Changing carbonate chemistry alters the toxicity of contaminants to marine invertebrates

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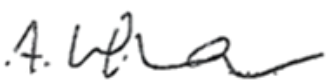

Abstract

The oceans are changing, globally and locally. Two stressors already impacting marine life on both scales are changing carbonate chemistry, whether induced by the worldwide increases in atmospheric concentrations of carbon dioxide termed ocean acidification (OA) or by small-scale spatiotemporal fluctuations in coastal waters; and contamination, caused by the countless anthropogenically-produced chemicals that enter the marine environment each year. The potential for these two stressors to interact, causing novel toxicity outcomes for marine life, has only been acknowledged in recent years. In this thesis, I investigate interactions between carbonate chemistry alterations and contaminants to explore whether certain aspects of animal physiology and contaminant chemistry consistently determine outcomes for marine invertebrates.

Through a review of the state of knowledge on the biological impacts of these interactions, I highlighted key knowledge gaps. Whilst established models exist for the effect of freshwater pH on the toxicity of contaminants, this has rarely been investigated in a marine context. This was particularly significant for ionisable organic contaminants: in general, acidic compounds increase in toxicity within the OA-relevant pH range, whilst basic compounds decrease in toxicity. Additionally, there was strong potential for the fluctuations in carbonate chemistry which naturally occur in coastal habitats to alter the toxicity of pH-sensitive marine contaminants.

To address these knowledge gaps, I first exposed two marine invertebrates, the common mussel *Mytilus edulis* and the king ragworm *Alitta virens*, to the pH-sensitive contaminant copper under a fluctuating pCO_2/pH regime representative of coastal conditions and which was expected to increase its toxicity. Fluctuating pCO_2/pH induced an extracellular acidosis of 0.2 units for mussels, likely contributing to the twofold increases in oxidative stress and DNA damage induced by copper compared to in non-fluctuating conditions. For *A. virens*, its ability to maintain acid-base homeostasis in fluctuating conditions via accumulation of bicarbonate resulted in an extracellular alkalosis of 0.3 units. This mitigated copper toxicity which resulted in reduced DNA damage and oxidative stress compared to exposure to copper in non-fluctuating pCO_2/pH conditions.

Secondly, I assessed reproductive parameters in early life stages of the painted urchin *Lytechinus pictus* and the lugworm *Arenicola marina* when exposed to two ionisable pharmaceuticals in OA conditions: tolcapone, which behaves as an acid and hence may increase in toxicity, and fluoxetine, which behaves as a base and hence may decrease in toxicity. In OA treatments both with and without pharmaceuticals, measurements of sperm swimming speed (curvilinear velocity, straight-line velocity and average path velocity) increased twofold for urchins, but decreased by half for lugworms, compared to ambient pH conditions. Pharmaceuticals altered the magnitude of the OA effects but not consistently: both pharmaceuticals decreased velocity measurements for lugworms in OA conditions, whilst tolcapone increased and fluoxetine decreased these effects for urchins. Additionally, these swimming parameters were differentially linked to fertilisation success for each species, resulting in differing outcomes which appeared to be driven predominantly by the physiology and reproductive ecology of each species in response to OA.

Finally, I exposed adult common mussels, *Mytilus edulis*, and purple urchins, *Paracentrotus lividus*, to these same pharmaceuticals in OA conditions, hypothesising that differences in species acid-base physiology would impact toxicity changes alongside the theorised ionisation changes. OA increased the effect of tolcapone on oxidative stress by 50 % in urchins, but not mussels. OA also increased the effect of fluoxetine on urchin antioxidant activity by 183 % and decreased ammonia excretion rate by 57 %; however, mussel antioxidant activity decreased by 73 % whilst oxygen uptake increased by 81 %. Importantly, the direction of OA-induced effects on these responses did not correspond to theorised changes to pharmaceutical ionisation, and the magnitude of effects did not correspond to differences in acid-base physiology.

Taken together, my findings demonstrate the importance of differences between species and life stages in determining their responses to the combination of altered carbonate chemistry and contaminants, and that the final toxicity outcomes for marine invertebrates cannot be estimated based solely on theorised chemical changes to contaminants in OA conditions. Whilst our knowledge of contaminants and changing carbonate chemistry as single stressors is increasing, marine organisms will experience both concurrently and this work contributes to our understanding of how these global and local stressors will interact in a future ocean.

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Acknowledgements

First and foremost, I would like to thank my lead supervisor, Ceri, for her unwavering support and guidance on this journey. From the first stages of my undergraduate project to the final days of my PhD, Ceri pushed me to think bigger and do better, and I never would have dreamed of turning my ideas into a PhD project without her encouraging me to do so. I would not be the scientist I am today without her influence and I will forever be grateful for this. Ceri and Tamara welcomed me into their research group, providing me with a friendly and dynamic environment to grow and develop, and thank you to all the members of this group who have supported me through the highs, the lows and the in-betweens. I would also like to thank my secondary supervisor, Rod, for his always helpful advice and feedback in both experimental work and writing, and his incredible knowledge of physiology.

Many, many thanks to the wonderful collaborators and technicians who have helped me at all stages of this project. I would particularly like to thank Katie for always having an ear to listen and a horde of helpful advice, both academic and non; Sharon for being endlessly kind and supportive; Greg and Paul for helping me to bring some very ambitious experiments to fruition; and John Dowdle for his dry jokes which always helped to improve even the worst lab disaster. Additional thanks go to Steve the master worm-digger, and to Adam, Rich and Alex for having to deal with the great accidental urchin spawning incident. My gratitude to all our technicians and support staff has been increased a hundred times over during the pandemic and I would not have completed this project without all of your efforts.

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I would also like to thank my friends and family outside of the university for their unwavering support during my PhD. Thank you to the Beans and the Better Pizzaboeufs, or whatever we call ourselves now, for always being there. Most of all, thank you to B^2 and M^2 for putting up with my stress, minor and major meltdowns, and for always being my hype men.

This work would not have been possible without the University of Exeter's Vice Chancellor's Scholarship which provided the funding for my stipend, research, training and travel. I am also grateful to the Challenger Society for Marine Science for providing me with a grant to travel to their conference, and the Malacological Society of London for awarding me with a grant to carry out additional research.









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List of abbreviations and acronyms

AChE Acetylcholinesterase

ASW Artificial seawater

CASA Computer Aided Sperm Analysis

CO_2 Carbon dioxide

CO2SYS Software for calculating carbonate chemistry parameters

Cl^- Chloride ion

Cu^{2+} Copper free ion

DIC Dissolved inorganic carbon

DNA Deoxyribonucleic acid

g Grams

H^+ Hydrogen ion

HCO_3^- Bicarbonate ion

IPCC Intergovernmental Panel on Climate Change

K^+ Potassium ion

L Litres

Log D Distribution coefficient

Log *K*ow Logarithm of the octanol/water partition coefficient

LPO Lipid peroxidation

mg Milligrams

mL Millilitres

Na^+ Sodium ion

O_2 Oxygen

OA Ocean acidification

OECD Organisation for Economic Cooperation and Development

pCO_2 Partial pressure of carbon dioxide

pH_e Extracellular pH

pH_i Intracellular pH

pH_{NBS} pH measured on the National Bureau of Standards scale

pK_a Acid dissociation constant

RCP Representative Concentration Pathway

ROS Reactive oxygen species

SEM Standard error of the mean

SOD Superoxide dismutase

SSP Shared Socioeconomic Pathway

TA Total alkalinity

VAP Average path velocity

µg Micrograms

µL Microlitres

µM Micromolar

COVID-19 impact

I am fortunate that the COVID-19 pandemic has not impacted my health during the past 18 months. I am extremely grateful to my colleagues, both academics and support staff, who have continued to work during the pandemic and supported me in my research in so many ways. The initial uncertainty meant that I was unable to carry out laboratory work between March and July 2020 and was unable to collect animals from the field until August 2020. I am very grateful to the University of Exeter for agreeing to a six-month extension to my living stipend and research grant due to this lost time, without which I would not have been able to complete the original goals of my PhD.

The greatest impact for me is that I have not been able to carry out full seawater chemistry for Chapters 3 and 4. This is also partly due to a laboratory fire in February 2021, which thankfully caused no injuries but damaged an essential and unique piece of equipment used to measure dissolved inorganic carbon. Due to the time required to process the insurance claim, and the lack of equivalent equipment available elsewhere, I have incomplete carbonate chemistry data for Chapter 3.

In addition, I have been working with a laboratory at the University of Exeter to develop Liquid Chromatography-Mass Spectrometry (LC-MS) techniques to quantify the pharmaceuticals used in Chapters 3 and 4. As this laboratory was closed for several months, much of the equipment was shut down for long periods and then had to be re-activated over the course of months due to the sensitive nature of the instruments. I was also not able to access the laboratory for training due to social distancing requirements, nor was I able to carry out preparatory work in my usual laboratory due to damage from the fire, and reduced capacity meant that others could not complete this work for me. Unfortunately, the time required to complete the chemistry was far beyond the time remaining to finish my PhD. We are continuing to work with this laboratory to develop methods, and it is my hope that we will eventually be able to quantify the pharmaceuticals so that the remaining chapters can be published. For the purposes of this thesis, I am confident that the nominal concentrations described within are close to accurate due to my experiments being conducted over short timescales in closed systems.

Author declaration

This thesis is presented as five chapters in a consistent style for ease of reading. All work in this thesis was planned, implemented, analysed and written by myself. **Chapter 2** has been published in the journal Science of the Total Environment, with full details of the publication, co-authors and acknowledgements available at the start of this chapter. Contributions of others are similarly described at the beginning of **Chapter 3** and **Chapter 4**, including acknowledgements. Work from this thesis was also presented at the Challenger Society for Marine Science conference in September 2019, the Society for Environmental Toxicology and Chemistry Europe 30th Annual Meeting in May 2020, and the Marine Biological Association Postgraduate Conference in April 2021.

Chapter 1. How will changing carbonate chemistry alter the toxicity of marine contaminants?

1.1. Introduction

The world's oceans have absorbed at least one third of all carbon dioxide (CO_2) released since the industrial revolution (Bindoff et al., 2019; Bromhead et al., 2015), driving a mean worldwide surface ocean pH decline of 0.1 units via the hydrolysis of CO_2 in seawater (Equation 1), with a further decline of up to 0.4 units anticipated by the year 2100 (IPCC, 2021; Kwiatkowski et al., 2020). This ocean acidification (OA) is predicted to drive major shifts in marine ecosystems (Linares et al., 2015) and affect human society via disruption of ecosystem services (Gattuso et al., 2015). Whilst open ocean pH is steadily declining over decades, greater changes in carbonate chemistry occur in some coastal habitats over far shorter timescales. Some sites experience daily pH fluctuations of over 1 unit, well above the maximum expected end-of-century open ocean decline, linked to fluctuations in pCO_2, alkalinity and associated carbonate chemistry parameters (Baumann, 2019; Duarte et al., 2013; Torres et al., 2021). There is a growing consensus of the importance of this variability to marine biota (Hofmann et al., 2015; Vargas et al., 2017), with recent studies demonstrating that biological responses can be altered when organisms experience a fluctuating pCO_2/pH regime as opposed to a stable one (Eriander et al., 2016; Hannan et al., 2021; Laubenstein et al., 2020; Mangan et al., 2017).

Additionally, many of the contaminants present in the marine environment are sensitive to pH within the range of near-future OA and present-day coastal fluctuations, such that their bioavailability and toxicity could be altered. Certain metals are known to undergo speciation changes within these pH ranges (Millero et al., 2009; Stockdale et al., 2016), and this could also occur for pH-sensitive ionisable organic contaminants due to differences in uptake and toxicity between their neutral and ionised forms (Rendal et al., 2011). Altered bioaccumulation and toxicity in OA conditions has been demonstrated for a limited number of metal and organic contaminants (e.g. Freitas et al., 2016; Ivanina et al., 2016; Moreira et al., 2018; Munari et al., 2018; Serra-Compte et al., 2018), but the mechanisms behind these alterations remain poorly understood. These effects may be felt most strongly in coastal regions, due to their greater pH extremes and higher levels of contamination resulting from their proximity to human activity. Whether short-term fluctuations in carbonate

chemistry alter the toxicity of contaminants in a meaningful way for coastal organisms is yet to be investigated.

Our current understanding of how animals will respond to the combination of marine contaminants and changing carbonate chemistry, whether related to OA or natural fluctuations, is limited. Here, I will discuss current knowledge of the biological effects of OA and fluctuating pCO_2/pH and the effects of marine contaminants. I will review what is known about the behaviour of contaminants as pH changes and consider how this may affect organisms in an OA scenario. I will consider whether observed biological effects observed in experiments combining OA and contaminants align with theorised alterations to contaminant behaviour as pH changes. Finally, I will discuss what these findings mean for organisms in coastal habitats, which will be subjected to a combination of OA, fluctuating pH and increasing contamination in a future ocean, and highlight the knowledge gaps which will be addressed in experimental chapters.

1.2. Carbonate chemistry changes over varying timescales

Ocean acidification (OA) is defined as the decline in ocean pH and associated changes in carbonate chemistry caused by increasing concentrations of carbon dioxide (CO_2). Over the past 30 years, ocean uptake of atmospheric CO_2 has doubled, from 1.2 Pg carbon per year in the 1980s to 2.0-2.8 Pg carbon per year between 2010 and 2015 (Landschützer et al., 2017; Le Quéré et al., 2018; Rödenbeck et al., 2014); approximately 20-30 % of global CO_2 emissions from burning of fossil fuels, cement production and land-use change since the mid-1980s has been taken up by the oceans (Bindoff et al., 2019). This CO_2 reacts with water (H_2O) to form carbonic acid (H_2CO_3), which dissociates into hydrogen ions (H^+) and bicarbonate anions (HCO_3^-):

$$CO_2 + H_2O \rightarrow H_2CO_3 \rightarrow H^+ + HCO_3^- \quad (1)$$

This release of hydrogen ions decreases the pH of the seawater, as pH is the negative logarithm of the hydrogen ion concentration. A pH decrease can also be described as an increase in acidity, hence the term OA. In tandem with increasing atmospheric and oceanic CO_2 concentrations, surface ocean pH declined by 0.018 ± 0.004 units between 1991 and 2011 in 70 % of ocean biomes, with long-term monitoring at eight

global locations showing mean declines of 0.017-0.027 pH units per decade since the 1980s (Bindoff et al., 2019). In addition, carbonate ions (CO_3^{2-}) react with the increasing concentration of H^+ at lower pH. This reduction in carbonate ions in the face of no change in calcium ion concentration reduces the saturation state of calcite and aragonite, the bioavailable forms of carbonate (Orr et al., 2005). This is occurring faster in some ocean regions, such as polar regions due to the greater capacity of colder waters to absorb CO_2 and additional freshwater input from sea-ice melting (Eyre et al., 2018; Yamamoto-Kawai et al., 2009).

The Intergovernmental Panel on Climate Change (IPCC) has modelled four potential pathways of greenhouse gas emissions and atmospheric concentrations for the 21st century, known as Representative Concentration Pathways (RCPs) (IPCC, 2014). These range from a stringent mitigation scenario (RCP 2.6) to a "business as usual" scenario with no efforts to constrain emissions (RCP 8.5). The projected CO_2 emissions with the associated mean surface ocean pH values for each of these pathways are shown in Figure 1. The projections from the RCP 8.5 scenario, approximately 1200 µatm CO_2 and surface ocean pH of 7.73 by the year 2100, are frequently targeted as a "future OA" scenario in studies assessing the biological effects of OA (Doney et al., 2020). Whilst such pCO_2 and pH values are not unique in the earth's geological history, the rate of change in global ocean carbonate chemistry is unprecedented (Hönisch et al., 2012).

A new set of emissions projections were published by the IPCC in 2021, the Shared Socio-economic Pathway (SSP) scenarios, based on socio-economic trends (Figure 2) (IPCC, 2021). Whilst the report states that these are similar to the nominally corresponding RCP projections (i.e. RCP 8.5 and SSP5-8.5), the report also highlights differences arising due to higher climate sensitivity and higher radiative forcing (the difference between planetary incoming and outgoing radiation) in the new SSP scenarios. For experimental OA studies, a key difference is that the scenario nominally associated with RCP 8.5, SSP5-8.5, projects a mean surface ocean pH of 7.66 for the year 2100, whilst the next lowest emissions scenario, SSP3-7.0, projects a mean surface ocean pH of approximately 7.73 (Bopp et al. 2013). The likelihood of different scenarios does not form part of the 2021 report, therefore it is not yet possible to

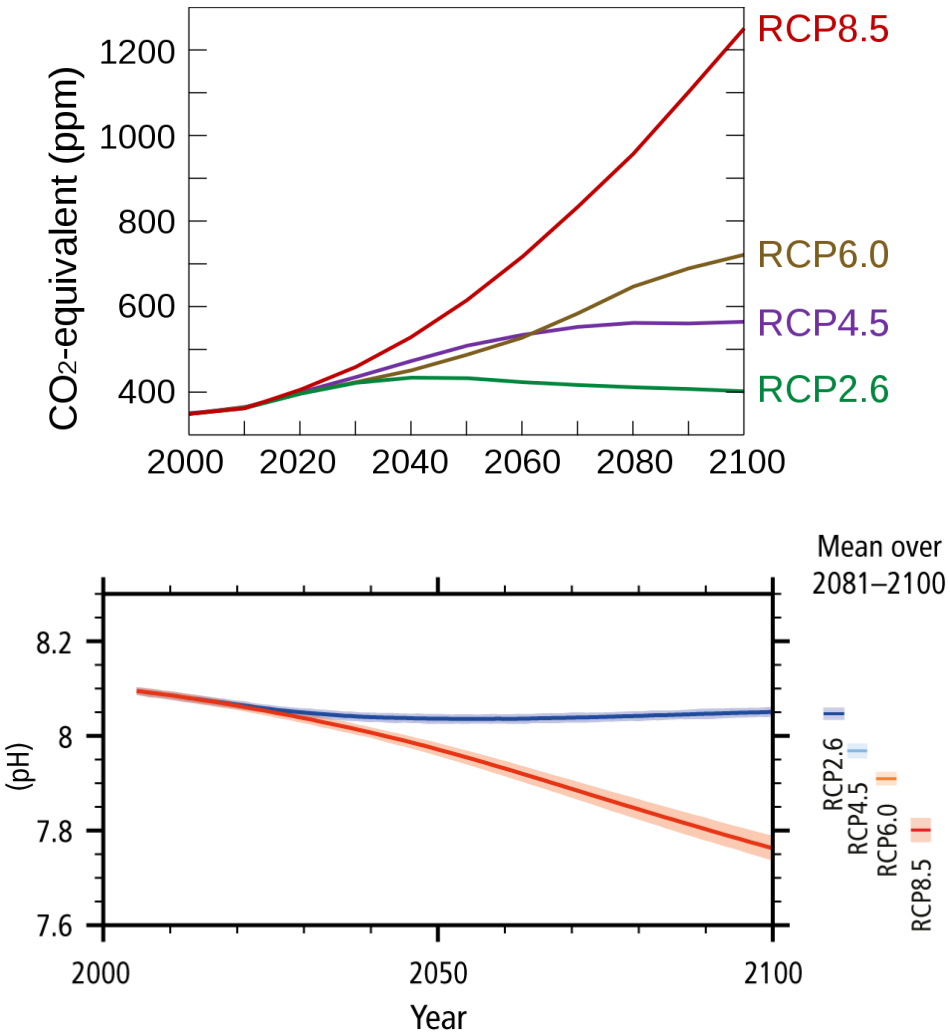

Figure 1. (Top) The 2014 Intergovernmental Panel on Climate Change (IPCC) projections for the total concentration of carbon dioxide present in the atmosphere up to the year 2100 under four different Representative Concentration Pathway (RCP) scenarios; (bottom) the mean global surface ocean pH associated with each RCP. Adapted from IPCC, 2014.

assess which, if any, may be most appropriate for experimental OA scientists to adopt as a target for future pH treatments. As well as the likelihood of SSP scenarios, future experimental OA studies choosing to target SSP-based OA projections will need to take into account the need for comparison with previous work which is primarily based on RCP 8.5.

