, D., Pörtner, H.-O., et al. 2016a. Combined effects of short-term exposure to elevated CO_2 and decreased O_2 on the physiology and energy budget of the thick shell mussel *Mytilus coruscus*. Chemosphere, 155: 207–216. http://www.ncbi.nlm.nih.gov/pubmed/27115845 (Accessed 1 May 2016).

Sui, Y., Kong, H., Shang, Y., Huang, X., Wu, F., Hu, M., Lin, D., *et al.* 2016b. Effects of short-term hypoxia and seawater acidification on hemocyte responses of the mussel *Mytilus coruscus*. Marine pollution bulletin: 1–7. Elsevier Ltd. http://www.ncbi.nlm.nih.gov/pubmed/27207025 (Accessed 26 May 2016).

The Sven Lovén Centre for Marine Sciences: Kristineberg: http://www.assemblemarine.org/the-sven-lov-n-centre-for-marine-sciences-kristineberg/, 2017.

Vaquer-Sunyer, R., and Duarte, C.: Thresholds of hypoxia for marine biodiversity PNAS, 105, 15452 - 15457, 10.1073/pnas.0803833105, 2008.

Vaquer-Sunyer, R., and Duarte, C.: Sulfide exposure accelerates hypoxia-driven mortality, Limnol Oceanogr, 55, 1075 - 1082, 10.4319/lo.2010.55.3.1075, 2010.

Vaquer-Sunyer, R., and Duarte, C.: Temperature effects on oxygen thresholds for hypoxia in marine benthic organisms, Glob Change Biol, 17, 1788 - 1797, 10.1111/j.1365-2486.2010.02343.x, 2011.

Wallace, R. B., Baumann, H., Grear, J. S., Aller, R. C., & Gobler, C. J. Coastal ocean acidification: The other eutrophication problem. Estuar Coast Shelf S, 148, 1-13. 10.1016/j.ecss.2014.05.027, 2014

Wei, L., and Gao, K.: A marine secondary producer respires and feeds more in a high CO_2 ocean, Mar Pollut Bull, 64, 699 - 703, 10.1016/j.marpolbul.2012.01.033, 2012.

Whiteley, N.: Physiological and ecological responses of crustaceans to ocean acidification, Mar Ecol Prog Ser, 430, 257 - 271, 10.3354/meps09185, 2011.

Deleted: Sabine, C. L., Feely, R. A., Gruber, N., Key, R. M., Lee, K., Bullister, J. L., Wanninkhof, R., Wong, C. S., Wallace, D. W. R., Douglas, W. R., Tilbrook, B., Millero, F. J., Peng, T.-H., Kozyr, A., Ono, T., and Rios, A. F.: The oceanic sink for anthropogenic CO_2, Science, 305, 367 - 371, 2004.¶
Seibel, B., Girguis, P., and Childress, J.: E-Letter: Critique of Respiration Index, Science, 324, 347 - 348, 2009.¶
Seibel, B., and Childress, J.: The real limits to marine life: a further critique of the Respiration Index, Biogeosciences, 10, 2815 - 2819, 10.5194/bg-10-2815-2013, 2013.¶

Deleted: Somero, G. N.: The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers', J Exp Biol, 213, 912 - 920, 10.1242/jeb.037473, 2010.¶

Deleted: Thomsen, J., and Melzner, F.: Moderate seawater acidification does not elicit long-term metabolic depression in the blue mussel *Mytilus edulis*, Mar Biol, 157, 2667 - 2676, 10.1007/s00227-010-1527-0, 2010.

Deleted: ¶

Deleted: Wood, H. L., Spicer, J. I., and Widdicombe, S.: Ocean acidification may increase calcification rates, but at a cost, P Roy Soc B-Biol Sci, 275, 1767 - 1773, 10.1098/rspb.2008.0343, 2008.¶

Table Headings

Table 1. Species used in the experiment along with the habitat where they were collected and the characteristic pH and O_2 levels in these habitats. pH and O_2 values at the habitats from Dorey et al. (2013), Hu et al. (2014), and Grans et al. (2014).