Although OA is affecting mean open ocean conditions over decades and centuries, coastal regions can undergo far greater changes in carbonate chemistry over far shorter timescales. Seawater carbonate chemistry, including pH, is dependent on

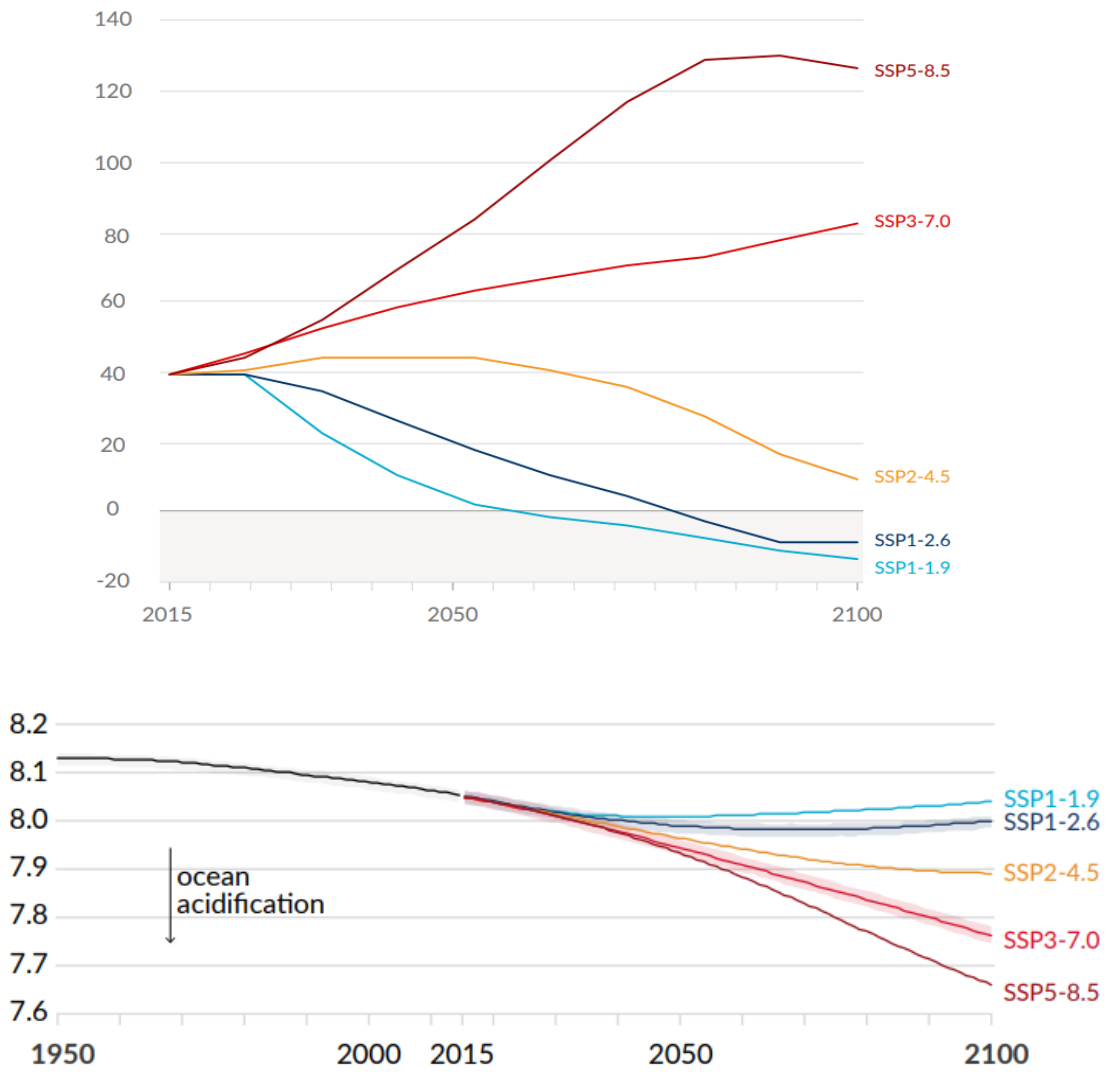

Figure 2. (Top) The 2021 Intergovernmental Panel on Climate Change (IPCC) projections for the total concentration of carbon dioxide present in the atmosphere up to the year 2100 under five different Shared Socio-economic Pathway (SSP) scenarios; (bottom) the mean global surface ocean pH associated with each SSP. Note differing scales on X axes. Adapted from IPCC, 2021.

multiple factors: biological uptake and release of CO_2 via photosynthesis, respiration and calcification; freshwater input, from terrestrial or atmospheric sources; temperature; the presence of upwellings; and exchange of CO_2 at the air-sea interface (Blackford et al., 2017). In shallow coastal ecosystems, temporal fluctuations in these processes combined with high biological activity and often lower buffering capacity (Duarte et al., 2013; Silbiger and Sorte, 2018), cause these habitats to experience greater natural variation than open ocean ecosystems, often in distinct site and season-specific patterns (Baumann, 2019; Guadayol et al., 2014; Hofmann et al.,

2011; Middelboe and Hansen, 2007). Typical daily pH ranges are in the region of 0.2 units (Duarte et al., 2013; Torres et al., 2021), yet ranges far exceeding this are present in coastal habitats worldwide (Baumann et al., 2015; Hofmann et al., 2011; Morris and Taylor, 1983; Thomsen et al., 2013). Diel ranges of 0.5 to more than 1 pH units have been recorded in coastal ecosystems such as tide pools (Silbiger and Sorte, 2018), seagrass meadows (Semesi et al., 2009), coral reefs (Santos et al., 2011), and macroalgal beds (Menéndez et al., 2001). The combination of these daily and seasonal fluctuations can result in low pH extremes exceeding the RCP 8.5 estimate for the end of this century: examples include an extreme low of pH 7.59 in a seagrass bed (Pacella et al., 2018) and lows of pH 7.66, 7.55 and 7.48 in different months in an Arctic lagoon (Miller et al., 2021).

The high variance of, and numerous influences on, coastal carbonate chemistry makes the prediction of future trends a significant challenge. Some regions, particularly coastal, may experience basification due to global trends of increase in river alkalinity (Duarte et al., 2013). Alternatively, declines in pH may already be occurring faster in coastal regions due to additional drivers; Waldbusser et al. (2011) measured declines in seawater pH of 0.1 and 0.3 units during different seasons in Chesapeake Bay, USA, between 1985 and 2008, which exceeded the decline measured in the open Pacific Ocean over the same period (Doney et al., 2009), suggested to be due to the combined impacts of global OA and local eutrophication. Wootton et al. (2008) measured an even greater mean annual decline of 0.045 pH units between 2000 and 2007 at Tatoosh Island, USA, driving mean pH values from approximately 8.4 to 8.1 and minimum values from approximately 8.0 to 7.4. Both studies took place in regions of high biological activity: Duarte et al. (2013) hypothesise that due to such biological activity, i.e. respiration, photosynthesis and calcification, potentially having greater effects on seawater CO_2, local fluctuations in seawater carbonate chemistry could outweigh future OA-induced changes. Corroborating this, Carstensen and Duarte (2019) found that respiratory control of pH was the strongest driver of variability in coastal habitats. This suggests that the high biological activity which is often characteristic of coastal regions may drive pH to lower extremes through additional production of CO_2, in combination with other coastal concerns such as eutrophication (Waldbusser et al., 2011).

As well as coastal pH ranges “shifting”, they may also broaden: predicted increases in extreme weather events (Bindoff et al., 2019) could lead to greater carbonate chemistry minima and maxima (Burger et al., 2020). Additionally, Melzner et al. (2020) predict that oxygen declines, expected to occur in the global ocean concurrently with OA, will lead to greater relative increases in pCO_2 in systems with high biological activity; this would exacerbate high pCO_2/low pH extremes in coastal regions. Downscaled models, covering a far smaller spatial scale than those used to make global predictions, also predict greater variability in coastal pH as mean atmospheric pCO_2 increases (Fagundes et al., 2020). Crucially, many coastal areas already regularly reach low pH extremes which are only expected to occur in the open ocean past the end of this century (Bindoff et al., 2019; Doney et al., 2009), and these are likely to become more extreme as global climate change continues.

1.3. Ocean acidification impacts marine organisms

The biological impacts of alterations in CO_2, pH and associated carbonate chemistry parameters are many and varied. Much research has focused on the effects of OA on calcifying organisms due to the associated reduction in seawater carbonate ions (for extensive reviews, see: Orr et al., 2005; Ries et al., 2009; Schönberg et al., 2017). As carbonate is a component of the biogenic calcium carbonate ($CaCO_3$) structures formed by calcifying organisms, they may struggle to produce and maintain components such as shells and skeletons if current trends continue; net dissolution is predicted in some ecosystems by the end of this century (Eyre et al., 2018; Yamamoto-Kawai et al., 2009). Although these effects are significant for many organisms and ecosystems, this section of the review will focus on biological effects of OA that are applicable to both calcifying and non-calcifying organisms, particularly effects with potential for toxic interactions with marine contaminants.

As well as affecting seawater chemistry, CO_2 can alter the internal chemistry of marine organisms. Animals possess regulatory mechanisms to cope with the CO_2 generated through normal cellular metabolism, but internal acid-base disturbances can occur in OA conditions due to the need to maintain a sufficient pCO_2 gradient between tissues and seawater to facilitate CO_2 excretion, and the diffusion of additional CO_2 into tissues from seawater (Melzner et al., 2020). Excess CO_2 can lead to hydrogen (H^+)

ion production (see Equation 1), facilitated by the enzyme carbonic anhydrase, and hence to pH decline internally (Melzner et al., 2009). In order to limit or prevent disruption of biochemical processes, organisms can compensate for the decrease in pH by increasing quantities of buffering substances, increasing proton-equivalent ion transport, or suppressing metabolism to wait out periods of high CO_2 (Fabry et al., 2008). It has been suggested that some organisms could increase ventilation rates in order to aid the removal of CO_2, however this may not be effective for marine animals in OA scenarios due to the small diffusion gradients between body fluids and the surrounding water (Melzner et al., 2009).

Buffering of H^+ ions by partially protonated amino acid side chains, amino groups of proteins or organic/inorganic phosphate groups is usually the earliest line of defence that limits the magnitude of pH change but cannot prevent it entirely (Melzner et al., 2020, 2009). The hydration of CO_2 by carbonic anhydrase also produces a small amount of bicarbonate (HCO_3^-). Whilst this acts as a pH buffer itself, the bicarbonate produced by this reaction does not restore internal pH. However, some organisms can accumulate additional bicarbonate via ion transport processes in the gills and/or other tissues such as kidney or gut (Melzner et al., 2020). Some organisms possessing biogenic calcium carbonate structures accumulate extracellular fluid bicarbonate via dissolution of these structures at high pCO_2, such as the mussel *Mytilus edulis* at approximately 10,000 µatm (Lindinger et al., 1984), although it is worth noting that these CO_2 concentrations are unlikely to be found outside of the most extreme environments, even during future OA. Surplus H^+ ions can be eliminated via trans-epithelial ion exchange: V-type H^+ ATPases coupled energetically to Na^+ channels, or electroneutral Na^+/H^+ exchangers, use the Na^+ gradient between seawater and cytosol generated by the ATP-consuming Na^+/K^+ pump to remove H^+ ions (Melzner et al., 2009). The degree of pH compensation is generally greater in intracellular compartments than extracellular spaces in marine organisms (Pörtner et al., 2004), likely in order to protect the most important biochemical processes.

Effectiveness of compensation for hypercapnia varies between taxa, with a combination of bicarbonate and non-bicarbonate buffering and ion transport proving most successful in compensating internal pH in an OA scenario (Melzner et al., 2009). Therefore, organisms with limited ability to perform one or more of these processes

may be at most risk of OA-induced acid-base disturbances. Marine ectothermic animals with high metabolic rates and active modes of life (i.e. high metabolic production of CO_2) generally have a high capacity for both bicarbonate and non-bicarbonate buffering even if they do not encounter environmental hypercapnia, and have therefore been suggested to be resilient against future OA (Fabry et al., 2008; Melzner et al., 2020, 2009). Less active animals, such as bivalves and many echinoderms, are generally unable to accumulate sufficient bicarbonate to counteract OA are often unable to fully compensate extracellular pH in OA conditions (Melzner et al., 2020). Pörtner et al. (2000) suggest that these bicarbonate-dependent mechanisms may become more important during times of energy deficiency, which could be significant for organisms which undergo metabolic depression in OA conditions, such as many sessile invertebrates (Melzner et al., 2009). Additionally, non-bicarbonate buffering is strongly linked to extracellular protein content, which is highest in organisms with extracellular respiratory pigments, such as crustaceans and cephalopods, and low in bivalves and echinoderms (Melzner et al., 2020). Taken together, these findings suggest that sessile invertebrates with low capacity for both bicarbonate and non-bicarbonate buffering and a reliance on metabolic depression may be most vulnerable to OA.

Acid-base disturbances induced by OA can lead to metabolic depression in some organisms, namely invertebrates (Michaelidis et al., 2005; Reipschläger and Pörtner, 1996), which may be an adaptation for living in tidal environments. The peanut worm *Sipunculus nudus* regulates intracellular pH via ion transport, but also undergoes metabolic depression in response to low extracellular pH, thought to be due to reduced energetic demand of intracellular pH regulation caused by a switch in ion transport exchange mechanisms (Portner et al., 2000); this may be a survival mechanism for the short periods of hypercapnia and hypoxia often present in tidal habitats. Some sessile littoral organisms vastly reduce ventilation rate during aerial exposure, such as bivalves (Scanes et al., 2017; Shick et al., 1986) and anemones (Zamer, 1986), therefore reducing metabolic rate reduces both oxygen use and CO_2 build-up to enable survival during periods of tidal exposure. This can be achieved by suppression of energetically expensive processes, such as protein synthesis (Langenbuch and Portner, 2002). Subsequently, it has been suggested that species exposed to such short-term CO_2 extremes could be more tolerant to future OA (Hofmann et al., 2010);

however, metabolic suppression can reduce growth (Michaelidis et al., 2005) and reproductive potential (Fabry et al., 2008). Organisms in such naturally variable environments may therefore be at high risk as OA drives pH and pCO_2 to greater extremes.

The impacts of uncompensated CO_2-induced acidification on marine organisms are well-documented and have been reviewed extensively (Doney et al., 2020, 2009; Fabry et al., 2008; Melzner et al., 2020). These impacts include alterations to metabolic rate as described above (Michaelidis et al., 2005; Wood et al., 2016); hampering blood oxygen transport by reducing extracellular pH (Melzner et al., 2020); decreasing the rate of calcium carbonate precipitation in calcifying organisms (Schönberg et al., 2017); interference with neuronal receptors, altering behaviour (Nilsson et al., 2012); and increasing oxidative stress (Wood et al., 2016). Even when organisms can effectively compensate for acidosis, these processes can be energetically expensive and hence affect growth and fitness (Fabry et al., 2008). As well as effects relating to internal acid-base disturbances, OA has other organism-level impacts, such as altering the ability of organisms to build and maintain calcified structures (Ries et al., 2009; Schönberg et al., 2017); weakening the attachment strength of mussel byssal threads (O'Donnell et al., 2013); and altering rates of photosynthesis (Mackey, 2015). Through these many effects, OA is predicted to have ecosystem-level impacts: these include alterations to pelagic food-webs; reductions in biogenic reefs formed by calcifying organisms such as mussels and oysters; and disruption of ocean ecosystem services such as fisheries and tourism related to coral reefs (Doney et al., 2020).

An additional potential source of population-level impacts is via the effects of OA on marine gametes, which can be particularly sensitive to high pCO_2/low pH conditions (Kroeker et al., 2010). Many marine species reproduce by releasing gametes directly into the water column, directly exposing these single-celled life stages to altered seawater chemistry, including in variable environments such as estuaries and coral reefs (Duarte et al., 2013). A change in external pCO_2 will likely cause greater changes in gamete internal pCO_2, and hence potentially acid-base homeostasis, than multi-cellular developmental stages due to their greater surface area to volume ratio (Melzner et al., 2009). Sperm activation, swimming and fertilisation reactions are all at

least partially pH-dependent, and the extent of gametes' ability to regulate internal pH is not well understood (Nishigaki et al., 2014).

Sperm are especially susceptible to environmental stressors: they lack DNA and cellular repair mechanisms (Aitken et al., 2004), and contain polyunsaturated fatty acids that can act as substrate for reactive oxygen species, making them vulnerable to oxidative stress (Nissen and Kreysel, 1983; Quijano et al., 2016). However, the impacts of OA on sperm swimming and fertilisation success are variable, from decreases in both parameters (Morita et al., 2010; Nakamura and Morita, 2012; Schlegel et al., 2015, 2014, 2012; Uthicke et al., 2013; Vihtakari et al., 2013), to no effects (Havenhand and Schlegel, 2009; Sung et al., 2014), and even increases in both (Caldwell et al., 2011; Smith et al., 2019). It is important to note that increases in sperm swimming measurements are not necessarily positive findings: there may be trade-offs between longevity and swimming speed for sperm, such that slower sperm live for longer. For some species, such as the urchin *Lytechinus pictus*, slower swimming speed may allow sperm more time to locate their small eggs (Levitan, 2000). OA can also change which sperm are most successful in fertilising eggs: Smith et al. (2019) found that at current mean open ocean pH (8.1), *L. pictus* sperm with low-to-medium speed and low path linearity have highest fertilisation success, but at pH 7.7 sperm with higher speed and path linearity had more success. The inverse occurred for the urchin *Heliocidaris erythrogramma*: high speed and path linearity were important at pH 8.1, whereas low speed and less linearity were more important at pH 7.7. Similarly, Campbell et al. (2016) observed that the strength of the association of sperm swimming speed and number of motile sperm with fertilisation success was reduced by OA for the urchin *Paracentrotus lividus*, resulting in changes to the performance of males between current and future OA conditions in terms of fertilisation success. These shifts demonstrate that unaltered mean fertilisation rates could hide changes in the genetic contribution of different males, from multiple species, to future populations. The physiology of sperm can also impact offspring survival: eggs fertilised by long-lived sperm are more likely to develop in the ascidian *Styela plicata* (Crean et al., 2012) and Atlantic salmon *Salmo salar* (Immler et al., 2014), therefore changes to the characteristics of successful sperm could also affect future populations.

1.4. Biological effects of fluctuating carbonate chemistry

Our current understanding of how organisms will respond to pH fluctuations when experienced under OA, or indeed at present atmospheric CO_2 levels, is limited, as the vast majority of experiments have compared stable pCO_2/pH regimes based on projections for the open ocean (Boyd et al., 2018). There have been several calls for studies that incorporate these natural fluctuations, to improve their environmental relevance (Gunderson et al., 2016; Hofmann et al., 2015); in a 2016 review, only 11 % of 324 experimental OA studies used fluctuating conditions (Wahl et al., 2016). Direct comparison of the biological effects of fluctuating versus static carbonate chemistry regimes is complex due to the vast differences in current pH and pCO_2 means, ranges, and rate of change between different natural habitats (Duarte et al., 2013) and methodological differences in experiments (Kapsenberg and Cyronak, 2019). Here, I will discuss some of the overarching findings from these experiments and their relevance to coastal organisms.

Table 1 presents a summary of experiments comparing biological responses for animals in static and fluctuating pCO_2/pH regimes. It is immediately apparent that there are differences in the values and ranges of pCO_2 and pH employed between experiments, which is to be expected as the natural fluctuations in carbonate chemistry, upon which most studies are based, vary widely depending on the site, season and even time of day (Duarte et al., 2013; Torres et al., 2021). Whether laboratory-manipulated regimes are relevant to what organisms will experience in their natural environment will depend on these factors, but very few studies included measurements of carbonate chemistry from the organisms' natural habitat (e.g. Mangan et al., 2019).

There are also differences in the patterns of fluctuations utilised in experiments: some manipulate carbonate chemistry in a high-frequency manner throughout the experiment (e.g. to mimic tidal cycles; Mangan et al., (2017)), whilst some use less frequent variation, such as day-to-day or day/night differences (e.g. Alenius and Munguia (2012)). These are relevant to different environments: whilst in many coastal habitats measurable carbonate chemistry changes occur on the order of hours (Duarte

Species	Stable treatment		Fluctuating treatment		Effects (compared to static control)	Reference
	pCO_2 (µatm)	pH	pCO_2 (µatm)	pH		
Abudefduf whitleyi (Fish)	1082 ± 108$^{\sigma}$	7.75 ± 0.06$^{\sigma}$	979 ± 152$^{\sigma}$; (708 ± 11$^{\sigma}$ to 1249 ± 25$^{\sigma}$)$^{\Psi}$	7.70 ± 0.04$^{\sigma}$; (7.61 ± 0.00$^{\sigma}$ to 7.82 ± 0.01$^{\sigma}$)$^{\Psi}$	= Critical swimming speed = Maximum oxygen uptake rate = Minimum oxygen uptake rate = Aerobic scope = Blood lactate = Blood glucose = Blood haemoglobin concentration ↑ Haematocrit = Mean corpuscular haemoglobin concentration	(Hannan et al., 2021)
Acanthochromis polyacanthus (Fish, juvenile)	990 ± 46$^{\sigma}$	7.75 ± 0.02$^{\sigma}$	961 ± 195$^{\sigma}$; (681 ± 21 to 1304 ± 102)$^{\Psi}$	7.77 ± 0.08$^{\sigma}$; (7.64 ± 0.03 to 7.89 ± 0.01)$^{\Psi}$	= Absolute lateralisation = Relative lateralisation	(Jarrold et al., 2017)
Acanthochromis polyacanthus (Fish, juvenile)	990 ± 46$^{\sigma}$	7.75 ± 0.02$^{\sigma}$	934 ± 389$^{\sigma}$; (482 ± 19 to 1591 ± 98)$^{\Psi}$	7.8 ± 0.16$^{\sigma}$; (7.57 ± 0.02 to 8.01 ± 0.01)$^{\Psi}$	↑ Absolute lateralisation = Relative lateralisation	(Jarrold et al., 2017)
Acanthochromis polyacanthus (Fish, juvenile)	748 ± 9$^{\sigma}$; 721-773$^{\Psi}$	NR	788 ± 203$^{\sigma}$; (527 ± 27 to 1025$^{\sigma}$ ± 86$^{\sigma}$)$^{\Psi}$	NR	↑ Absolute lateralisation = Relative lateralisation	(Jarrold et al., 2017)
Acanthochromis polyacanthus (Fish, juvenile)	994 ± 23$^{\sigma}$; 926-1060$^{\Psi}$	NR	1024 ± 256$^{\sigma}$; (667 ± 33$^{\sigma}$ to 1328 ± 78$^{\sigma}$)$^{\Psi}$	NR	= Absolute lateralisation = Relative lateralisation	(Jarrold et al., 2017)
Acanthochromis polyacanthus (Fish, juvenile)	990 ± 46$^{\sigma}$	7.75 ± 0.02$^{\sigma}$	961 ± 195$^{\sigma}$; (681 ± 21$^{\sigma}$ to 1304 ± 102$^{\sigma}$)$^{\Psi}$	7.77 ± 0.08$^{\sigma}$; (7.64 ± 0.03$^{\sigma}$ to 7.89 ± 0.01$^{\sigma}$)$^{\Psi}$	= Growth = Survival = Otolith development	(Jarrold and Munday, 2018a)
Acanthochromis polyacanthus (Fish, juvenile)	990 ± 46$^{\sigma}$	7.75 ± 0.02$^{\sigma}$	934 ± 389$^{\sigma}$; (482 ± 19$^{\sigma}$ to 1591 ± 98$^{\sigma}$)$^{\Psi}$	7.8 ± 0.16$^{\sigma}$; (7.57 ± 0.02$^{\sigma}$ to 8.01 ± 0.01$^{\sigma}$)$^{\Psi}$	= Growth = Survival = Otolith development	(Jarrold and Munday, 2018a)
Acanthochromis polyacanthus (Fish, juvenile)	992 ± 19$^{\sigma}$	NR	1009 ± 25$^{\sigma}$ (653 ± 25$^{\sigma}$ to 1329 ± 89$^{\sigma}$)	NR	= Survival ↑ Wet weight ↑ Standard length = Behavioural lateralisation = Active time = Swimming speed = Boldness = Response to stimulus = Locomotor traits	(Jarrold and Munday, 2018b)

Acanthochromis polyacanthus (Fish, juvenile)	992 ± 19$^{\sigma}$	NR	1060 ± 34$^{\sigma}$ (521 ± 43$^{\sigma}$ to 1544 ± 80$^{\sigma}$)	NR	= Survival ↑ Wet weight = Standard length ↑ Behavioural lateralisation = Active time = Swimming speed = Boldness = Response to stimulus = Locomotor traits	(Jarrold and Munday, 2018b)
Acanthochromis polyacanthus (Fish)	1163.8 ± 12.6$^{\varphi}$	7.612 ± 0.004$^{\varphi}$	806.4 ± 50.5$^{\varphi}$; 451.1-1202.3$^{\Psi}$	7.783 ± 0.025$^{\varphi}$; 7.588-7.969$^{\Psi}$	↓ Oxygen uptake rate ↓ Minimum oxygen uptake rate = Maximum oxygen uptake rate = Aerobic scope ↓ Factorial aerobic scope = Cell haemoglobin concentration = Whole blood glucose concentration	(Hannan et al., 2020)
Acanthochromis polyacanthus (Fish)	1163.8 ± 12.6$^{\varphi}$	7.612 ± 0.004$^{\varphi}$	842.7 ± 49.0$^{\varphi}$; 465.8-1264.9$^{\Psi}$	7.764 ± 0.023$^{\varphi}$; 7.582-7.965$^{\Psi}$	↓ Oxygen uptake rate ↓ Minimum oxygen uptake rate = Maximum oxygen uptake rate = Aerobic scope ↓ Factorial aerobic scope = Cell haemoglobin concentration = Whole blood glucose concentration	(Hannan et al., 2020)
Acanthochromis polyacanthus (Fish)	992 ± 19$^{\sigma}$ (956 ± 74$^{\sigma}$ to 1035 ± 95$^{\sigma}$)$^{\Psi}$	NR	1060 ± 34$^{\sigma}$ (521 ± 43$^{\sigma}$ to 1544 ± 80$^{\sigma}$)$^{\Psi}$	NR	↓ Resting oxygen uptake rate = Maximum oxygen uptake rate = Factorial aerobic scope = Absolute aerobic scope	(Laubenstein et al., 2020)
Amblyglyphidodon curacao (Fish)	1163.8 ± 12.6$^{\varphi}$	7.612 ± 0.004$^{\varphi}$	806.4 ± 50.5$^{\varphi}$; 451.1-1202.3$^{\Psi}$	7.783 ± 0.025$^{\varphi}$; 7.588-7.969$^{\Psi}$	= Oxygen uptake rate = Minimum oxygen uptake rate = Maximum oxygen uptake rate = Aerobic scope = Factorial aerobic scope = Cell haemoglobin concentration = Whole blood glucose concentration	(Hannan et al., 2020)
Amblyglyphidodon curacao (Fish)	1163.8 ± 12.6$^{\varphi}$	7.612 ± 0.004$^{\varphi}$	842.7 ± 49.0$^{\varphi}$; 465.8-1264.9$^{\Psi}$	7.764 ± 0.023$^{\varphi}$; 7.582-7.965$^{\Psi}$	= Oxygen uptake rate = Minimum oxygen uptake rate = Maximum oxygen uptake rate = Aerobic scope = Factorial aerobic scope = Cell haemoglobin concentration = Whole blood glucose concentration	(Hannan et al., 2020)

Amphiprion melanopus (Fish, juvenile)	964 ± 50$^{\sigma}$	7.75 ± 0.03$^{\sigma}$	957 ± 233$^{\sigma}$; (681 ± 63$^{\sigma}$ to 1270 ± 127$^{\sigma}$)$^{\Psi}$	7.76 ± 0.09$^{\sigma}$; (7.63 ± 0.06$^{\sigma}$ to 7.88 ± 0.03$^{\sigma}$)$^{\Psi}$	↑ Survival = Wet weight = Standard length	(Jarrold and Munday, 2019)
Amphiprion percula (Fish, juvenile)	990 ± 46$^{\sigma}$	7.75 ± 0.02$^{\sigma}$	961 ± 195$^{\sigma}$; (681 ± 21$^{\sigma}$ to 1304 ± 102$^{\sigma}$)$^{\Psi}$	7.77 ± 0.08$^{\sigma}$; (7.64 ± 0.03$^{\sigma}$ to 7.89 ± 0.01$^{\sigma}$)$^{\Psi}$	↓ Time spent in predator cue	(Jarrold et al., 2017)
Amphiprion percula (Fish, juvenile)	990 ± 46$^{\sigma}$	7.75 ± 0.02$^{\sigma}$	934 ± 389$^{\sigma}$; (482 ± 19$^{\sigma}$ to 1591 ± 98$^{\sigma}$)$^{\Psi}$	7.8 ± 0.16$^{\sigma}$; (7.57 ± 0.02$^{\sigma}$ to 8.01 ± 0.01$^{\sigma}$)$^{\Psi}$	↓ Time spent in predator cue	(Jarrold et al., 2017)
Amphiprion percula (Fish, juvenile)	748 ± 9$^{\sigma}$; 721-773$^{\Psi}$	NR	788 ± 203$^{\sigma}$; (527 ± 27 to 1025$^{\sigma}$ ± 86$^{\sigma}$)$^{\Psi}$	NR	= Time spent in predator cue	(Jarrold et al., 2017)
Amphiprion percula (Fish, juvenile)	994 ± 23$^{\sigma}$; 926-1060$^{\Psi}$	NR	1024 ± 256$^{\sigma}$; (667 ± 33$^{\sigma}$ to 1328 ± 78$^{\sigma}$)$^{\Psi}$	NR	= Time spent in predator cue	(Jarrold et al., 2017)
Amphiprion percula (Fish, juvenile)	990 ± 46$^{\sigma}$	7.75 ± 0.02$^{\sigma}$	961 ± 195$^{\sigma}$; (681 ± 21$^{\sigma}$ to 1304 ± 102$^{\sigma}$)$^{\Psi}$	7.77 ± 0.08$^{\sigma}$; (7.64 ± 0.03$^{\sigma}$ to 7.89 ± 0.01$^{\sigma}$)$^{\Psi}$	= Growth = Survival = Otolith development	(Jarrold and Munday, 2018a)
Amphiprion percula (Fish, juvenile)	990 ± 46$^{\sigma}$	7.75 ± 0.02$^{\sigma}$	934 ± 389$^{\sigma}$; (482 ± 19$^{\sigma}$ to 1591 ± 98$^{\sigma}$)$^{\Psi}$	7.8 ± 0.16$^{\sigma}$; (7.57 ± 0.02$^{\sigma}$ to 8.01 ± 0.01$^{\sigma}$)$^{\Psi}$	= Growth = Survival = Otolith development	(Jarrold and Munday, 2018a)
Caesio cuning (Fish)	1082 ± 108$^{\sigma}$	7.75 ± 0.06$^{\sigma}$	979 ± 152$^{\sigma}$; (708 ± 11$^{\sigma}$ to 1249 ± 25$^{\sigma}$)$^{\Psi}$	7.70 ± 0.04$^{\sigma}$; (7.61 ± 0.00$^{\sigma}$ to 7.82 ± 0.01$^{\sigma}$)$^{\Psi}$	↑ Critical swimming speed = Maximum oxygen uptake rate = Minimum oxygen uptake rate = Aerobic scope ↑ Blood lactate = Blood glucose = Blood haemoglobin concentration ↑ Haematocrit = Corpuscular haemoglobin concentration	(Hannan et al., 2021)
Cheilodipterus quinquelineatus (Fish)	1082 ± 108$^{\sigma}$	7.75 ± 0.06$^{\sigma}$	979 ± 152$^{\sigma}$; (708 ± 11$^{\sigma}$ to 1249 ± 25$^{\sigma}$)$^{\Psi}$	7.70 ± 0.04$^{\sigma}$; (7.61 ± 0.00$^{\sigma}$ to 7.82 ± 0.01$^{\sigma}$)$^{\Psi}$	↑ Critical swimming speed ↓ Maximum oxygen uptake rate = Minimum oxygen uptake rate ↓ Aerobic scope ↑ Blood lactate = Blood glucose ↑ Blood haemoglobin concentration ↑ Haematocrit = Corpuscular haemoglobin concentration	(Hannan et al., 2021)

Lutjanus fulviflamma (Fish)	1082 ± 108$^{\sigma}$	7.75 ± 0.06$^{\sigma}$	979 ± 152$^{\sigma}$; (708 ± 11$^{\sigma}$ to 1249 ± 25$^{\sigma}$)$^{\Psi}$	7.70 ± 0.04$^{\sigma}$; (7.61 ± 0.00$^{\sigma}$ to 7.82 ± 0.01$^{\sigma}$)$^{\Psi}$	↑ Critical swimming speed ↑ Maximum oxygen uptake rate = Minimum oxygen uptake rate ↑ Aerobic scope = Blood lactate = Blood glucose = Blood haemoglobin concentration = Haematocrit = Corpuscular haemoglobin concentration	(Hannan et al., 2021)
Paralichthys dentatus (Fish, juvenile)	NR	7.49 ± 0.002$^{\varsigma}$	NR	(7.22 ± 0.001$^{\varsigma}$ to 7.78 ± 0.001$^{\varsigma}$)$^{\Psi}$	= Growth rate	(Davidson et al., 2016)
Paralichthys dentatus (Fish, juvenile)	NR	7.49 ± 0.002$^{\varsigma}$	NR	(6.87 ± 0.007$^{\varsigma}$ to 7.99 ± 0.005$^{\varsigma}$)$^{\Psi}$	= Growth rate	(Davidson et al., 2016)
Argopecten irradians (Mollusc, larvae)	516 ± 9.0$^{\sigma}$	7.91 ± 0.02$^{\sigma}$	1880 ± 1390$^{\sigma}$	7.47 ± 0.23$^{\sigma}$	↓ Survival = Growth rate = Development	(Clark and Gobler, 2016)
Crassostrea virginica (Mollusc)	1043.2 ± 36.7^ (upper and mid values NR)	(7.81 ± 0.00$^{\varphi}$; 7.82 ± 0.01$^{\varphi}$; 7.93 ± 0.02$^{\varphi}$)$^{\Psi}$	7343.8 ± 606.1^ (upper and mid values NR)	(6.98 ± 0.00$^{\varphi}$; 7.79 ± 0.01$^{\varphi}$; 7.98 ± 0.02$^{\varphi}$)$^{\Psi}$	= Infection prevalence = Infection intensity = Phagocytosis ↑ Reactive oxygen species production ↓ Rate of apoptosis = Granular haemocytes = Dead granular haemocytes = Agranular haemocytes ↓ Dead agranular haemocytes = Phagocytic granular haemocytes = Phagocytic haemocytes	(Keppel et al., 2015)
Crassostrea virginica (Mollusc, larvae)	522 ± 2.7$^{\sigma}$	7.85 ± 0.04$^{\sigma}$	1900 ± 1540$^{\sigma}$	7.54 ± 0.26$^{\sigma}$	= Survival ↓ Growth rate	(Clark and Gobler, 2016)
Haliotis discus hannai (Mollusc)	721.3 ± 19.5$^{\sigma}$	7.79 ± 0.01$^{\sigma}$	788.2 ± 232.3$^{\sigma}$; 420.3-1189.0$^{\Psi}$	7.77 ± 0.12$^{\sigma}$; 7.60-8.00$^{\Psi}$	= Survival = Malformation = Shell length	(Onitsuka et al., 2018)
Haliotis discus hannai (Mollusc)	1175.3 ± 19.6$^{\sigma}$	7.60 ± 0.01$^{\sigma}$	1122.5 ± 251.9$^{\sigma}$; 707.1-1537.1$^{\Psi}$	7.63 ± 0.09$^{\sigma}$; 7.49-7.80$^{\Psi}$	= Survival = Malformation ↓ Shell length	(Onitsuka et al., 2018)

Haliotis discus hannai (Mollusc)	450.8 ± 10.7$^{\sigma}$	7.98 ± 0.01$^{\sigma}$	483.9 ± 49.7$^{\sigma}$; 409.3-573.6$^{\Psi}$	7.96 ± 0.04$^{\sigma}$; 7.89-8.02$^{\Psi}$	= Survival ↑ Malformation = Shell length	(Onitsuka et al., 2018)
Haliotis discus hannai (Mollusc)	719.5 ± 14.5$^{\sigma}$	7.8 ± 0.01$^{\sigma}$	779.6 ± 145.7$^{\sigma}$; 583.8-1035.0$^{\Psi}$	7.78 ± 0.07$^{\sigma}$; 7.66-7.88$^{\Psi}$	= Survival = Malformation ↓ Shell length	(Onitsuka et al., 2018)
Haliotis discus hannai (Mollusc)	719.5 ± 14.5$^{\sigma}$	7.8 ± 0.01$^{\sigma}$	780.6 ± 235.4$^{\sigma}$; 490.6-1243.9$^{\Psi}$	7.79 ± 0.11$^{\sigma}$; 7.59-7.95$^{\Psi}$	= Survival = Malformation ↓ Shell length	(Onitsuka et al., 2018)
Mercenaria mercenaria (Mollusc, larvae)	509 ± 70$^{\sigma}$	7.97 ± 0.07$^{\sigma}$	1870 ± 1470$^{\sigma}$	7.43 ± 0.65$^{\sigma}$	↓ Survival = Growth rate ↓ Development	(Clark and Gobler, 2016)
Mytilus californianus (Mollusc, larvae)	399 (range NR)	8.04 (range NR)	482 ± 155$^{\Psi}$	7.97 ± 0.12$^{\Psi}$	= Development	(Frieder et al., 2014)
Mytilus californianus (Mollusc, larvae)	1542 (range NR)	7.51 (range NR)	1542 ± 574$^{\Psi}$	7.51 ± 0.15$^{\Psi}$	= Survival = Development = Larval shell length	(Frieder et al., 2014)
Mytilus edulis (Mollusc)	441 ± 8$^{\sigma}$	8.13 ± 0.01$^{\sigma}$	NR	7.92 ± 0.2$^{\sigma}$; 7.66-8.23$^{\Psi}$	= Haemolymph pCO_2 = Haemolymph pH = Haemolymph bicarbonate ↑ Metabolic rate ↑ SOD activity ↑ Neutral red retention = TBARS activity	(Mangan et al., 2017)
Mytilus edulis (Mollusc)	1276 ± 72$^{\sigma}$	7.70 ± 0.02$^{\sigma}$	NR	7.70 ± 0.3$^{\sigma}$; 7.66-8.23$^{\Psi}$	= Haemolymph pCO_2 = Haemolymph pH = Haemolymph bicarbonate ↑ Metabolic rate ↑ SOD activity ↑ Neutral red retention = TBARS activity	(Mangan et al., 2017)
Mytilus galliprovincialis (Mollusc, larvae)	1637 (range NR)	7.48 (range NR)	1426 ± 533$^{\Psi}$	7.54 ± 0.15$^{\Psi}$	= Survival = Development ↑ Larval shell length	(Frieder et al., 2014)
Mytilus galliprovincialis (Mollusc, larvae)	784-886$^{\Psi}$	7.78-7.82$^{\Psi}$	436-1252$^{\Psi}$	7.64-8.05$^{\Psi}$	= Shell length ↑ Abnormal development	(Kapsenberg et al., 2018)

Mytilus galliprovincialis (Mollusc, larvae)	784-886$^{\Psi}$	7.78-7.82$^{\Psi}$	320-2322$^{\Psi}$	7.39-8.16$^{\Psi}$	= Shell length ↑ Abnormal development	(Kapsenberg et al., 2018)
Mytilus galliprovincialis (Mollusc, larvae)	784-886$^{\Psi}$	7.78-7.82$^{\Psi}$	311-2432$^{\Psi}$	7.37-8.17$^{\Psi}$	= Shell length ↑ Abnormal development	(Kapsenberg et al., 2018)
Mytilus galliprovincialis (Mollusc, larvae)	364-386$^{\Psi}$	8.09-8.11$^{\Psi}$	350-3147$^{\Psi}$	7.26-8.13$^{\Psi}$	= Shell length ↑ Abnormal development	(Kapsenberg et al., 2018)
Mytilus galliprovincialis (Mollusc, larvae)	2204-2253$^{\Psi}$	7.40-7.41$^{\Psi}$	320-2295$^{\Psi}$	7.39-8.16$^{\Psi}$	= Shell length ↓ Abnormal development	(Kapsenberg et al., 2018)
Mytilus galliprovincialis (Mollusc, larvae)	373-389$^{\Psi}$	8.09-8.11$^{\Psi}$	352-3193$^{\Psi}$	7.25-8.13$^{\Psi}$	= Shell length ↑ Abnormal development	(Kapsenberg et al., 2018)
Mytilus galliprovincialis (Mollusc, larvae)	2081-2242$^{\Psi}$	7.40-7.43$^{\Psi}$	421-2226$^{\Psi}$	7.40-8.06$^{\Psi}$	= Shell length ↓ Abnormal development	(Kapsenberg et al., 2018)
Acropora formosa (Coral)	392 ± 55$^{2\sigma}$	8.02 ± 0.05$^{2\sigma}$	487 ± 397$^{2\sigma}$	7.97 ± 0.27$^{2\sigma}$	↑ Calcification rate	(Chan and Eggins, 2017)
Acropora hyacinthus (Coral)	366 ± 4$^{\varphi}$	8.07 ± 0.004$^{\varphi}$	Day: 257 ± 5$^{\varphi}$; Night: 507 ± 9$^{\varphi}$	Day: 8.20 ± 0.006$^{\varphi}$; Night: 7.96 ± 0.006$^{\varphi}$	= Calcification rate	(Comeau et al., 2014)
Acropora hyacinthus (Coral)	638 ± 11$^{\varphi}$	7.88 ± 0.006$^{\varphi}$	Day: 507 ± 9$^{\varphi}$; Night: 983 ± 15$^{\varphi}$	Day: 7.96 ± 0.006$^{\varphi}$; Night 7.71 ± 0.006$^{\varphi}$	= Calcification rate	(Comeau et al., 2014)
Acropora hyacinthus	983 ± 15$^{\varphi}$	7.71 ± 0.006$^{\varphi}$	Day: 366 ± 4$^{\varphi}$; Night: 1839 ± 24$^{\varphi}$	Day: 8.07 ± 0.004$^{\varphi}$; Night: 7.47 ± 0.007$^{\varphi}$	↑ Calcification rate	(Comeau et al., 2014)
Goniopora sp. (Coral)	343–419$^{\varphi}$	8.03-8.10$^{\varphi}$	238–1252$^{\varphi}$	7.62-8.22$^{\varphi}$	= Calcification rate = Photosynthetic rate = Respiration rate = Calcifying fluid carbonate chemistry	(Cornwall et al., 2018)
Goniopora sp. (Coral)	932–1270$^{\varphi}$	7.62-7.74$^{\varphi}$	376–3323$^{\varphi}$	7.23-8.07$^{\varphi}$	= Calcification rate = Photosynthetic rate = Respiration rate = Calcifying fluid carbonate chemistry	(Cornwall et al., 2018)