Table 2. Realised carbonate chemistry and oxygen concentrations for the four treatments ($H_{O2}L_{CO2}$, $L_{O2}L_{CO2}$, $H_{O2}H_{CO2}$, and $L_{O2}H_{CO2}$). Values are averages ± SE of measurements and calculations (using CO2SYS). Respiration Index (RI) as defined by Brewer and Peltzer (2009) (see section 2.4 for details).

Table 3. Respiration Rate in mg L^{-1} O_2 min^{-1} L^{-1} g DW^{-1} (± SE) and results of the General Linear Model (GLM) off all tested species (pooled data where we had data of different days). Levels not connected by the same letter are significantly different (after Student's T and Tukey HSD tests). Numbers written in red color highlight significant differences.

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Table 1

Species	Sampling site	pH	Oxygen (mg L^{-1})
Ciona intestinalis	Mooring rope, surface	Highly variable (8.7-7.6)	High (8)
Pagurus bernhardus	Gravels, 30m	Variable (8.1-7.7)	High (8)
Littorina littorea	Rocky shore, surface	Highly variable (8.7-7.6)	High (8)
Tarebia granifera	Soft sediment, 30m, infaunal	Low (7.6)	Low (1.6)
Mytilus edulis	Rocky shore, surface	Highly variable (8.7-7.6)	High (8)
Ophiocomina nigra	Gravels, 30m	Variable (8.1-7.7)	High (8)
Ophiothrix fragilis	Gravels, 30m	Variable (8.1-7.7)	High (8)
Amphiura filiformis	Soft sediment, 30m, infaunal	Low (7.6)	Low (1.6)
Asterias rubens	Rocky shore, surface	Highly variable (8.7-7.6)	High (8)
Psammechinus miliaris	Gravels, 30m	Variable (8.1-7.7)	High (8)
Brissopsis lyrifera	Soft sediment, 30m, infaunal	Low (7.6)	Low (1.6)

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Table 2

	$H_{O2}L_{CO2}$		$L_{O2}H_{CO2}$		$H_{O2}H_{CO2}$		$L_{O2}H_{CO2}$	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Temperature (°C)	15.4	0.2	15.4	0.2	15.4	0.2	15.4	0.2
Oxygen (mg L^{-1})	9.43	0.06	2.91	0.20	9.60	0.06	3.05	0.24
pH_{NBS}	8.02	0.01	8.06	0.02	7.58	0.03	7.61	0.04
Salinity	32.16	0.12	32.16	0.12	32.16	0.12	32.16	0.12
Total Alkalinity (µmol kg^{-1})	2245.9	11.2	2249.5	9.1	2249.5	7.2	2258.8	11.5
pCO_2 (µatm)	591.9	22.4	536.7	26.6	1783.5	131.7	1687.7	146.7
HCO_3^- (µmol kg^{-1})	1975.4	15.9	1951.8	16.7	2143.6	9.0	2144.8	16.6
CO_3^{2-} (µmol kg^{-1})	111.3	3.0	120.4	4.1	44.5	2.9	47.6	3.5
Ω Aragonite	1.73	0.05	1.87	0.06	0.69	0.04	0.74	0.05
Ω Calcite	2.71	0.07	2.93	0.10	1.08	0.07	1.16	0.08
RI	1.60	0.02	1.15	0.03	1.14	0.03	0.69	0.04

Table 3.