Seriatopora caliendrum (Coral, larvae/recruits)	456.0 ± 71.3$^{\varphi}$	8.00 ± 0.05$^{\varphi}$	Day: 420.4 ± 38.0$^{\varphi}$; Night: 596.8 ± 57.3$^{\varphi}$	Day: 8.02 ± 0.03$^{\varphi}$; Night: 7.90 ± 0.04$^{\varphi}$	↑ Calcification	(Dufault et al., 2012)
Seriatopora caliendrum (Coral, larvae/recruits)	464.0 ± 14.1$^{\varphi}$	7.98 ± 0.01$^{\varphi}$	Day: 448.2 ± 10.6$^{\varphi}$; Night: 845.0 ± 8.0$^{\varphi}$	Day: 8.00 ± 0.01$^{\varphi}$; Night: 7.76 ± 0.00$^{\varphi}$	↑ Survival	(Dufault et al., 2012)
Acropora cervicornis (Coral)	NR	8.05 ± 0.00	NR	8.05 ± 0.10	↑ Calcification rate	(Enochs et al., 2018)
Acropora cervicornis (Coral)	NR	7.80 ± 0.00	NR	7.80 ± 0.10	= Calcification rate	(Enochs et al., 2018)
Acropora cervicornis (Coral)	NR	7.80 ± 0.00	NR	7.80 ± 0.20	= Calcification rate	(Enochs et al., 2018)
Arbacia lixula (Echinoderm)	608.8 ± 19.1$^{\varphi}$; 492.5-779.3$^{\Psi}$	8.04 ± 0.01$^{\varphi}$; 7.95-8.12$^{\Psi}$	1742.3 ± 352.7$^{\varphi}$; 599-5497.4$^{\Psi}$	7.72 ± 0.07$^{\varphi}$; 7.14-8.04$^{\Psi}$	↓ Coelomic fluid pH ↑ Coelomic fluid pCO_2 ↓ Coelomic fluid bicarbonate	(Small et al., 2016)
Paracentrotus lividus (Echinoderm)	608.8 ± 19.1$^{\varphi}$; 492.5-779.3$^{\Psi}$	8.04 ± 0.01$^{\varphi}$; 7.95-8.12$^{\Psi}$	1742.3 ± 352.7$^{\varphi}$; 599-5497.4$^{\Psi}$	7.72 ± 0.07$^{\varphi}$; 7.14-8.04$^{\Psi}$	↓ Coelomic fluid pH ↑ Coelomic fluid pCO_2 ↑ Coelomic fluid bicarbonate	(Small et al., 2016)
Paracentrotus lividus (Echinoderm, larvae)	415.5 (range NR)	Hour 0: 8.02 ± 0.02$^{\sigma}$; Hour 12: 8.00 ± 0.02$^{\sigma}$	1038.1 (range NR)	Hour 0: 7.67 ± 0.05$^{\sigma}$; Hour 12: 8.00 ± 0.04$^{\sigma}$	= Survival = Growth = Stomach volume ↑ Development speed	(García et al., 2018)
Paracentrotus lividus (Echinoderm, larvae)	332.0 (range NR)	Hour 0: 8.11 ± 0.02$^{\sigma}$; Hour 12: 8.12 ± 0.02$^{\sigma}$	1062.5 (range NR)	Hour 0: 7.67 ± 0.04$^{\sigma}$; Hour 12: 8.00 ± 0.04$^{\sigma}$	↓ Swimming = Settled ↓ Survival	(García et al., 2018)
Strongylocentrotus purpuratus (Echinoderm, larvae)	Day 1: 317.3; Day 4: 300.1; Day 7: 311.0	Day 1: 8.10; Day 4: 8.10; Day 7: 8.10	Day 1: 315.3; Day 4: 861.2; Day 7: 1065.5	Day 1: 8.09; Day 4: 7.70; Day 7: 7.62	= Survival ↓ Length = Genetic responses	(Garrett et al., 2020)

Strongylocentrotus purpuratus (Echinoderm, larvae)	Day 1: 1444.0; Day 4: 1406.9; Day 7: 1417.8	Day 1: 7.50; Day 4: 7.50; Day 7: 7.50	Day 1: 1362.0; Day 4: 2644.4; Day 7: 2331.1	Day 1: 7.51; Day 4: 7.23; Day 7: 7.30	↑ Survival = Length = Genetic responses	(Garrett et al., 2020)
Strongylocentrotus purpuratus (Echinoderm, larvae)	Day 1: 4760.7; Day 4: 4588.0; Day 7: 5203.4	Day 1: 7.00; Day 4: 7.00; Day 7: 6.95	Day 1: 4604.1; Day 4: 8007.5; Day 7: 7140.6	Day 1: 7.01; Day 4: 6.77; Day 7: 6.82	↑ Survival ↓ Length ↕ Genetic responses	(Garrett et al., 2020)
Balanus improvisus (Crustacean)	970 (range NR)	7.70 ± 0.03$^{\sigma}$	Day: 625 ± 1$^{\sigma}$; Night 1665 ± 20$^{\sigma}$	Day: 7.90 ± 0.02$^{\sigma}$; Night: 7.50 ± 0.03$^{\sigma}$	= Mean growth rate ↑ Variance in growth rate = Mean survival = Variance in survival = Mean shell mineral composition ↑ Variance in shell mineral composition = Mean shell strength = Variance in shell strength	(Eriander et al., 2016)
Balanus improvisus (Crustacean)	359 ± 37^$^{\sigma}$	8.10 ± 0.08$^{\sigma}$	Day: 100 ± 54^$^{\sigma}$; Night: 978 ± 60^$^{\sigma}$	8.03 ± 0.39$^{\sigma}$	= Growth rate = Final size	(Johnson et al., 2020)
Balanus improvisus (Crustacean)	988 ± 63^$^{\sigma}$	7.72 ± 0.07$^{\sigma}$	Day: 313 ± 54^$^{\sigma}$; Night: 1758 ± 159^$^{\sigma}$	7.83 ± 0.32$^{\sigma}$	= Growth rate = Final size	(Johnson et al., 2020)
Balanus improvisus (Crustacean)	392 ± 47^$^{\sigma}$	8.05 ± 0.09$^{\sigma}$	Day: 169 ± 49^$^{\sigma}$; Night: 992 ± 97^$^{\sigma}$	8.04 ± 0.32$^{\sigma}$	= Growth rate = Final size	(Johnson et al., 2020)
Balanus improvisus (Crustacean)	1077 ± 73^$^{\sigma}$	7.74 ± 0.09$^{\sigma}$	Day: 342 ± 46^$^{\sigma;}$ Night: 1980 ± 103^$^{\sigma}$	7.82 ± 0.33$^{\sigma}$	= Growth rate = Final size	(Johnson et al., 2020)
Paradella dianae (Crustacean)	NR	7.60 ± 0.005$^{\varphi}$	NR	7.61 ± 0.027$^{\varphi}$	↓ Survival ↓ Oxygen uptake rate ↓ Time spent swimming ↓ Swimming speed ↑ Time spent crawling = Time spent resting = Time spent conglobating = Sheltering	(Alenius and Munguia, 2012)

Electra pilosa (Bryozoan)	359 ± 37^σ	8.10 ± 0.08σ	Day: 100 ± 54^σ; Night: 978 ± 60^σ	8.03 ± 0.39σ	= Growth rate = Growth efficiency	(Johnson et al., 2020)
Electra pilosa (Bryozoan)	988 ± 63^σ	7.72 ± 0.07σ	Day: 313 ± 54^σ; Night: 1758 ± 159^σ	7.83 ± 0.32σ	= Growth rate = Growth efficiency	(Johnson et al., 2020)
Electra pilosa (Bryozoan)	392 ± 47^σ	8.05 ± 0.09σ	Day: 169 ± 49^σ; Night: 992 ± 97^σ	8.04 ± 0.32σ	= Growth rate = Growth efficiency	(Johnson et al., 2020)
Electra pilosa (Bryozoan)	1077 ± 73^σ	7.74 ± 0.09σ	Day: 342 ± 46^σ; Night: 1980 ± 103^σ	7.82 ± 0.33σ	= Growth rate = Growth efficiency	(Johnson et al., 2020)

Table 1: Experimental studies assessing the effect of fluctuating carbonate chemistry conditions on marine animals. Studies were selected from a Web of Science literature search with the search term "TOPIC: (fluctuati* OR varia* OR oscillati* OR cycl*) AND (pH OR CO2 OR carbon dioxide OR pCO2) AND (marine OR ocean)" from the past 10 years. Studies were omitted if they did not compare responses of an animal species between static and fluctuating carbonate chemistry regimes, or if there was no overlap between the pH and/or pCO_2 levels in each regime (i.e. a comparison between a present-day static pH regime and an acidified fluctuating regime would not be included). Studies including multiple experimental species/regimes are included as separate rows. Note the differences in pH/pCO_2 values, ranges and manner of reporting between studies. Under treatment conditions: "^" = pCO_2 reported as ppm; "NR" = Not reported; "σ" = mean ± standard deviation; "φ" = mean ± standard error; "ς" = mean ± coefficient of variation; "ψ" = minimum to maximum or mean ± range. Under "Effects of fluctuating pH", "↑" = increase in parameter; "↓" = decrease in parameter; "↕" = change in parameter for which direction cannot be determined; "=" = no change in parameter; blue = no change in any parameter; pale orange = a change in fewer than half of the parameters measured; dark orange = a change in half or more than half of the parameters measured. Changes in parameters are classed according to whether the authors of the original study found them to be statistically significant.

et al., 2013; Torres et al., 2021), day/night variation is more applicable to habitats where cycles of photosynthesis/respiration and calcification/dissolution are sunlight-mediated, such as coral reefs (Enochs et al., 2018). It is worth nothing that Guadayol et al. (2014) measured more frequent hourly fluctuations of 0.05–0.19 pH units across a coral reef, therefore incorporating more high-frequency variation into experimental regimes for these habitats may improve their environmental relevance, although this is methodologically challenging. The pattern of fluctuations is likely key in determining biological responses, as time spent above tolerance limits can be fundamental in determining the severity of biological effects: pCO_2 fluctuations induced additional negative impacts on the calcifying abalone *Haliotis discus hannai* when sustained for longer periods above levels associated with aragonite saturation state < 1.1 (1000-1300 µatm pCO_2) (Onitsuka et al., 2018).

Additionally, the predictability of events can influence the ability of intertidal organisms to adapt to them (Branch et al., 1987). Hence, studies utilising random variation (e.g. Alenius and Munguia, (2012)) may produce more severe biological effects than would occur in the natural environment currently, although as the variability of carbonate chemistry fluctuations is predicted to increase with climate change (Burger et al., 2020; McNeil and Sasse, 2016), particularly in coastal regions (Fagundes et al., 2020), combining more random variation with acidified regimes may be more relevant to future ocean conditions. Other studies utilise carbonate chemistry variation *in situ* (e.g. Small et al. (2016)), which is of course more environmentally relevant but harder to control and therefore requires regular, accurate measurements to understand the conditions organisms are exposed to. This also makes it harder to distinguish the cause of biological effects, as carbonate chemistry often fluctuates in tandem with other parameters e.g. light and temperature (Blackford et al., 2017).

Not all studies in Table 1 report complete carbonate chemistry data: several neglect to report pH and/or pCO_2, and although not shown here many did not report parameters such as alkalinity or calcite/aragonite saturation state. These are considered key in understanding the biological impacts of OA (Melzner et al., 2020, 2009), particularly for calcifying species (Orr et al., 2005), and therefore are likely to also be important in understanding the impacts of other forms of altered carbonate chemistry. There is also much variation in how these parameters are reported (i.e.

standard deviation/error, coefficient of variation and/or range). Knowing the full range of conditions experienced by the organisms and the time over which these took place is key in order to account for the time spent above tolerance limits and the predictability of fluctuations as described above. Regular measurements of key carbonate chemistry parameters throughout the duration of the experiment would therefore aid in interpreting biological responses: several studies report measurements at regular timepoints throughout the experiment and/or present these graphically (e.g. Laubenstein et al., 2020; Mangan et al., 2017; Small et al., 2016). It would also be beneficial to report the carbonate chemistry conditions at the exact time biological measurements are taken, or to take measurements at multiple points of a fluctuating regime, in order to establish whether responses are truly related to fluctuations or to conditions at the point of sampling.

The species considered in fluctuating pCO_2/pH experiments over the past ten years, as outlined in Table 1, are biased towards fish and molluscs (62.7 **%** of total experiments), and within these there are only 8 species of fish and 7 species of mollusc. Note that “experiment” may refer to regimes within the same publication which differed either in species or carbonate chemistry treatments, which have been allocated their own row in Table 1. This may be partially due to selection and publication bias as molluscs are generally sensitive to OA-induced carbonate chemistry alterations (Byrne, 2011), and almost all (18/21) experiments on molluscs demonstrated differing responses between a static and fluctuating regime. However, this was not the case for fish as almost half (12/26) of experiments reported no effects of fluctuating pCO_2/pH. Research into fish has focused on those living on coral reefs, which undergo strong day/night fluctuations in carbonate chemistry due to light-mediated photosynthesis/respiration and calcification/dissolution processes (Enochs et al., 2018). Similarly, all molluscs in Table 1 can be found in coastal regions where carbonate chemistry can be strongly influenced by tidal flux (Blackford et al., 2017). However, there are other taxa in these habitats which have not yet been considered: for example, polychaete worms are commonly found in intertidal habitats and undergo different responses to seawater acidification compared to other marine invertebrates (Nielson et al., 2019; Ricevuto et al., 2015), and therefore could be expected to respond differently to fluctuating carbonate chemistry.

The number of experiments where a fluctuating pCO_2/pH regime induced biological effects differed greatly between taxa (Figure 3). Effects have not been classed as "positive" or "negative" due to the complexity of categorising responses; for example, an increased growth rate could correspond to greater competitiveness but also greater food requirements. Overall, 40 % of experiments reported no differences in endpoints between static and fluctuating regimes, 38.6 % resulted in less than half of measured endpoints measured being affected, and 21.4 % resulted in half or more measured endpoints being affected. Echinoderms and molluscs had the greatest proportion of experiments demonstrating biological impacts of fluctuations (85.7 % for both), although this is based on only three studies for echinoderms. The least sensitive taxa were bryozoans and crustaceans, for which 100 % and 66.7 % of fluctuating regimes respectively reported no biological effect, but it would be speculative to draw conclusions for a higher taxonomic rank than the species studied here, as only one species of bryozoan and two species of crustacean were studied. Determining whether effects are comparable between experiments is complex due to the variation in methodology as described above; here, I will present an overview of the findings.

Approximately half of experiments on fish measured some effect of fluctuating pCO_2/pH (Figure 3), often on metabolic parameters or behaviour, although these same endpoints were found to be affected differently in the same species between different fluctuating regimes. Jarrold et al. (2017) observed no behavioural alterations in juvenile *Acanthochromis polyacanthus* exposed to fluctuating pCO_2 ranges of 681-1304 µatm or 667-1328 µatm compared to a static pCO_2 regime, but a wider range of 482-1591 µatm induced an increase in absolute lateralisation behaviour despite all regimes having similar mean pCO_2 levels and pattern of fluctuations. This supports the hypothesis that time spent above tolerance limits may be a key determinant of responses to fluctuating conditions (Onitsuka et al., 2018), although there is currently significant debate over the methodology used to assess the effect of high pCO_2/low pH conditions on fish behaviour (Clark et al., 2020a, 2020b; Munday et al., 2020). In contrast, other fish species show near-identical responses to narrower and wider pCO_2 ranges in terms of growth rate (Davidson et al., 2016), development and survival (Jarrold and Munday, 2018). Fish appear to be broadly tolerant to pCO_2/pH fluctuations, potentially due to being active ectothermic animals (Melzner et al., 2009) and as the majority of studied species are from coral reefs where diel fluctuations are

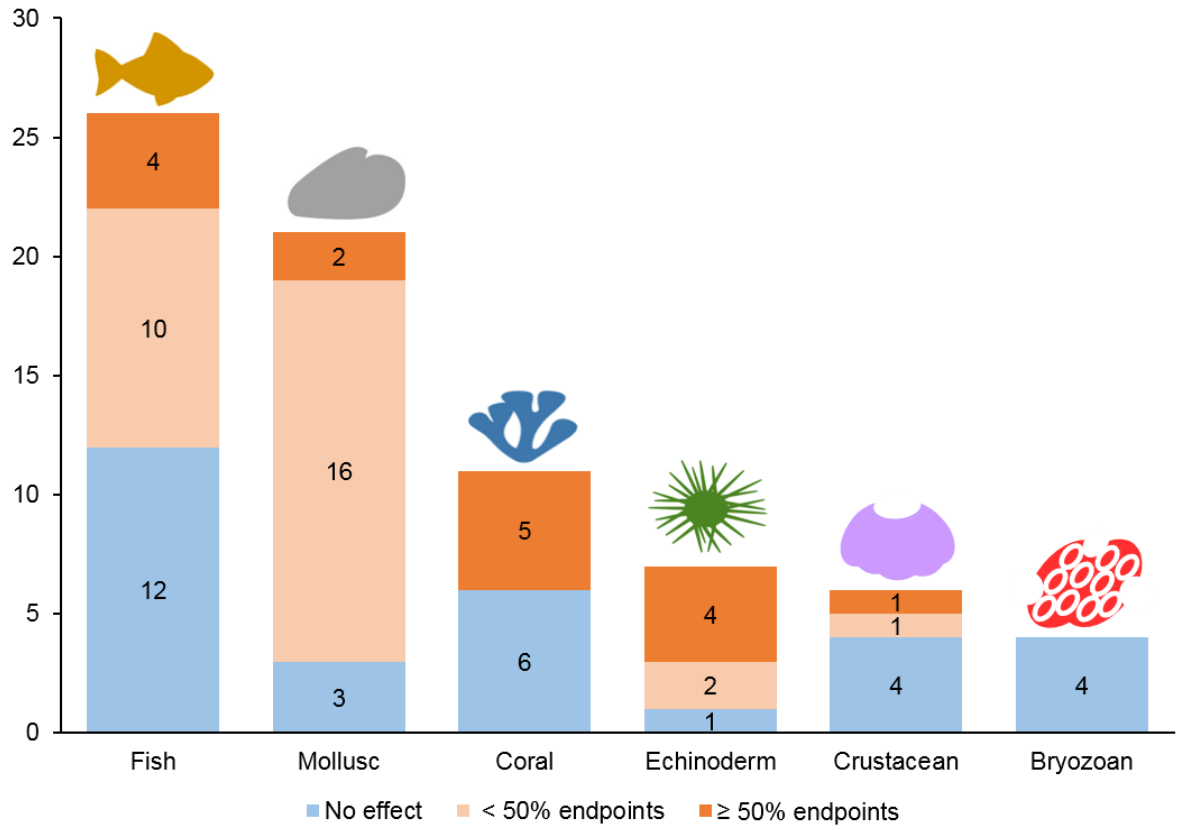

Figure 3: Number of experimental regimes from Table 1 demonstrating no effect, effects on less than 50 % of endpoints measured, or effects on 50 % or more endpoints measured. Colours for each taxa and category correspond to Table 1.

common (Enochs et al., 2019; Torres et al., 2021). Fluctuating pCO_2/pH conditions may also be protective to fish in OA conditions: several regimes report "recovery" of physiological and behavioural endpoints in fluctuating versus static OA regimes (Hannan et al., 2021, 2020; Laubenstein et al., 2020). This may be partly due to the reduced time spent in OA conditions as these studies utilised fluctuations above and below OA-predicted mean values.

In contrast, 85.7 % of experiments on molluscs measured some effect of a fluctuating carbonate chemistry regime (Figure 3); this mirrors findings from OA studies which generally find adverse effects for molluscs due to their activity as calcifiers and general poor acid-base regulatory ability (Kroeker et al., 2010; Melzner et al., 2009). As poor acid-base regulators, such as the mussel *Mytilus edulis* (Mangan et al., 2019), tend to mirror seawater acid-base changes in their internal environment, they may therefore be adversely affected by fluctuations in a similar manner to OA. Although the only

study to assess acid-base status in a fluctuating experiment for molluscs found no effects (Mangan et al., 2017), measurements were taken at the low pCO_2 point of the fluctuating cycle, therefore haemolymph pCO_2 would be expected to be similarly low. This demonstrates the importance of taking measurements at different points of a fluctuating cycle as some biological responses may only coincide with certain pCO_2/pH values. The increase in metabolic rate in *M. edulis* induced by a fluctuating regime (Mangan et al., 2017) may indicate changes in energy demand from intracellular acid-base regulation (de Vooys, 1987), rather than extracellular, in order to protect key physiological processes; the closely related *Mytilus galloprovincialis* fully compensates intracellular pH in OA conditions (Zittier et al., 2018). However, it is unclear whether this occurs during the short-term fluctuations characteristic of coastal habitats as full intracellular pH compensation takes several days during exposure to a stable low pH regime (Michaelidis et al., 2005).

Few other studies have investigated the acid-base regulatory responses of organisms to fluctuating pCO_2/pH regimes (Table 1), despite this being considered a significant factor in determining resilience to OA-induced carbonate chemistry changes (Widdicombe and Spicer, 2008). The magnitude of effects of fluctuating pCO_2/pH may be partly dependent on the method by which organisms regulate their internal acid-base status: non-bicarbonate protein buffering and respiratory control by the urchin *Arbacia lixula* resulted in greater extracellular pH compensation in a fluctuating regime than the bicarbonate buffering employed by *Paracentrotus lividus* (Small et al., 2016). This is in contrast to the paradigm that organisms lacking strong bicarbonate-buffering abilities are the most at risk from OA (Melzner et al., 2009). Animals with multiple mechanisms of acid-base compensation may therefore prove most successful in future acidified but variable coastal regions.

Another common impact of OA-induced carbonate chemistry alterations is reduced calcification, but few studies in Table 1 measured this endpoint in fluctuating regimes. The effect of fluctuating carbonate chemistry on mollusc or echinoderm calcification activity is yet to be investigated, but reductions in growth rate and length were common in these taxa, suggesting that calcification could be impacted similarly to OA regimes. In contrast, all experiments with corals found either no effect or an increase in calcification rates in fluctuating regimes; all of these regimes utilised low pCO_2 during

the day and high pCO_2 at night as naturally occurs on coral reefs. Chan and Eggins (2017) hypothesise that this effect could be due to light-enhanced calcification or night-time carbon storage facilitating daytime calcification when dissolved inorganic carbon is lower (Dufault et al., 2012). Despite this, results in OA scenarios for corals were mixed, with some species demonstrating increased calcification (Comeau et al., 2014) and others showing no effect (Cornwall et al., 2018; Enochs et al., 2018) between static acidified and fluctuating acidified regimes. This suggests that the benefits of fluctuating carbonate chemistry for coral calcification may not be maintained for all species in future OA conditions. The phenomenon of light-enhanced calcification is not known to occur in non-photosynthesising calcifiers, therefore the overall lower bioavailability of carbonate throughout natural fluctuating regimes compared to static mean open ocean pH (Duarte et al., 2013) may mean that calcification rates are lower for these taxa in their natural environment than in experiments utilising static pCO_2/pH regimes.