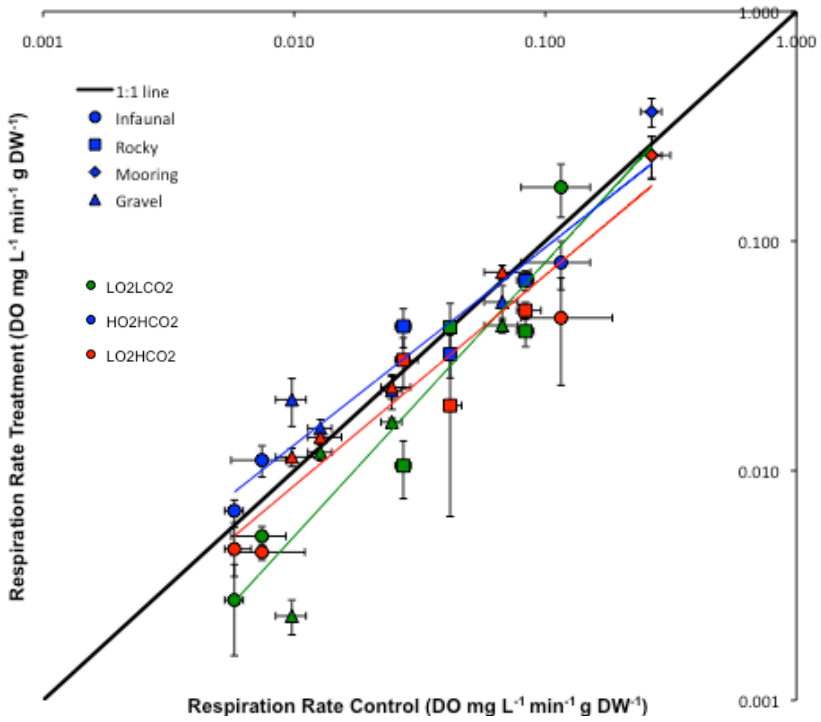
Species	Taxa	day	Prob. > F		average respiration rate (± SE) $H_{O2}L_{CO2}$	$L_{O2}L_{CO2}$	$H_{O2}H_{CO2}$	$L_{O2}H_{CO}$	General Linear Model (GLM) $CO_2{*}O_2$
Pagurus bernhardus *n = 12*	Crustacean	3/6	0.0417	Average	0.067	0.043	0.054	0.073	-0.0434
				(± SE)	0.010	0.003	0.010	0.005	
				Students' T	A	B	AB	A	
				Tukey HSD	AB	B	AB	A	
Ciona intestinalis *n = 24*	Tunicata	3/5	0.1578	Average	0.265	0.236	0.366	0.236	0.1001
				(± SE)	0.025	0.050	0.052	0.049	
				Students' T	A	A	A	A	
				Tukey HSD	A	A	A	A	
Brissopsis lyrifera *n = 12*	Echinoidea	3	0.0715	Average	0.0058	0.0027	0.0067	0.0046	-0.0009
				(± SE)	0.0005	0.0012	0.0008	0.0011	
				Students' T	A	B	A	AB	
				Tukey HSD	A	A	A	A	
Psammechinus miliaris *n = 12*	Echinoidea	3/6	0.1202	Average	0.024	0.016	0.022	0.023	-0.0090
				(± SE)	0.002	0.001	0.004	0.003	
				Students' T	A	B	AB	AB	
				Tukey HSD	A	A	A	A	
Amphiura filiformis *n = 12*	Echinoidea	3	0.1678	Average	0.115	0.172	0.081	0.046	0.0904
				(± SE)	0.035	0.045	0.019	0.023	
				Students' T	AB	A	AB	B	
				Tukey HSD	A	A	A	A	
Ophiothrix fragilis *n = 12*	Echinoidea	3	0.0023	Average	0.0098	0.0023	0.0204	0.0115	0.0015
				(± SE)	0.0014	0.0004	0.0048	0.0011	
				Students' T	B	C	A	B	
				Tukey HSD	BC	C	A	AB	
Ophiocomina nigra *n = 24*	Echinoidea	3 2/4/6	0.2054	Average	0.013	0.012	0.015	0.014	0.0006
				(± SE)	0.001	0.001	0.001	0.001	
				Students' T	AB	B	A	AB	
				Tukey HSD	A	A	A	A	
Mytilus edulis *n = 12*	Bivalve	3/6	0.0063	Average	0.027	0.011	0.043	0.030	-0.0045
				(± SE)	0.002	0.003	0.008	0.008	
				Students' T	A	B	A	A	
				Tukey HSD	AB	B	A	AB	
Asterias rubens *n = 12*	Gastropoda	3	0.3302	Average	0.042	0.042	0.032	0.019	0.0133
				(± SE)	0.002	0.011	0.007	0.013	
				Students' T	A	A	A	A	
				Tukey HSD	A	A	A	A	
Littorina littorea *n = 24*	Gastropoda	3/6	< 0.0001	Average	0.083	0.040	0.067	0.050	-0.0254
				(± SE)	0.006	0.006	0.007	0.004	
				Students' T	A	B	A	B	
				Tukey HSD	A	C	AB	BC	
Tarebia granifera *n = 12*	Gastropoda	3/6	0.0073	Average	0.007	0.005	0.011	0.004	0.0044
				(± SE)	0.002	0.001	0.002	0.000	
				Students' T	AB	B	A	B	
				Tukey HSD	AB	B	A	B	