Conversely, a recent review found that exposure to a variable environmental history reduced sensitivity to OA for 76 % of biological responses for non-photosynthesising calcifiers (Kapsenberg and Cyronak, 2019), which are often suggested to be most at risk from future OA (Kroeker et al., 2013; Orr et al., 2005); therefore, populations of these organisms living in variable coastal regions may be more tolerant to future OA. Götze et al. (2014) suggest that adaptation to extremes during pCO_2/pH fluctuations are responsible for the tolerance of the bivalves *Crassostrea virginica* and *Mercenaria mercenaria* to long-term exposure to elevated pCO_2. Despite this, many coastal species are thought to exist close to physiological tolerance limits (Tomanek and Helmuth, 2002), and animals already living at the limits of their tolerance to hypercapnia may be the most threatened by OA (Widdicombe et al., 2011), therefore adaptations to short-term exposure may not always be sufficient for longer term hypercapnic events. For example, the peanut worm, *Sipunculus nudus*, undergoes bicarbonate accumulation and metabolic depression to survive short periods of hypercapnia, which allow it to survive during low tide (Langenbuch and Portner, 2002; Portner et al., 2000), but over longer-term exposures these adaptations are ineffective (Langenbuch and Pörtner, 2004). Although the exact physiological mechanisms are unclear for many species, it is clear that they must have adaptations to cope with the pCO_2/pH fluctuations which occur in their natural habitats. Crucially, the findings from

studies reported in Table 1 suggest that the static regimes most commonly used when assessing the impacts of OA may not fully reflect the complexity of biological responses for organisms inhabiting variable environments, particularly if the combination of future OA and increased variation drives carbonate chemistry to greater extremes (Angeles Gallego et al., 2018; Kwiatkowski and Orr, 2018; McNeil and Sasse, 2016).

1.5. Changing carbonate chemistry affects the chemistry of coastal contaminants

Alongside OA, fluctuating carbonate chemistry, and a host of other stressors, coastal organisms must contend with chronic and emerging contaminants. These vary from trace metals released from mining and industrial activities, to pesticides from farmland run-off, to pharmaceutical and illegal drugs from sewage waste, encompassing a vast range of organic and inorganic compounds (Nyström, 2019). Many are toxic to marine life even at low concentrations (Mearns et al., 2019). For some contaminants, changes in water carbonate chemistry can alter their chemical behaviour, in turn altering their bioavailability and subsequently toxicity (Rendal et al., 2011; Stockdale et al., 2016). Mechanisms of toxicity, organism defence strategies, and how these are affected by ocean carbonate chemistry will be discussed.

1.5.1. Ocean acidification alters the biological uptake potential of metals

Many trace metals present in the marine environment are essential for biological function, but some are toxic to marine life in certain forms or at high concentrations, or are toxic at low concentrations with no known biological function (Shah, 2021). Marine animals can take up metals through tissues exposed to the water, such as the gills or dermis, or from the gut via ingested material. Uptake into cells occurs via dedicated trans-membrane transporters, open-gate chemiosmotic pores, or endocytosis (Kim et al., 2008). Metals in the body are then handled by metal-binding ligands, trans-membrane transport elements and/or sequestration, with biologically essential metals regulated and transported by chaperones and proteins to prevent toxicity (O'Halloran and Culotta, 2000).

Mechanisms of metal toxicity can be broadly divided into two categories, both requiring free metal ions in solution. Redox-active metals (iron, copper, chromium, vanadium

and cobalt) catalyse the production of reactive oxygen species, which cause oxidative stress, via cycling between redox states (Valko et al., 2005). For example, in the presence of hydrogen peroxide (H_2O_2), which is present in cells as a by-product of metabolism, copper ions undergo Fenton-like redox cycling between Cu^+ and Cu^{2+}, converting H_2O_2 into superoxide (O_2^-) and catalysing the production of the strong oxidant Cu^{3+} and hydroxyl radicals which damage proteins, lipids and DNA (Pham et al., 2013). A similar Fenton process occurs for iron, which cycles between Fe^{3+} and Fe^+, generating reactive oxygen species in the process (Stohs and Bagchi, 1995). Reactive oxygen species damage lipids, proteins and DNA via oxidation (Lesser, 2006).

The second major mechanism of metal toxicity is covalent binding to functional groups, disrupting the structure and function of essential macromolecules, with some non-essential metals further complicating their removal by binding competitively (Nies, 1999). The primary route of toxicity for mercury, cadmium and nickel is binding to the sulfhydryl groups of proteins and depletion of glutathione (Valko et al., 2005). Excessive metal concentrations can even disrupt metalloproteins by displacing or substituting essential metal cofactors, preventing these metal regulatory mechanisms from functioning (O'Halloran and Culotta, 2000). Metals can also interfere with ionic regulatory mechanisms: for example, copper has high affinity for the sulfhydryl groups contained in Na^+/K^+ ATPases, disrupting acid-base homeostasis and osmoregulation (Katranitsas et al., 2003; Shekh et al., 2019; Viarengo et al., 1996). Excess copper ions also have high affinity for nuclear DNA due to copper's role in strand folding (Linder, 2012); as well as causing DNA damage via strand breakage (Lewis et al., 2016), this can depress or degrade key genes, such as those related to olfaction in fish (Julliard et al., 1996; Tilton et al., 2008).

Mechanisms of metal detoxification differ between taxa: for example, copper is generally sequestered by bivalves, barnacles and aquatic insects, and actively regulated by fish, decapod crustaceans and algae (Brix and Deforest, 2000). The major defensive element against metal toxicity in cells is the tripeptide glutathione (GSH), the most abundant cellular thiol (Ercal et al., 2001). Its thiolate sulphur atom has high affinity for many heavy metal ions, forming complexes with many metals soon after they enter cells. This ligand binding restricts the ability of metals to bind to cellular

components or to become involved in Fenton-like cycling (Corazza et al., 1996). Another metal detoxification mechanism is metallothionein, a cysteine-rich protein identified in virtually all living organisms (Coyle et al., 2002). Metallothioneins can sequester metals such as cadmium and mercury, although this function appears secondary to its role in regulating zinc homeostasis (Vignesh and Deepe, 2017). Metal binding allows expulsion or transportation to tissues better equipped to detoxify the metal, such as the mineralised granules in the dermal tissue of metal-resistant *Nereis diversicolor* which permanently immobilise metals (Kim et al., 2008). Cellular efflux pumps can be specific, such as Znt1 for zinc (Palmiter, 2004), or remove a range of metals, such as the multixenobiotic resistance protein in freshwater clams (Achard et al., 2004). Antioxidant compounds, such as glutathione and the enzyme superoxide dismutase, can remediate oxidative stress, but excessive levels of ROS or of metals themselves can overwhelm or disrupt these defence mechanisms (Lesser, 2006). The different biochemical environments within membrane-bound organelles are also essential in regulating metal behaviour, providing controlled redox potential, ion composition and pH for specific metal-ligand reactions (Mason et al., 1995).

Changes in carbonate chemistry, including those associated with OA, affect the distribution of metals amongst different chemical forms, known as their speciation (Stockdale et al., 2016). Different metal species vary in their potential for uptake by and toxicity to marine organisms, with free ions generally the most bioavailable as they are not already bound to other matter. There is a growing consensus that metals dominantly complexed by hydroxide (e.g. iron and aluminium) or carbonate (e.g. copper and rare earths) will be influenced by the OA-associated decline in these ligands, predicted to be 82 % and 77 % respectively, whereas metals that exist mainly as complexes with chloride (e.g. cadmium and silver) or in free form (e.g. manganese) will be far less affected (Byrne et al., 1988; Gledhill et al., 2015; Millero et al., 2009). Concentrations of organically complexed forms of metals are also set to change, although these typically show much lower sensitivity to OA (Stockdale et al., 2016), and are less biologically available due to already being complexed by organic matter. It has been suggested that the increased protonation of binding sites on organic material at a lower pH may also reduce the availability of binding sites for metals, thereby increasing the proportion of metals existing in their free form (Millero et al., 2009); however, others suggest that changes in organic complexation will have only

mild to moderate effects on speciation (Gledhill et al., 2015; Stockdale et al., 2016). Additionally, decreasing pH increases the solubility of metals such as iron, thereby increasing its bioavailability (Millero et al., 2009).

There is a growing body of evidence that changes in ocean pH have the potential to alter the biological uptake of metals via effects on speciation, but experimental investigations into the toxicity of metals to marine biota in OA conditions are limited in number. There have been several studies in recent years concerning the toxicity of copper in OA conditions (e.g. Cao et al., 2019; Gao et al., 2017; Lewis et al., 2016; Marangoni et al., 2019; Nielson et al., 2019; Scanes et al., 2018), although these have mostly considered marine bivalves; results from these studies are discussed below. Free ion concentrations of aluminium, which complexes with hydroxide, may increase by a factor of 21 (Stockdale et al., 2016), but there has been little investigation into the toxicity of aluminium to marine biota in an OA scenario despite free ions generally being among the most biologically available forms of metals (Paquin et al., 2000). The most bioavailable form of lead, the free ion Pb^{2+}, is also set to increase by between 10 and 50 % (Millero et al., 2009; Stockdale et al., 2016). Although Stockdale et al. (2016) suggest that this change is not sufficient to increase toxic effects in open ocean environments, this may still be a concern for contaminated coastal habitats: bioconcentration of lead in soft tissues of the mussel *Mytilus edulis* shows significant correlation with decreasing pH between pH 7.94 and 7.16 (Belivermiş et al., 2020), posing a risk for consumers of seafood harvested from coastal areas where pH reaches such low levels. This highlights the issue that the majority of work concerning metal toxicity in future OA conditions focuses on changes in open ocean environments, rather than the coastal habitats where pH regularly reaches lower extremes (Duarte et al., 2013; Torres et al., 2021) and metal contamination is often more prevalent (Landrigan et al., 2020; Nriagu and Pacyna, 1988).

1.5.2. Can changing carbonate chemistry affect the uptake and accumulation potential of other contaminants?

As well as metals, an enormous variety of other contaminants are present in the marine environment, including pharmaceuticals, pesticides, fertilisers, oils, fuels, industrial chemicals and physical debris (Alves et al., 2021). Some have been entering

the oceans for many years, such as oil from spills, but marine organisms are regularly exposed to new contaminants due to the speed and diversity of chemicals developed for anthropogenic use (Nyström, 2019; Tang et al., 2019). The effects of these contaminants are extremely wide-ranging due to their variety, from impacts at a genetic and transcriptomic level to organism or population-level impacts (Mearns et al., 2019). In freshwater field and laboratory studies, there is compelling evidence supporting the paradigm that changes in pH can alter the uptake, toxicity, bioconcentration and accumulation of certain non-metal contaminants (Rendal et al., 2011; Simon and Beevers, 1952). Here, I will discuss how OA, specifically the associated decrease in seawater pH, could be expected to similarly alter the chemical behaviour of pH-sensitive non-metal contaminants.

The principle site of contaminant uptake for aquatic organisms is the gills (Stott et al., 2015), although uptake through the skin may also occur for some taxa (Karlsson et al., 2017). Transport from the water into the gills, and between biological compartments within the organism, occurs via transporter-mediated processes, such as the ATP-binding cassette and solute-carrier transporter families (Dobson and Kell, 2008), although these processes are not well understood in aquatic animals (Armitage et al., 2017), and passive diffusion via electrostatic interactions with lipid membranes, which may be dominant (Chang et al., 2019). Traditionally, the octanol-water partition coefficient log K_{ow} is used to calculate the bioconcentration potential of contaminants as a measure of the extent to which they concentrate into lipids (Hermens et al., 2013). However, log K_{ow} does not account for the effect of the pH of the aquatic medium on the biological uptake of contaminants, which is considered to be a key factor in determining their bioconcentration and toxicity (Chang et al., 2019; Rendal et al., 2011). The pH-corrected log D_{OW} is not sufficient to account for this as it does not account for partitioning across biological membranes (Stott et al., 2015). Additional chemical parameters must be considered to fully appreciate pH-induced alterations to the uptake and toxicity of aquatic contaminants, particularly those which are ionisable.

Many of the contaminants present in the marine environment are ionisable in solution (Hermens et al., 2013; Patel et al., 2019), meaning that their molecules are able to exist in both ionised (either positively or negatively charged) and unionised (neutral, with no charge) forms. The ratio of ionised to unionised molecules of a chemical is

dependent on many factors, such as collisions between molecules, the presence of other compounds, temperature and the pH of the aquatic media. For an ionisable compound, the effect of changes in external pH are in part dependent on its strength as an acid in solution (a concept which applies to both acids and bases), known as its acid dissociation constant K_a (Henderson, 1908). K_a can be quantified as the equilibrium constant of the reaction

$$HA \leftrightharpoons H^+ + A^- \quad (2)$$

where HA is the neutral form of the acid that dissociates into a hydrogen ion, H^+, and the dissociated conjugate base of the acid, A^-. Due to the many orders of magnitude present in this equation, the acid dissociation constant is often more simply expressed as pK_a which equates to

$$pK_a = -\log_{10} \frac{[HA]}{[H^+][A^-]} \quad (3)$$

where the quantities in square brackets represent the concentrations of those species at equilibrium. When the pK_a value of an acid or base is equal to the external pH, the concentration of the acid and conjugate base will be equal, meaning that half of the chemical is in a dissociated (ionised) form and half in neutral form. Outside of this value, the relative concentrations of ionised and neutral forms will differ, as described by the Henderson-Hasselbach equation (Henderson, 1908)

$$\alpha_{ion} = \alpha_{neutral} \times 10^{i(pH-pK_a)} \quad (4)$$

where α is the fraction of the neutral or ionic form of the compound, i is 1 for acids and -1 for bases.

The potential for biological uptake and toxicity differs between these ionised and neutral forms of acids and bases, as observed by Simon and Beevers in their 1952 paper. They demonstrated that the concentration of a range of weak acids and bases required to produce a standard toxicity response was strongly correlated with the degree of ionisation of the compound, as calculated by the pK_a of the compound and pH of the media (Henderson, 1908; Simon and Beevers, 1952). This was because the fraction in neutral form, compared to the ionised fraction, of an ionisable acid increases when pH is below pK_a. Neutral forms are less polar, and hence more lipophilic and

better able to permeate membranes and lipids than the ionised portion (Camenisch et al., 1996). Therefore, as pH decreases towards the pK_a of acids, their uptake potential increases. This relationship is the inverse for bases: their degree of ionisation decreases as pH increases above pK_a, meaning that more of the base is present in neutral form and hence has greater uptake potential. This was further explored in a review of 117 studies where the toxicity and/or bioconcentration of acids or bases were tested at more than one pH level (Rendal et al., 2011). They found that changes in bioconcentration factors and toxicity were strongest when pH minus pK_a was between -1 to 3 for acids and -3 to 1 for bases. The toxicity and bioconcentration of acids was always greatest at the lowest exposure pH, whereas for bases toxicity and bioconcentration factor were almost always highest at the highest pH; the only bases for which this was not the case were complex amphoteric sulfonamides.

Based on this extensive review, and more recent studies of multiple contaminants in varying pH conditions which demonstrate similar findings (e.g. Karlsson et al., 2017; Sun et al., 2020), it appears that, as a general rule, a reduction in aquatic pH will lead to an increase in the biological uptake and toxicity of acids to aquatic organisms, and to a decrease in the same measurements for bases. In an OA scenario, we could therefore postulate that acids will become more toxic, and bases less so, to marine organisms. Specifically, acidic compounds with pK_a lower than the pH of a future acidified ocean may increase most significantly in uptake as the pH of the ocean decreases (Figure 4)**.** Above pK_a, changes in pH have negligible effects (Simon and Beevers, 1952), therefore for acids with pK_a above future ocean pH, declining seawater pH may have negligible effects on uptake. For bases in an OA scenario, their potential for uptake may decline, and bases with pK_a below current ocean pH may be minimally affected. Although pK_a has been demonstrated to be one of the key chemical properties determining pollutant uptake in freshwater environments with differing pH conditions (Chang et al., 2019), it has rarely been considered in OA studies.

Changes to contaminant membrane permeation invoked by the pH-pK_a relationship can also result in the ion trap effect. This occurs when unionised molecules cross biological membranes and then become ionised due to different pH conditions, trapping ions due to their lower membrane permeability and potentially leading to high concentrations of compounds (Rendal et al., 2011). This is utilised to improve

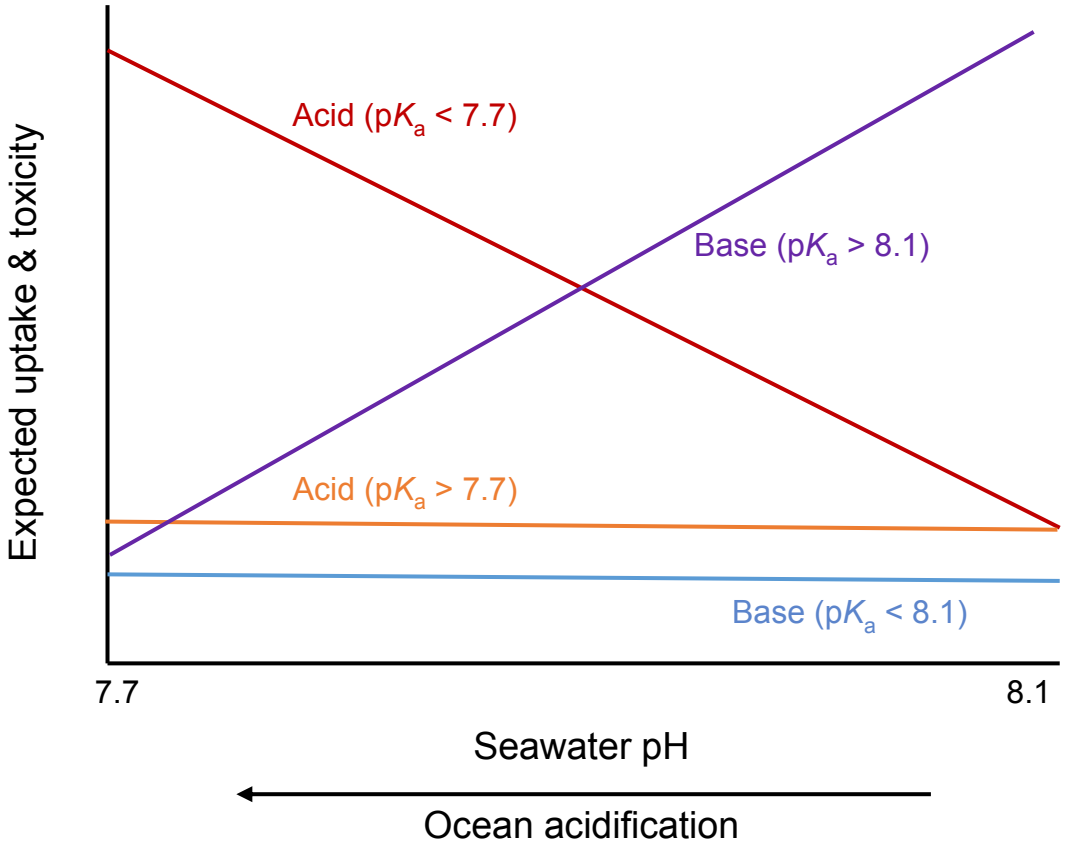

Figure 4**.** Schematic representation of how the uptake of weakly acidic and basic compounds may be affected by future OA, based on their predicted chemical behaviour. Weak acids become less ionised as pH declines below their pK_a, and weak bases become more ionised. As the unionised fractions of compounds are more readily taken up through biological membranes, we would expect the uptake, and hence toxicity, of weak acids and bases to increase, decrease or remain constant based on their pK_a as shown.

pharmaceutical efficacy (Mateus et al., 2013; Zheng et al., 2011): for example, basic anti-malarial drugs are designed to accumulate in lysosomes where they can be most effective (Trapp et al., 2008). Conversely, this may increase exocytosis of basic compounds from cells (Zhitomirsky and Assaraf, 2017) hence reducing their efficiency. Most marine organisms display a continuously increasing pH gradient from intracellular spaces, to extracellular environments and then to seawater, due to CO_2 generated during respiration readily diffusing outwards across biological barriers following a decreasing gradient (Melzner et al., 2020). A theoretical representation of how this would affect acidic and basic compounds is presented in Figure 5. For acidic compounds with pK_a < extracellular pH, more of the compound will become unionised

as pH decreases towards their pK_a, therefore less of the compound will be ionised. The unionised forms of compounds cross biological membranes more readily than ionised forms, thereby further increasing the relative concentration of the unionised form in compartments with lower pH. The total concentration of the acid will decrease with movement into each compartment due to the ionised fraction being less able to travel through. Basic compounds with pK_a > extracellular pH become more ionised as pH decreases, therefore it is likely that a lower total amount of this compound will cross each boundary layer as the amount of the unionised form decreases as pH declines. However, due to the ion trap effect (Rendal et al. 2011), more of this ionised form may become "trapped" within each compartment as pH decreases. The movement of ionisable compounds into organelles is poorly understood, as are the pH conditions of the organelles of marine organisms (Melzner et al. 2020), therefore the relative ionisation state and toxicity of compounds here cannot be estimated. Given the body of research demonstrating that OA conditions can cause extracellular, and sometimes intracellular, pH changes for a number of invertebrate taxa (Melzner et al., 2020), this raises the question of whether these changes could alter the internal movement and accumulation of contaminants; this is discussed in the next section.