Figure Headings

Figure 1. Respiration rate (Average ± SE) control vs. treatments of all tested species: green – $L_{O2}L_{CO2}$ (y = 0.9571x – 0.0046, R^2 = 0.90), blue – $H_{O2}H_{CO2}$ (y = 1.2905x – 0.0122, R^2 = 0.93) and red – $L_{O2}H_{CO2}$ (y = 0.8301x – 0.0032, R^2 = 0.92). The 1:1 line represents where treatment metabolism is equal to ambient metabolism.

Figure 2. The Ln Effect Size of the response ratios for invertebrate species and phyla in response to three treatments: low O_2 ($L_{O2}L_{CO2}$), low pH ($H_{O2}H_{CO2}$), and coupled low O_2 and low pH ($L_{O2}H_{CO2}$) compared to control levels ($H_{O2}L_{CO2}$). LnRR = ln(treatment)-ln(control) ± Bias-corrected bootstrapped 95% confidence interval (after Kroeker et al., 2010; Hedges et al., 1999; Gurevitch and Hedges, 1999). The zero line indicates no effect, and significance of mean effects is determined when the 95% confidence interval does not overlap zero (significant results marked with '*'). Grey background was added to summarize the species by phyla.

Figure 3. Realised oxygen concentration and pH conditions for a manipulation experiment for 11 invertebrate species from four different natural habitats in the Gullmar Fjord; gravel, infaunal, mooring & rocky. Average and extreme (maximum & minimum) O_2 and pH conditions during experimental exposure are show in black and with dotted line, respectively. The natural O_2 and pH conditions expected for each habitat are shown in the white boxes.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HO2HCO2
LO2HCO2
Respiration Rate Treatment (DO mg L-1 min-1 g DW-1)
Respiration Rate Control (DO mg L-1 min-1 g DW-1)

Figure 1.

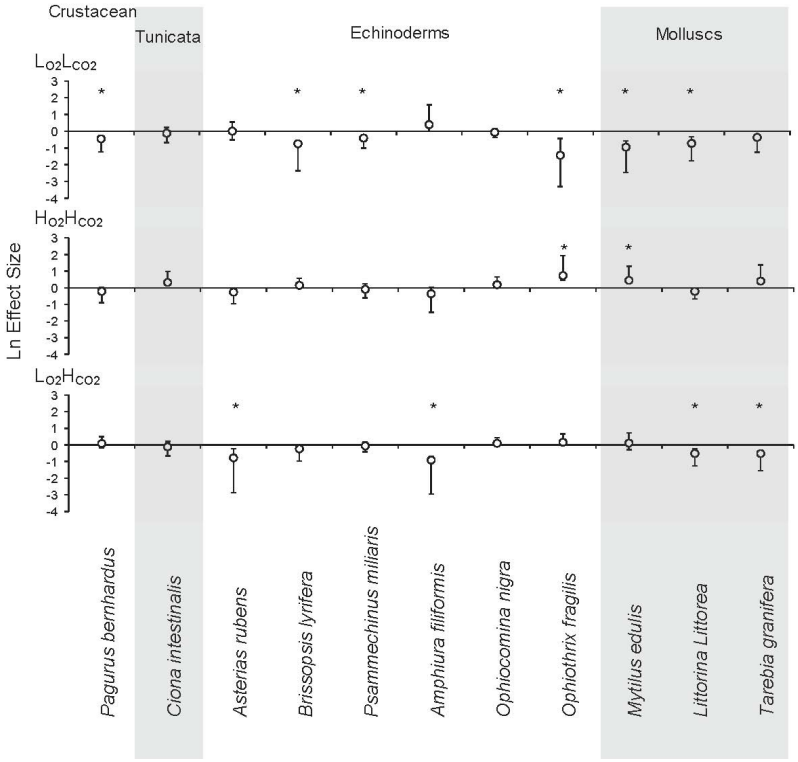
Crustacean
Tunicata
Echinoderms
Molluscs
LO2LCO2
HO2HCO2
LO2HCO2
Ln Effect Size
Pagurus bernhardus
Ciona intestinalis
Asterias rubens
Brissopsis lyrifera
Psammechinus miliaris
Amphiura filiformis
Ophiocomina nigra
Ophiothrix fragilis
Mytilus edulis
Littorina Littorea
Tarebia granifera

Figure 2.

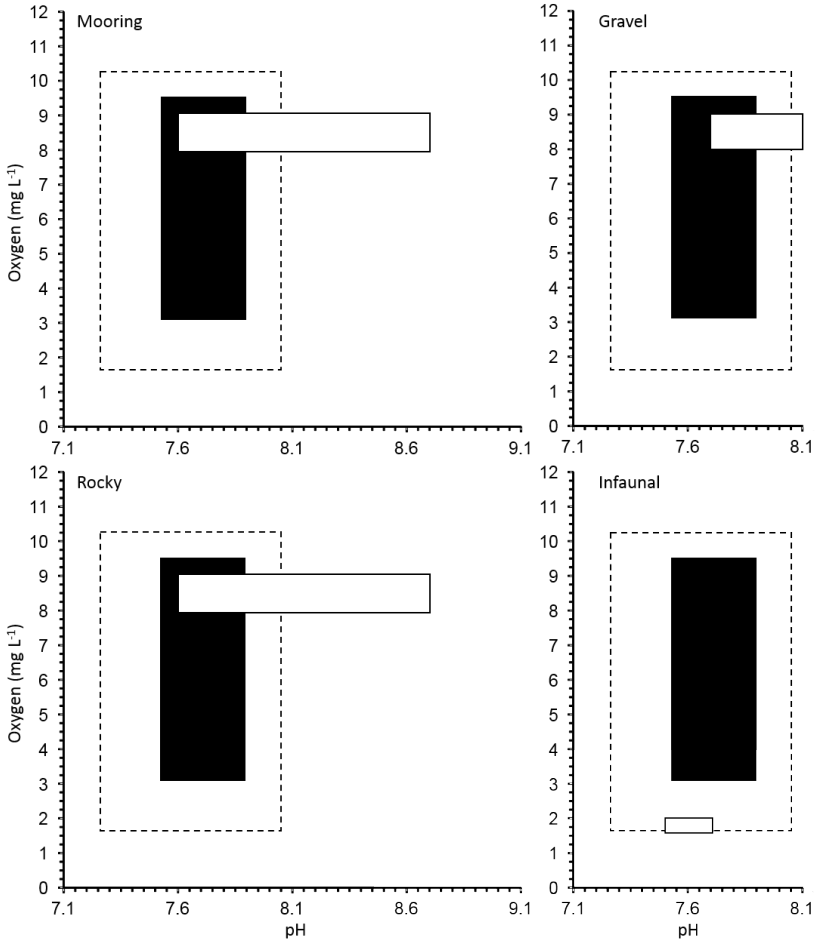
Mooring
Gravel
Rocky
Infaunal
Oxygen (mg L-1)
pH
0
1
2
3
4
5
6
7
8
9
10
11
12
7.1
7.6
8.1
8.6
9.1

Figure 3.