There are additional complexities when considering the influence of the pH-pK_a relationship on contaminant toxicity in an OA scenario. Calculation of pK_a becomes complex when molecules contain multiple ionisable components or groups with both acidic and basic properties. Similarly, some molecules and functional groups can act as either acids or bases, depending on external conditions, or form amphoteric compounds in seawater. Determining which of the four categories in Figure 4 a marine contaminant best fits into is therefore complex. Where acid-base properties can be estimated or experimentally confirmed, there are additional challenges. Ionised compounds can pair with other ions which facilitates membrane transport (Neubert, 1989), and hydroxide and carbonate ions are expected to decrease in concentration by approximately 50 % by the year 2100 under the RCP 8.5 scenario, alongside changes in their organic complexation (Stockdale et al., 2016). Changes in pH may also affect partitioning of contaminants between water and sediment, hence impacting bioavailability (Branchet et al., 2020). Additionally, given the OA-induced physiological changes observed within the extracellular fluids of many marine animals, it might be

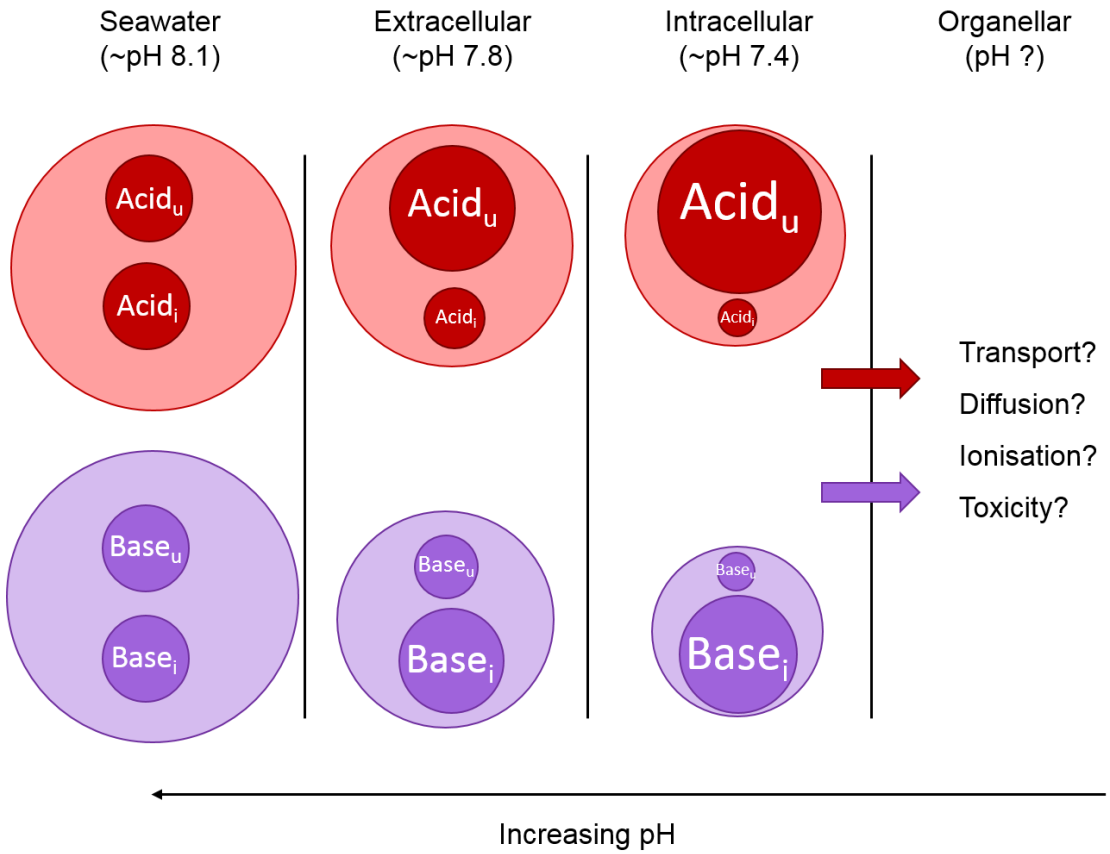

Figure 5**:** A representation of how the total quantity and the relative proportions of ionised (i) and unionised (u) molecules of acidic and basic compounds may theoretically be altered between different biological compartments. The pH values for different compartments for a theoretical marine invertebrate are adapted from Melzner et al. (2020).

predicted that further pH-induced speciation changes to contaminants occur once they have entered the bodies of marine animals. This could potentially alter the movement and accumulation of contaminants between different physiological compartments, alongside speciation-related uptake changes.

1.6. How does ocean acidification influence physiological responses to contaminants for marine organisms?

As discussed in the previous sections, speciation changes to metals and ionisable organic contaminants are likely to occur in a future OA scenario, altering their potential biological availability to marine organisms (Rendal et al., 2011; Stockdale et al., 2016). However, biological availability alone is not enough to determine toxicity outcomes for

marine organisms: even the simplest are highly complex beings, possessing physiological mechanisms to facilitate survival in a range of ocean conditions (Dahlhoff, 2004; Lesser, 2006; Sanford and Kelly, 2011). They are able to both protect against and repair damage through multiple, often interacting, processes, which are employed in response to OA, contaminants or both (Gunderson et al., 2016; Przeslawski et al., 2015). The effect of OA on one mechanism may influence how this mechanism responds to a contaminant, and vice versa. Different species and life-stages, possessing different mechanisms, are also likely to vary in their susceptibility to this combination of stressors.

Many marine invertebrates exercise physiological control over uptake and accumulation of trace metals due to their biological importance, which can be as influential as changes in metal bioavailability. The proportion of copper present as the free ion, Cu^{2+}, is projected to more than double by the year 2100 due to the OA-associated reduction in carbonate (Millero et al., 2009; Stockdale et al., 2016). As free ions of metals are generally highly bioavailable (Paquin et al., 2000), this is projected to increase uptake and bioaccumulation of copper in marine organisms, demonstrated in the clam *Mercenaria mercenaria* (Ivanina et al., 2013) and the oyster *Crassostrea gigas* (Cao et al., 2019). However, Götze et al. (2014) found that copper accumulation was also mediated by physiological mechanisms in marine bivalves, such as metal uptake and elimination. They found that maximum copper tissue concentration occurred at different pCO_2 values for two species, whereas if copper accumulation was facilitated purely by metal speciation then a linear increase in copper would be expected. Similarly, Ivanina et al. (2013) found that hypercapnia increased copper accumulation in the clam *Mercenaria mercenaria* but there was no difference in total copper concentration between clams exposed to 1.52 or 3.01 kPa pCO_2. However, intracellular levels of Cu^{2+} did increase between these treatments due to the increase in this form of copper induced by OA, demonstrating that the overall outcome was due to the combined effects of physiology and copper speciation.

Similarly to accumulation, organisms would be expected to suffer greater toxicity effects of copper in an OA scenario based on its speciation behaviour alone, but experimental evidence demonstrates that this is not always the case. Increased toxicity has been observed for a range of species and life stages exposed to copper

at current and future mean open ocean pH, such as the gametes and larvae of the lugworm *Arenicola marina* (Campbell et al., 2014); adult mussels *Mytilus edulis* and sea urchins *Paracentrotus lividus* (Lewis et al., 2016); and the foraminifer *Amphistegina gibbosa* (Marques et al., 2017). However, not all species experience greater toxic effects of copper at lower pH. Antagonistic effects of copper exposure and low pH were observed for the copepod *Amphiascoides atopus* (Pascal et al., 2010) and for the coral *Mussismilia harttii* (Marangoni et al., 2019), suggested to be due to competition between H^+ and copper ions for binding sites. Different responses to the combination of OA and copper even occur between life stages of the same species: for the polychaete *Pomatoceros lamarckii*, there was no additional effect of copper at pH 7.6 or 7.4 on fertilisation success, but this same combination decreased larval survival from approximately 77 % to 15 % after 72 hours (Lewis et al., 2013). Similarly for the lugworm *Arenicola marina*, the negative effects of copper on sperm motility, larval survivorship and DNA integrity were greater under OA conditions, but toxicity to fertilisation success remained the same (Campbell et al., 2014); if there was no effect of physiology, we would expect a similar level of increase in all life-stages in line with the increase in the free ion. These findings suggest that copper toxicity in an OA scenario can be mediated by physiological differences between species and life stages, as well as changes in copper speciation.

Another potential interaction between copper and OA is the disruption of acid-base homeostasis mechanisms. Copper has high affinity for Na^+/K^+ ATPases (Shekh et al., 2019; Viarengo et al., 1996) which are involved in acid-base homeostasis (Melzner et al., 2009). Copper also disrupts the activity of the acid-base regulatory enzyme carbonic anhydrase by displacing the native zinc co-factor at the active site (Lionetto et al., 2016). Several studies have observed increases in copper-induced acid-base disturbances under OA conditions (Duckworth et al., 2017; Lewis et al., 2016; Marangoni et al., 2019), although it is not clear whether this is due to increased copper bioavailability caused by speciation change, or due to copper and OA impacting the same endpoints. For some species, acid-base alterations in response to OA may influence responses to copper. Lewis et al. (2016) observed that acid-base physiology affected the DNA damage induced by copper to the mussel *Mytilus edulis* and the urchin *Paracentrotus lividus* in OA conditions, with urchins' greater capacity to accumulate bicarbonate in response to acid-base disturbance resulting in a smaller

increase in DNA damage than mussels. Bicarbonate can complex with free copper ions (Stiff, 1971), and moderate the toxicity of reactive oxygen species (Ramirez et al., 2005); therefore, organisms which buffer against acid-base disturbances by accumulating bicarbonate may be more resilient against copper toxicity (Lewis et al., 2016). Similarly, OA can induce an increase in glutathione (Sun et al., 2017) which may be protective against copper as a side effect. However, copper can convert reduced glutathione (GSH) into oxidised glutathione (GSSG); lowering the GSH:GSSG ratio can suppress mitosis (Stauber and Florence, 1987), and the generation of protons may induce acid-base disturbances. Copper can also decrease tissue total glutathione content, stimulate GST activity and inhibit GSH synthesis rate, which may be a defensive mechanism against metal toxicity (Canesi et al., 1999). The physiological ratio of copper to GSH is likely key in determining the toxicity of copper here.

These findings reveal that physiology plays a critical role in determining the final copper toxicity outcome. The nature and level of toxicity change to copper under high CO_2/low pH conditions, whilst in part dependent on speciation behaviour, are also highly influenced by species and life stage. However, the importance of seawater chemistry cannot be understated: as well as being affected by pH, copper toxicity has been widely shown to be affected by water hardness, salinity and dissolved organic carbon concentrations, due to increased competition for biological binding sites from cations and the formation of organic complexes with copper (Okocha and Adedeji, 2012). Both animal physiology and multiple aspects of changing ocean chemistry must be considered when assessing the toxicity of copper in a future ocean.

In contrast to copper, the metal cadmium is chloride speciation-dominated, and hence its speciation is not predicted to be altered in end-of-century OA conditions even under the most extreme RCP scenario predicting mean open ocean pH of approximately 7.7 (Stockdale et al., 2016). However, OA has been demonstrated to alter the effects of cadmium through physiological mechanisms. Although cadmium accumulation was not affected by increasing pCO_2 in haemolymph and haemocytes of the clam *Mercenaria mercenaria* and oyster *Crassostrea virginica* (Ivanina et al., 2014), separate studies found that elevated pCO_2 increased accumulation of cadmium in mantle cells of the same species (Götze et al., 2014), and cadmium uptake into

organelles and cytosolic enzymes increased at elevated pCO_2 (Hawkins and Sokolova, 2017). Increased cadmium accumulation was also observed in three other bivalve species in OA conditions, suggested to be due to either increased influx through calcium channels; epithelial damage resulting in easier penetration; or hampered excretion of cadmium by OA (Shi et al., 2016). These studies also found interactive effects of OA and cadmium on stress biomarkers, potentially via additive effects of reduced metabolic activity and mitochondrial function rather than a mechanistic interaction between the two stressors. Interestingly, the displacement of Zn^{2+} ions by Cd^{2+} from binding sites in *M. mercenaria* was rectified by increasing pCO_2, indicating that hypercapnia may protect clams from this aspect of cadmium toxicity, although this effect has only been demonstrated in isolated mantle cells and not whole organisms (Ivanina et al., 2013). These findings reveal that OA may enhance cadmium toxicity, suggesting a physiological component to uptake of this metal in an OA scenario and demonstrating that organisms may experience altered toxicity effects in OA conditions even for metals which do not undergo speciation changes.

Relative changes in toxicity under OA conditions have also been observed to occur for ionisable contaminants which do not become more neutral in lower pH conditions, and hence do not increase in membrane permeability at a chemical level. The pharmaceutical drug carbamazepine is a base with pK_a 15.96 (Chang et al., 2019), and therefore would be expected to decrease in uptake and toxicity in an OA scenario. No changes in bioconcentration of carbamazepine were observed at pH 7.6 for the mussel *Mytilus galloprovincialis* (Serra-Compte et al., 2018) or at pH 7.1 for the clam *Scrobicularia plana* (Freitas et al., 2016), but the latter study observed increased oxidative stress and mortality at pH 7.1 with carbamazepine, attributed to the two stressors impacting the same physiological endpoints. Notably, carbamazepine led to increased intracellular Na^+ concentration indicating possible disruption of acid-base homeostasis via Na^+/H^+ exchangers and/or Na^+-dependent HCO^{3-} transporters; acidosis is also reported as an effect of carbamazepine overdose in humans (Hughes et al., 1985). Although this study was conducted at a pH value well below the predicted end-of-century value of 7.7, this work highlights another potential pathway for harmful interactions between OA and non-metal contaminants: alterations to acid-base homeostasis induced by OA may be further impacted by drugs that affect ionoregulation. The pharmaceutical drugs diclofenac and clofibric acid alter plasma

concentrations of Na^+, Cl^- and K^+ in the carp *Cirrhinus mrigala* (Saravanan and Ramesh, 2013), all of which are involved in acid-base homeostasis via exchange of H^+ and HCO^{3-} (Griffith, 2017). Additionally, both drugs are acids with pK_a values well below 7.7 (Adeyeye and Li, 1990; Rosal et al., 2009), and therefore increased uptake in OA conditions could further increase these effects; the toxicity of diclofenac has been observed to increase at pH associated with OA (Munari et al., 2020, 2016). The potential for marine contaminants to increase in uptake in an OA scenario and to increase in effects via impacts on the same biological structures and endpoints must be considered in combination.

Changes to acid-base homeostasis, induced by OA conditions, contaminants or a combination of the two, could further influence contaminant toxicity. For example, a reduction in fish gill pH could decrease the pH of water at the gill surface due to an increase in the elimination of acids (Armitage et al., 2017); as this is a site of uptake for contaminants, this would increase the bioaccumulation potential for acidic compounds with the opposite predicted for bases. Such changes in uptake could theoretically also occur for poor acid-base regulators that are unable to maintain pH of tissues in an OA scenario, such as the mussel *Mytilus edulis* for which extracellular pH decreases below seawater pH in OA conditions (Lewis et al., 2016; Zittier et al., 2018). However, measurements of OA-induced pH changes specifically in gills and other potential sites of uptake are limited in marine organisms (Melzner et al., 2020).

Additionally, the pH of intracellular spaces can be affected by OA although this varies between species: from a reduction in intracellular pH (Gibbin et al., 2014) to regulation at control levels (Zittier et al., 2018) and even an increase above control values (Esbaugh et al., 2012). For example, extracellular pH in *M. edulis* decreases in OA conditions whilst pH of mantle cells is not affected (Zittier et al., 2018), suspected to be a common response for marine invertebrates to protect important cellular processes (Melzner et al., 2020). If extracellular pH were to drop below intracellular pH, this could result in an ion trap effect for acidic contaminants which, combined with the increase in the unionised fraction in the extracellular space, could lead to an increase in the intracellular concentration of acids (Figure 5) (Rendal et al., 2011). Concurrently, the ion trap effect would be lessened for bases; even a slight decrease in extracellular pH could reduce the unionised fraction of bases and hence reduce their

movement across cell membranes (Rendal et al., 2011). This could result in different toxicological effects due to the different biological components contained within these different compartments: for example, the basic pharmaceutical drug fluoxetine is suspected to interact with the extracellular site of bivalve serotonin and serotonin reuptake receptors (Fong et al., 2003), therefore an increase in the extracellular concentration could increase this mode of action. Greater understanding of the impact of OA on acid-base homeostasis within intracellular and extracellular compartments would aid our understanding of changes in uptake, accumulation and toxicity of pH-sensitive contaminants in these compartments, due to the complexity of measuring intracellular pharmaceutical concentrations directly (Gordon et al., 2016).

As well as affecting uptake and accumulation, physiological processes can also alter accumulation and elimination of non-metal contaminants, thereby affecting toxicity. OA increased bioconcentration of the contaminant sulfamethoxazole in the mussel *Mytilus galloprovincialis*, whilst decreasing the bioconcentration of venlafaxine, citalopram, sotalol and carbamazepine (Serra-Compte et al., 2018). Sulfamethoxazole is acidic with pK_a below 7 (Christaki, 2017), whilst venlafaxine, carbamazepine and citalopram are basic with pK_a values greater than 8.1 (British Pharmacopoeia Commission, 2007; Chang et al., 2019; Li et al., 2018; Vasskog et al., 2006), all of which correspond to the above theory of acids and bases, although it is not possible to state whether lower pH alone was the cause of these changes. However, bioconcentration of triclosan decreased in OA conditions: triclosan is acidic and becomes fully neutral at pH 6 (Roberts et al., 2014), therefore its uptake would be expected to increase at lower pH. Similarly, triclosan concentrations dramatically decreased in muscle and liver at pH 7.6 compared to 8.1 for the seabream *Diplodus sargus*, but lipid peroxidation and heat shock protein/ubiquitin concentrations were greater (Maulvault et al., 2019). It was suspected that OA increased elimination of triclosan, although not quickly enough to avoid the toxic effects of increased uptake. Similarly, gene expression in the clam *Tegillarca granosa* showed that their ability to eliminate benzo[α]pyrene may be impaired by OA, resulting in greater accumulation, but the ability to eliminate chloramphenicol and nitrofurazone was enhanced (Su et al., 2017). Alternatively, elimination mechanisms may themselves have caused the additional stress, by increased metabolism increasing production of reactive oxygen species causing lipid peroxidation (Lesser, 2006), or by re-allocation of a finite energy

supply away from certain stress response processes; energetic re-allocation is suggested as a critical cause of synergistic effects in multi-stressor scenarios (Crain et al., 2008).

Whilst understanding changes to contaminant uptake and accumulation are certainly important, this does not present a complete picture of the impacts to marine organisms as uptake is not always clearly correlated with toxicity effects. As discussed above, Maulvault et al. (2019) and Freitas et al. (2016) found greater oxidative stress and heat shock protein concentrations induced by the combination of OA and ionisable organic contaminants despite there being no increase in tissue concentrations. Differential accumulation of ionisable compounds between biological compartments, which is impacted by pH, could also influence toxicity; those which accumulate in lysosomes may be removed from cells (Zhitomirsky and Assaraf, 2017), which would alter toxicity whilst not impacting total organism concentration or uptake. Similarly for metals, increased uptake and bioaccumulation is not always correlated with toxicity as metals may be sequestered, for example in metallothioneins (Hawkins and Sokolova, 2017) or insoluble granules (Vr and Nott, 1993), rendering them biologically inactive. For organisms which lack such sequestration or removal mechanisms, such as the early life-stages of marine invertebrates (Aitken et al., 2004; Marshall, 2006), the effects of OA on contaminant toxicity may be influenced most strongly by chemical changes to the compound. For more complex organisms, these studies highlight the importance of accounting for animal physiology alongside changes to the chemical behaviour of contaminants, and in exploring which aspects of physiology are impacted in OA-contaminant scenarios, to determine which organisms are most at risk in a future ocean.

1.6.1. How might fluctuating carbonate chemistry alter contaminant toxicity?

Although future OA is established as having the potential to alter the toxicity of contaminants for marine organisms, whether by chemical or physiological means, little is known about how current carbonate chemistry extremes in coastal regions affect contaminant toxicity, or the effects of potential short-term fluctuations in toxicity as opposed to gradual longer term increases. This is significant for coastal organisms due to the spatial overlap between high variance in carbonate chemistry (Duarte et al.,

2013; Torres et al., 2021) and high levels of contamination (Landrigan et al., 2020) in coastal regions.

Exposure to a pH-sensitive contaminant under fluctuating pH conditions could be compared to a pulsed exposure of the contaminant. For metals, particularly copper, pulsed exposures tend to result in similar outcomes to continuous time-averaged exposure (Amachree et al., 2013; Angel et al., 2015, 2010). Comparisons between pulsed and continuous exposures for non-metal contaminants are limited, although Jarvinen et al. (1988) found that relationships between exposure duration and pesticide concentration were fundamental in predicting toxicity outcomes for fathead minnow larvae (*Pimephales promelas*), rather than the nature of the pulses. Brief changes in toxicity induced by short-term changes in carbonate chemistry may be easier to contend with, as short-term upregulation of defences, metabolic depression or other coping mechanisms may not have long-term impacts on organism health, and it may be possible to repair and recover from damage before the next increased toxicity event.

However, as discussed above, both long-term and short-term changes in carbonate chemistry are an additional stressor for some species, and the addition of a second stressor generally reduces the ability to cope with either due to the associated energetic costs (Crain et al., 2008). Higher metabolic costs were observed by Mangan et al. (2017) for the mussel *M. edulis* in a fluctuating pH regime, which would reduce the energy available to respond to contaminants, for example via energetically expensive antioxidant mechanisms (Lesser, 2006). Additionally, some organisms experience altered acid-base physiology in response to fluctuating carbonate chemistry conditions (e.g. Mangan et al., 2019, 2017), which could alter their susceptibility to contaminants via changes to their chemical speciation within the body as discussed above. As future climate change will likely increase the unpredictability and intensity of fluctuations (Angeles Gallego et al., 2018; Bindoff et al., 2019), the potential for adaptation, to both carbonate chemistry fluctuations themselves and related changes in toxicity, may be reduced.

1.7. Research aims and hypotheses

This review has highlighted several overarching research questions concerning changes to the effects of contaminants on marine organisms induced by changes in carbonate chemistry:

• Do an organism's acid-base regulation abilities affect its responses to contaminants in altered carbonate chemistry conditions?

• Can fluctuations in carbonate chemistry, such as diel cycles of pH in coastal habitats, alter the toxicity of contaminants to marine organisms?

• Will ionisable compounds which behave as acids and with pK_a below 7.7 become more toxic to marine organisms in an OA scenario?

• Will ionisable compounds which behave as bases and with pK_a above 8.1 become less toxic to marine organisms in an OA scenario?

• Are changes to the toxicity of acids and bases in an OA scenario further altered by whether the organism is able to maintain acid-base homeostasis?

In order to address these knowledge gaps, I tested several pH-sensitive chemicals known or suspected to be present in the marine environment, and several marine invertebrate species and life stages. As discussed previously, the bioavailability and toxicity of the metal copper is known to vary within the OA-relevant pH range, and, by extension, within the fluctuating pH ranges which occur in certain coastal habitats. Interactions between OA and copper are already described for marine invertebrates (Lewis et al., 2016; Nielson et al., 2019), therefore known impacts of altered carbonate chemistry on copper toxicity could be targeted in a fluctuating scenario. In order to explore the effects of OA on ionisable compounds, one acidic and one basic compound were selected which would be expected to change in their potential for uptake and hence toxicity between current (pH 8.1) and future acidified (pH 7.7) ocean pH. The catechol-o-methyltransferase inhibitor tolcapone was selected as an acidic compound as it has a single pK_a value below 7.7 (Nissinen and Mannisto, 2010), and

the selective serotonin reuptake inhibitor fluoxetine was selected as a basic compound as it has a single pK_a value above 8.1 (Nakamura et al., 2008). Both compounds are pharmaceutical drugs with known oxidative stress impacts (Di Poi et al., 2016; Gonzalez-Rey and Bebianno, 2013; Grünig et al., 2017; Maser et al., 2017) which are detectable in a range of marine invertebrates and life stages and can lead to a multitude of organelle to organism-level effects (Lesser, 2006); both are soluble in seawater allowing experiments to be carried out without need for a solvent carrier; and during the conceptualisation of this thesis neither had been investigated previously in an ocean acidification context, although one such study has since been published for fluoxetine (Lo et al., 2021). The chosen organisms were selected due to their varying ability to maintain acid-base homeostasis in OA conditions, in order to explore whether differences in internal acid-base status impact the toxicity of pH-sensitive contaminants. Subsequently, I tested the following hypotheses:

Hypothesis 1: Fluctuating pH/pCO_2 will increase the toxicity of the coastal contaminant copper to a greater extent in a poor acid-base regulator, the blue mussel (*Mytilus edulis*), than in a strong acid-base regulator, the king ragworm (*Alitta virens*), compared to exposure to copper in static pCO_2/pH conditions.

Hypothesis 2: Ocean acidification (pH 7.7) will increase the toxicity of the acidic pharmaceutical tolcapone to gamete and adult marine organisms.

Hypothesis 3: Ocean acidification (pH 7.7) will decrease the toxicity of the basic pharmaceutical fluoxetine to gamete and adult marine organisms.

Hypothesis 4: The acidic pharmaceutical tolcapone will undergo larger toxicity increases in OA conditions in organisms which are poor acid-base regulators, the mussel *Mytilus edulis* and the gametes of marine invertebrates, than in a strong acid-base regulator, the urchin *Paracentrotus lividus*.

Hypothesis 5: The basic pharmaceutical fluoxetine will undergo larger toxicity decreases in OA conditions in organisms which are poor acid-base regulators, the mussel *Mytilus edulis* and the gametes of marine invertebrates, than in a strong acid-base regulator, the urchin *Paracentrotus lividus*.

1.8. References

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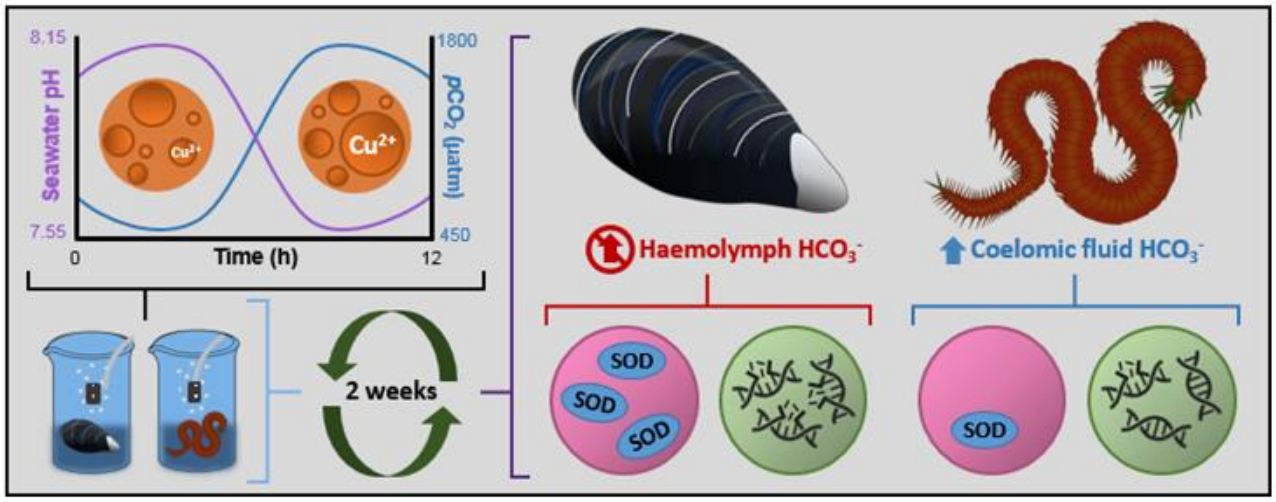
Chapter 2. Fluctuating seawater pCO_2/pH induces opposing interactions with copper toxicity for two intertidal invertebrates



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Science of the Total Environment CRediT authorship contribution statement

Alice Wilson-McNeal: Conceptualization, Project administration, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing.
Cameron Hird: Investigation, Conceptualization, Methodology, Review & editing.
Catherine Hobbs: Investigation, Resources, Methodology, Review & editing.
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2.1. Abstract

Global ocean pCO_2 is increasing as a result of anthropogenic CO_2 emissions, driving a decline in seawater pH. However, coastal waters already undergo fluctuations in pCO_2/pH conditions over far shorter timescales, with values regularly exceeding those predicted for the open ocean by the year 2100. The speciation of copper, and therefore its potential toxicity, is affected by changing seawater pH, yet little is known concerning how present-day natural fluctuations in seawater pH affect copper toxicity to marine biota. Here, we test the hypothesis that a fluctuating seawater pCO_2/pH regime will alter the responses of the mussel *Mytilus edulis* and the ragworm *Alitta virens* to sub-lethal copper, compared to a static seawater pCO_2/pH scenario. Mussels and worms were exposed to 0.1 and 0.25 µM copper respectively, concentrations determined to produce comparable toxicity responses in these species, for two weeks under a fluctuating 12-hour pCO_2/pH cycle (pH8.14–7.53, pCO_2 445–1747 µatm) or a static pH 8.14 (pCO_2 432 µatm) treatment. Mussels underwent a haemolymph acidosis of 0.1–0.2 pH units in the fluctuating treatments, alongside two-fold increases in the superoxide dismutase activity and DNA damage induced by copper, compared to those induced by copper under static pH conditions. Conversely, ragworms experienced an alkalosis of 0.3 pH units under fluctuating pH/pCO_2, driven by a two-fold increase in coelomic fluid bicarbonate. This mitigated the copper-induced oxidative stress to slightly reduce both antioxidant activity and DNA damage, relative to the static pH + copper treatment. These opposing responses suggest that differences in species acid-base physiology were more important in determining toxicity responses than the pH-induced speciation change. With variability in seawater chemistry predicted to increase as climate change progresses, understanding how fluctuating conditions interact with the toxicity of pH-sensitive contaminants will become more crucial in predicting their risk to coastal biota.

2.2. Introduction

As global climate change progresses, the seawater physico-chemical conditions under which coastal organisms experience anthropogenic contaminants are changing. A number of studies have demonstrated that the changes in seawater carbonate chemistry termed ocean acidification (OA), driven by the dissolution of increasing levels of atmospheric CO_2 in seawater, can alter the responses of marine species to pH-sensitive contaminants (Cao et al., 2019; Dorey et al., 2018; Lewis et al., 2016; Moreira et al., 2016). These global changes in carbonate chemistry are occurring over years to decades; however, in coastal areas, far greater changes in pCO_2 and pH occur over far shorter timescales, due to natural fluctuations in physical and biogeochemical processes (Baumann, 2019; Kapsenberg and Cyronak, 2019). Typical diel pH ranges are in the region of 0.3 units (Duarte et al., 2013), and ranges of 0.5 to more than 1 pH units have been recorded in tide pools (Silbiger and Sorte, 2018), seagrass meadows (Semesi et al., 2009), and salt marshes (Wolaver et al., 1986), driving coastal pH minima far below the predicted open-ocean end-of-century mean pH of 7.73 (Bopp et al., 2013). Furthermore, OA is shifting these ranges downwards, resulting in even lower pH extremes (Wootton et al., 2008), and fluctuations are predicted to increase in magnitude as climate change continues, via amplification of seasonal cycles (Kwiatkowski and Orr, 2018; McNeil and Sasse, 2016). It is therefore becoming increasingly important to understand how fluctuating seawater chemistry interacts with contaminant toxicity.

Many anthropogenic contaminants polluting coastal waters, including metals (Nriagu and Pacyna, 1988), undergo changes in speciation driven by changing carbonate chemistry (Stockdale et al., 2016). As metal species vary in their bioavailability (with the free hydrated ion, those which can release metal ions to bind with biological receptors, and those which can cross biological membranes being the most bioavailable; Sunda, 2012), these speciation changes are likely to contribute to changes in biological uptake, and potentially toxicity. Such changes may regularly occur in coastal areas where carbonate chemistry fluctuates, but knowledge of how short-term changes in metal speciation influence toxicity is lacking. One metal which may be affected is copper, a common coastal contaminant with concentrations in the English Channel ranging from 0.011 to as high as 1.61 µM (Bryan and Gibbs, 1983;

Pini et al., 2015). The mechanism of toxicity for elevated copper is well-described and includes the production of reactive oxygen species (ROS), inhibition of ion transport processes (including those associated with acid-base homeostasis; Viarengo et al., 1996) and disruption of carbonic anhydrase function (Duckworth et al., 2017). Copper undergoes speciation changes within pH ranges measured in coastal habitats, including an increase in the free ion Cu2+ as pH declines (Millero et al., 2009). Several studies have now demonstrated subsequent increases in copper toxicity under stable OA conditions (seawater pH ~7.7), in a species- and life stage-specific manner (Campbell et al., 2014; Lewis et al., 2016; Scanes et al., 2018).

Marine animals exposed to elevated pCO_2 over sustained periods (as in OA scenarios; Fabry et al., 2008) or fluctuating regimes (in both laboratory and field scenarios; Mangan et al., 2019, 2017; Small et al., 2016) can experience an extracellular acidosis. Circulatory CO_2 rises alongside external levels, generating additional protons (H+) which reduce extracellular pH. Uncompensated respiratory acidification can disrupt blood-oxygen transport, biochemical processes, and calcification (Wittmann and Pörtner, 2013).Whilst marine organisms possess various acid-base regulatory mechanisms, the mechanisms used, and degree to which they can compensate for elevated CO_2, vary significantly between species (Melzner et al., 2009). Key players in pH homeostasis during hypercapnia include pH-buffering compounds, transport of relevant ions (including the role of carbonic anhydrase in catalysing the inter-conversion of CO_2 and HCO_3^-) and suppressing metabolism (Fabry et al., 2008). Accumulating extracellular bicarbonate, retained from metabolic output or taken up from seawater, is a highly efficient acid-base regulatory mechanism which raises pH, and species lacking this ability are suggested to be the most vulnerable to hypercapnia (Melzner et al., 2009).

Whilst hypercapnia affects internal pH, elevated copper affects acid-base regulation processes, thus the interaction between these stressors will likely impact acid-base homeostasis. Recent work demonstrating differing responses to combined OA and copper in two invertebrate species supports this idea. The common mussel, *Mytilus edulis*, has limited ability to regulate its acid-base status in response to changes in seawater carbonate chemistry, and is adversely affected by copper which increases oxidative stress and exacerbates acid-base disturbances (Lewis et al., 2016).

Conversely, the ragworm *Alitta virens* does not undergo any significant acid-base disturbances in response to OA, and OA appears to mitigate copper-induced lipid peroxidation for this species (Nielson et al., 2019). Both species inhabit coastal areas where copper pollution can be prevalent (Bryan and Langston, 1992), and where large short-term changes in carbonate chemistry are observed (Mangan et al., 2019). *M. edulis* experiences acid-base disturbances both in situ across tidal cycles and in the laboratory under manipulated seawater pCO_2/pH fluctuations (Mangan et al., 2019, 2017; Shang et al., 2020). Compared to static scenarios, fluctuating seawater pCO_2/pH regimes can also affect growth and survival (Alenius and Munguia, 2012; Clark and Gobler, 2016; Frieder et al., 2014), calcification rate (Onitsuka et al., 2018), behaviour, and stress-related biomarkers (Mangan et al., 2017), in a range of species and life stages. Fluctuating seawater carbonate chemistry may also influence responses to contaminants, by affecting similar physiological processes or by changing their chemistry, and these interactions may vary between species with differing acid-base physiologies and hence internal environments.

Here, we investigate how a fluctuating pCO_2/pH scenario affects sublethal responses to copper for *M. edulis* and *A. virens*, compared to a static seawater pCO_2/pH regime, and how organism acid-base physiology influences this response.

2.3. Material and methods

2.3.1. Animal husbandry

Common mussels, *Mytilus edulis* (49–62 mm, 100 in total), were collected from Starcross, Exe estuary, UK (50°62′19″N, 3°44′35″W). Polychaete ragworms *Alitta* (formerly *Nereis*) *virens* (2.7–4.4 g wet weight, 93 in total) were obtained from Exeter Angling, Devon, sourced from a farm in the Netherlands. Animals were maintained in a 300 L recirculating artificial seawater system (Tropic Marin© salts 30 PSU, pHNBS 8.13, 15 ± 0.5 °C), with worms in additional sediment with macroalgae (Fucus spp.) collected from the Exe estuary, UK (50°40′3″N, 3°26′42″W).Mussels were fed ad libitum with Shellfish Diet 1800®. Animals were acclimated to these aquarium conditions for one week prior to experiments, and good health status was confirmed in all experimental animals before use (coloration indicating immaturity, normal feeding and behaviour, and response to physical stimuli).

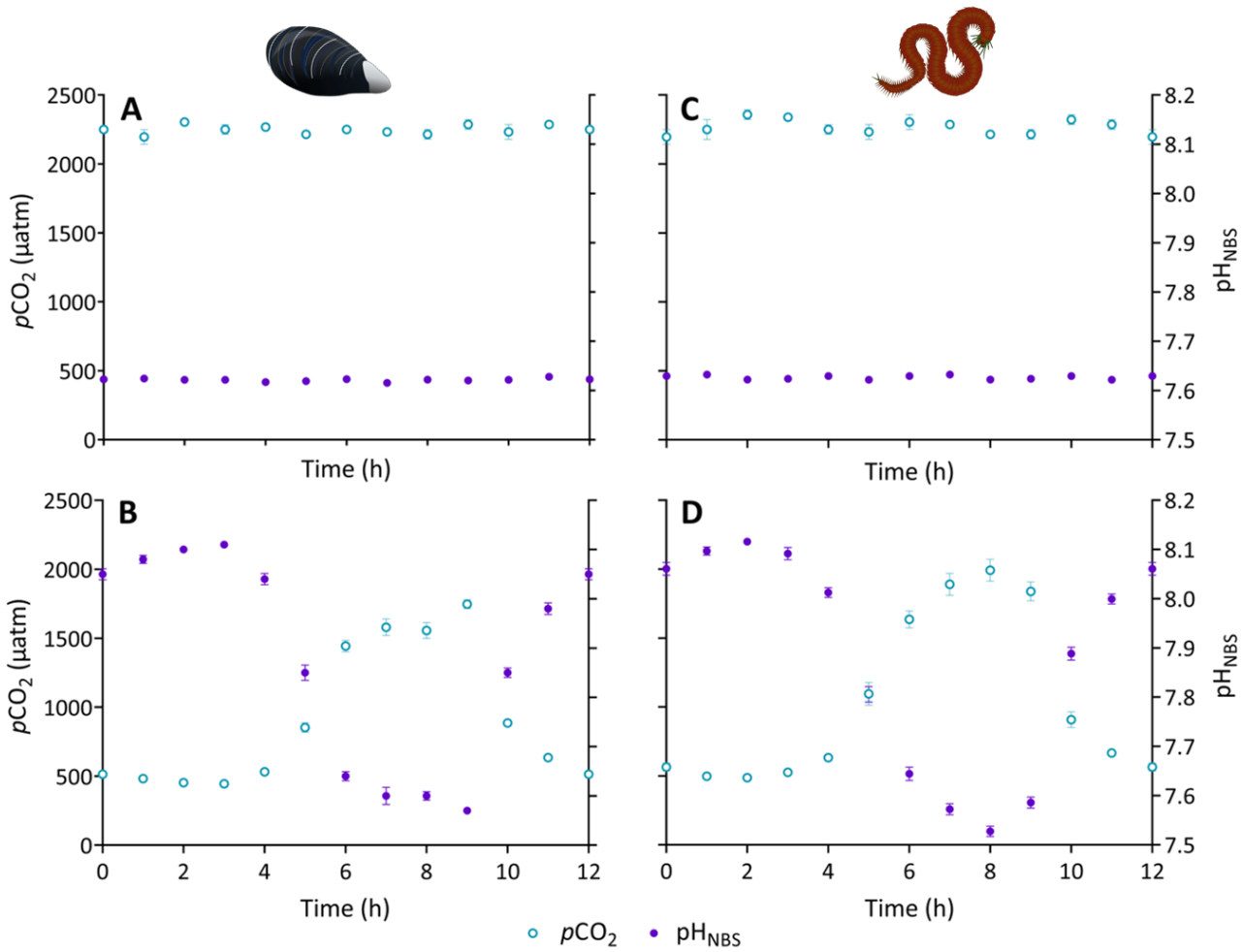

Figure 1. Seawater pH and pCO_2 (mean ± SEM) of static (A) and fluctuating (B) treatments for *Mytilus edulis*; and static (C) and fluctuating (D) treatments for *Alitta virens*, measured hourly in individual tanks.

2.3.2. Treatment conditions

For the 14-day exposure experiments, animals were exposed to one of four treatments in individual 2 L glass tanks (12 tanks per treatment each containing one animal) maintained at 15 °C (±0.3 °C) and 30 (± 0.2 PSU): 1. Static pH (pH_{NBS} 8.14 ± 0.004); 2. fluctuating pH (pH_{NBS} 8.14–7.53); 3. static pH + copper; or 4. fluctuating pH + copper. As mussels and ragworms exhibit different sensitivities to elevated copper, with mussels responding to much lower concentrations of copper than ragworms, copper concentrations were used that induce comparable levels of toxicity (using DNA damage for reference), to enable us to test our hypothesis regarding the relative impact of fluctuating seawater pCO2/pH conditions on these responses in these species.

Treatment	Temp. (˚C)	pH_{NBS}	Salinity (PSU)	Copper (µM)	TA (µmol/kg)	pCO_2 (µatm)	HCO_3^- (µmol/kg)	CO_3^{2-} (µmol/kg)	ΩCa	ΩAr
Static	15.6 (±0.06)	8.14 (±0.003)	30.1 (±0.05)	0.006 (±0.002)	2195 (±8)	433.2 (±3.2)	1873.4 (±6.3)	131.2 (±1.1)	3.2 (±0.03)	2.1 (±0.02)
Static + Cu	15.7 (±0.1)	8.14 (±0.004)	30.2 (±0.08)	0.06 (±0.002)	2184.8 (±7.4)	433.4 (±6.2)	1864.2 (±8.3)	130.7 (±0.6)	3.2 (±0.01)	2.0 (±0.01)
Fluc.	15.6 (±0.02)	7.87 (±0.04)	30.2 (±0.02)	0.005 (±0.003)	2098.6 (±13)	937.4 (±84.3)	1903.8 (±17.4)	78.8 (±5.3)	1.9 (±0.1)	1.2 (±0.1)
Fluc. + Cu	15.6 (±0.03)	7.88 (±0.04)	30.1 (±0.03)	0.06 (±0.002)	2089.9 (±13.8)	929.1 (±83.3)	1895.5 (±17.4)	78.6 (±5.4)	1.9 (±0.1)	1.2 (±0.1)
Static	15.3 (±0.02)	8.12 (±0.004)	30.9 (±0.06)	<0.001	2343 (±2.5)	445.5 (±8.9)	1995.6 (±6.3)	142.3 (±2.1)	3.5 (±0.05)	2.2 (±0.03)
Static + Cu	15.4 (±0.05)	8.14 (±0.003)	30.8 (±0.05)	0.2 (±0.003)	2350.1 (±6.7)	438.9 (±6.6)	1985.9 (±6.7)	149.4 (±1.8)	3.6 (±0.04)	2.3 (±0.03)
Fluc.	15.1 (±0.04)	7.86 (± 0.03)	30.9 (±0.05)	0.004 (±0.002)	2395.1 (±6.3)	1085.6 (±103.6)	2168.8 (±14.2)	93.1 (±6.5)	2.2 (±0.2)	1.4 (±0.1)
Fluc. + Cu	15.2 (±0.08)	7.87 (± 0.03)	30.8 (±0.08)	0.2 (±0.001)	2392.7 (±4.2)	1072.4 (±105.7)	2160.5 (±16.3)	95.5 (±7.1)	2.3 (±0.2)	1.5 (±0.1)

Table 1: Seawater carbonate chemistry and copper concentrations (mean ± SEM) for *Mytilus edulis* (top, white) and *Alitta virens* (bottom, grey) exposures.

For mussels, a nominal copper concentration of 0.1 µM was used to represent a polluted coastal site (Bryan and Gibbs, 1983), and in line with previous work (Lewis et al., 2016). A nominal concentration of 0.25 µM was chosen for ragworms, which induces a similar level of DNA damage to the mussel response at 0.1 µM under a static pCO_2/pH regime (Nielson et al., 2019).

The fluctuating pH regime was achieved by the constant equilibration of seawater with a temporally varying air/CO_2 mixture using timer-controlled mass flow controllers (AALBORG® TIO Totalizer Input/Output Flow Monitor/Controller connected to AALBORG® Mass Flow Controller GFC17). This treatment mimicked a pH range measured in the Exe estuary, UK (Mangan et al., 2019). The pH in the fluctuating treatments fell from 8.14 to 7.53 (pCO_2 445 to 1747 µatm) over 6 h, and rose back to 8.14 over a further 6 h, to simulate a tide-driven cycle (Figure 1). Static pH tanks were aerated with air to maintain seawater pCO_2 equivalent to current atmospheric levels (mean 432 µatm) and pH at 8.14. Mussels were exposed to a 12:12 day:night light cycle. Ragworm tanks were kept in near-total darkness to simulate sediment cover, with silicone tubes (10 cm length, 8 mm diameter), with holes to allow seawater exchange, for use as burrows in place of the sediment which they would naturally burrow into, in order to maintain normal behaviour.

Full seawater changes were performed every 48 h, with 1 mL of copper sulphate (obtained from Merck®) stock solution (200 µM and 500 µM for mussels and worms respectively) added to tanks immediately after each water change. Animals were starved throughout the exposure to avoid the uptake of copper bound to food. Temperature, salinity and pH_{NBS} were measured daily (YSI® 30 Salinity, Conductivity and Temperature System; HANNA® HI-98191 Professional Waterproof meter with HI-72911B electrode). Seawater samples (12 mL) from fluctuating treatments were taken every hour for 12 h (to encompass the full range of the cycle), and four times per day for static pH treatments (to ensure seawater conditions were not influenced by changes in the fluctuating cycle), on three separate days during each exposure for measurement of dissolved inorganic carbon (DIC) using a custom built system (Lewis et al., 2013). In total, 192 DIC samples were analysed. Seawater carbonate chemistry parameters were calculated using the CO2SYS program v2.1 (Pierrot et al., 2006),

with dissociation constant from Dickson and Millero (1987) and $KHSO_4$ from Dickson (1990) (Table 1).

To account for any loss of copper to the exposure system, water samples (50 mL) for copper analysis were taken 1, 24 and 48 h after water changes on every third day of each exposure period, with six replicate samples at each time point (24 samples), to ensure that copper concentration did not differ significantly between water changes. In addition, three samples were taken from the fluctuating treatments at hours 0, 3, 6 and 9 of the fluctuating cycle on days 2, 7 and 12 of each exposure, to account for any changes in total copper concentration throughout the fluctuating cycle (24 samples). In total, 48 samples were analysed for total copper concentration by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) using an Agilent 7900 Spectrometer utilizing a collision cell with helium as the collision gas to minimize polyatomic interferences, and operating in high matrix introduction (HMI) mode with a 25-times aerosol dilution factor. Instrument calibration was performed using a four-point series, matrix matched to the samples, at 1, 5, 10 and 20 μg L^{-1} respectively. Samples were prepared with 100 μg L^{-1} scandium as an internal standard. Calibration was validated using quality control standards at 1, 5 and 16 μg L^{-1}, prepared in an identical manner but from different reagent stocks to the instrument calibration standards. For 4 samples, copper was added to a portion of the sample to give an additional concentration of 10 μg L^{-1}, and the spiked sample analysed to determine copper recovery.

2.3.3. Ammonia excretion

Ammonia excretion rate was measured during the final 24 h of the experiment, between days 13 and 14. Water samples (2 mL) were taken at the beginning and end of a 24-hour period and analysed using a modified version of the colorimetric ammonia detection assay (Li et al., 2005). Solutions of 1.44 M sodium salicylate, 5.5 mM sodium nitroprusside, 0.031 M sodium dichloroisocyanurate and 2.7 M potassium hydroxide were added to water samples in a 96-well plate and analysed using a Tecan Infinite® M200 PRO plate reader at 660 nm. Ammonia excretion rates were calculated following Cooper and Wilson (2008) and are expressed as total ammonia flux (μmol) g wet weight^{-1} h^{-1}.

2.3.4. Acid-base parameters

Haemolymph and coelomic fluid samples were taken from mussels and ragworms respectively, at the end of the 14-day exposure, and during the high pH/low CO_2 point of the fluctuating cycle so that samples from all treatments were collected under similar seawater conditions. Mussel haemolymph (approximately 1 mL per animal) was drawn from the posterior adductor muscle, and ragworm coelomic fluid (approximately 200 µL per animal) from the posterior region of the body, avoiding the gut. The pH_{NBS} was immediately measured (HANNA® HI-98191 ProfessionalWaterproof pH/ORP/ISE meter with HI-1330B glass body refillable semi-micro electrode and HI-7662 temperature probe) and 50 µL of haemolymph or coelomic fluid was transferred into a glass micro-haematocrit tube for total CO_2 analysis (Corning 965 Total CO_2 Analyser). Acid-base parameters were calculated using a modified Henderson-Hasselbach equation, using values derived from Truchot (1976) for the first dissociation constant (pK) for carbonic acid and solubility constant for CO_2.

2.3.5. Oxidative stress

Oxidative stress analyses were performed using homogenised whole body tissue, minus shells for mussels. Superoxide dismutase (SOD) activity, a first-line defence enzyme against oxidative stress (Ighodaro and Akinloye, 2018), was quantified using the Nitrotetrazolium Blue (NBT) reduction assay (Parry and Pipe, 2004); the rate of color change of NBT, using xanthine oxidase to generate free radicals, was used to determine the concentration of SOD. Lipid peroxidation was quantified using the thiobarbituric acid reactive substances (TBARS) assay to quantify malondialdehyde (MDA), a by-product of oxidative stress to lipids, following Camejo et al. (1999). Results were normalised to protein content measured using the Bradford assay (Bradford, 1976).

2.3.6. DNA damage

DNA damage was measured as single-strand breaks using the Comet assay following Lewis and Galloway (2008). Mussel haemolymph (500 µL) or ragworm coelomic fluid (100 µL)was collected into syringes pre-loaded with 100 µL chilled phosphate-buffered saline as an anticoagulant and kept on ice until use. Samples were gently centrifuged,

then the cell concentrate vortexed with low melting point agarose and spread on glass slides. After 1 h in cell lysis solution (2.5 M NaCl, 0.1 MEDTA, 10mM Tris) and 45minute denaturation in electrophoresis solution (0.3M NaOH, 1mM EDTA), electrophoresis was carried out for 30 min at 25 V and 300 mA. Samples were pH-neutralised, stained with Sybr Safe (0.0001 % in TBE buffer), and 100 cells per replicate were analysed with a fluorescence microscope (excitation: 502 nm, emission: 530 nm) using COMET IV software (Perceptive Instruments Ltd.). DNA damage is expressed as percentage of total DNA present in the comet tail (% tail moment).

2.3.7. Statistical analysis

Datawere analysed in GraphPad Prism8.2 using two-way analysis of variance (ANOVA) with fixed factors “pH” and “copper”. Data that violated the homogeneity of variance assumption according to the Brown-Forsythe test, or the assumption of normality according to the Shapiro-Wilk test, were log-transformed prior to analysis. Multiple comparisons were performed using Tukey's post-hoc test. Results are presented as mean ± SEM. Full statistical results are presented in Table S1.

2.4. Results

2.4.1. Acid-base parameters

Mussel haemolymph pCO_2 under static pH conditions was 0.2 ± 0.01 kPa (Figure 2A). The addition of copper at static pH significantly increased haemolymph pCO_2 by 60 % to 0.32 ± 0.04 kPa (two-way ANOVA for copper, $F_{1,47} = 5.93$, $P = 0.02$, Tukey's post-hoc, $P = 0.025$). There was no effect of fluctuating pH on haemolymph pCO_2 (two-way ANOVA for pH, $F_{1,47} = 2.09$, $P = 0.16$). Haemolymph pCO_2 in the fluctuating pH + copper treatment was similar to that of the static and fluctuating pH treatments at 0.22 ± 0.02 kPa. Mussel haemolymph bicarbonate was 2.63 ± 0.07 mM under static pH conditions (Figure 2B), with no effect of any treatment (two-way ANOVA for pH, $F_{1,47}=0.01$, $P=0.91$; for copper, $F_{1,47}=1.351$, $P = 0.25$). Mussels displayed an acidosis of their haemolymph of 0.1–0.2 pH units in all fluctuating pH and copper treatment conditions, with pH reducing from 7.6 ± 0.02 in the static pH treatment to 7.44 ± 0.06, 7.51 ± 0.06 and 7.48±0.05 for the static pH + copper, fluctuating pH and fluctuating pH + copper treatments respectively (Figure 2C). There were no significant

treatment effects (two-way ANOVA for pH, F1,47=0.2, P =0.65; for copper, F1,47 =3.4, P=0.07).

Ragworm coelomic fluid pCO_2 did not differ significantly between treatments (two-way ANOVA for pH, F1,47 = 0.085, P = 0.77; for copper, F1,47=2.06, P=0.16; Figure 2D), although the addition of copper resulted in declines of 0.05–0.07 kPa under both pH conditions. In static pH conditions, ragworm coelomic fluid bicarbonate was double that of mussels (~5 versus 2.5 mM; Figure 2E). There was a two-fold increase in extracellular bicarbonate in both fluctuating pH treatments compared to the static pH treatment (11.92 ± 1.92 mM for fluctuating pH and 12.41 ± 2.47 mM for fluctuating pH + copper; two-way ANOVA for pH, F1,47 = 17.73, P = 0.001; Tukey's post-hoc P = 0.0025 and P = 0.0028 for fluctuating pH and fluctuating pH + copper respectively). There was a slight increase in bicarbonate in the static pH + copper treatment to 7.73±1.15mM, but there was no significant effect of copper overall (two-way ANOVA for copper, F1,47=1.44, P=0.24). Coelomic fluid pH_{NBS} in the static pH treatment was 7.86 ± 0.06 (Figure 2F), whilst exposure to fluctuating pH resulted in significant increases in coelomic fluid pH_{NBS} to 8.17 ± 0.06 and 8.14 ± 0.08 for the fluctuating pH and fluctuating pH + copper treatments respectively (two-way ANOVA for pH, F1,47=8.25, P=0.007). There was no significant effect of copper on coelomic fluid pH (two-way ANOVA for copper, F1,47 = 0.74, P = 0.39).

2.4.2. Ammonia excretion

Mussel ammonia excretion rate declined by almost half in response to copper under static pH conditions, from 0.41 ± 0.1 to 0.26 ± 0.07 μmol g^{-1} h^{-1}, and by two-thirds in combination with fluctuating seawater pH to 0.13 ± 0.03 μmol g^{-1} h^{-1} (Figure 4A). There was a significant effect of copper (two-way ANOVA for copper, F1,47 = 7.5, P = 0.01), but no effect of fluctuating pH (two-way ANOVA for pH, F1,47= 1.519, P = 0.23). Ammonia excretion rate for ragworms in the static pH treatment was more than double that of mussels at 0.93 ± 0.21 μmol g^{-1} h^{-1} (Figure 4B). There were non-significant increases to 1.68 ± 0.18 and 2.26±0.81 μmol g^{-1} h^{-1} for static pH+copper and fluctuating pH respectively. However, ammonia excretion rate in the fluctuating pH+copper treatment was almost identical to the static pH treatment (0.92±0.17 μmol

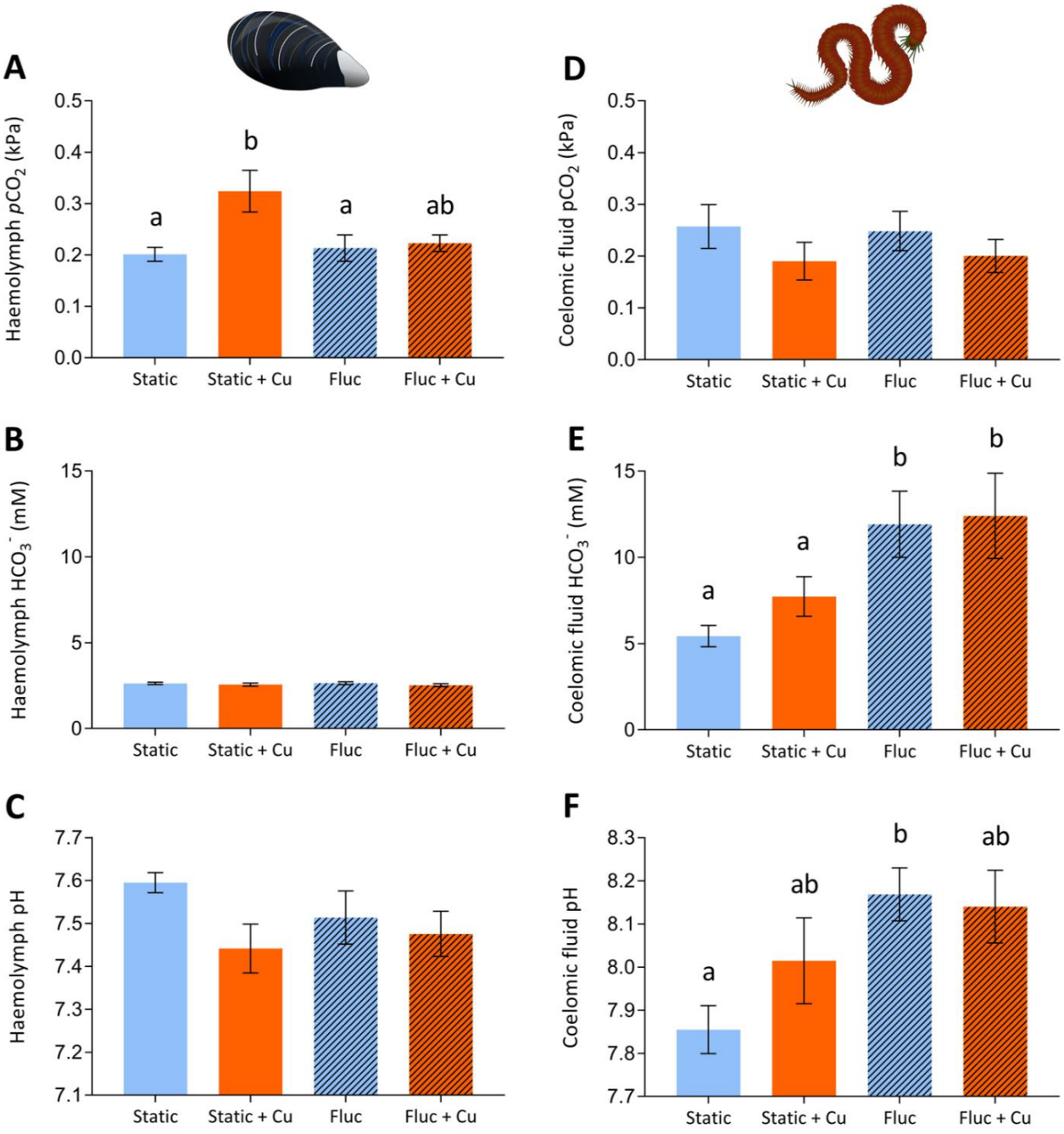

Figure 2. Acid-base parameters (±SEM) in *Mytilus edulis* haemolymph (A, B, C) and *Alitta virens* coelomic fluid (D, E, F) after 14 days exposure to a static or fluctuating pH regime with or without the addition of copper. Haemolymph and coelomic fluid pCO_2 (A, D); concentration of bicarbonate (B, E); pH (C, F). Note differing scales. Bars denoted by different letters represent significant differences ($P < 0.05$).

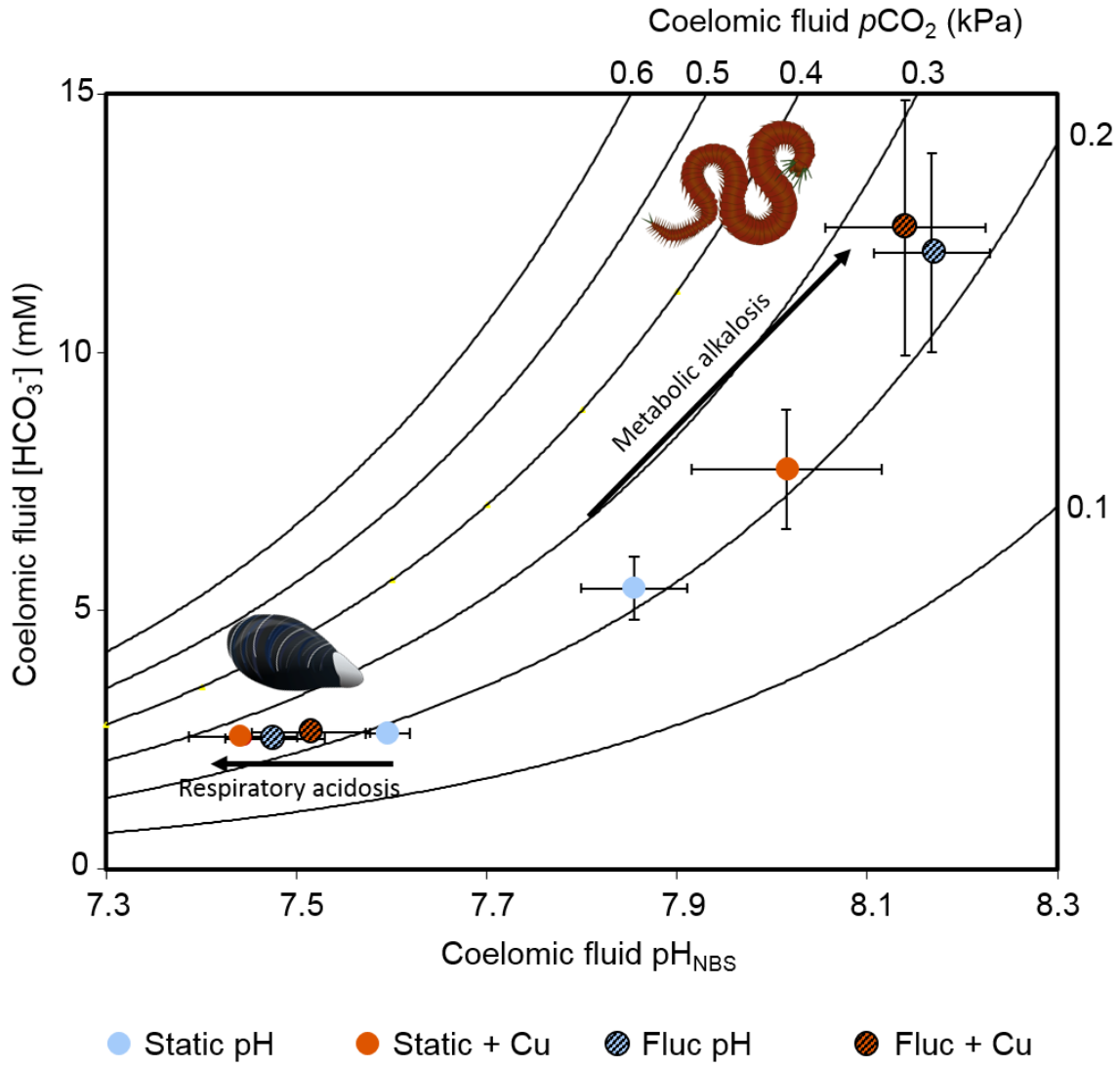

Figure 3. pH-bicarbonate diagrams visualising the relationship between pH, pCO_2 and bicarbonate ion concentration in the haemolymph of *Mytilus edulis* (bottom, left) and *Alitta virens* (top, right). Lines represent isopleths of equal pCO_2 (kPa).

g^{-1} h^{-1}), leading to a significant interaction term between the two stressors (two-way ANOVA for pH ∗ copper, $F_{1,47}=6.12$, $P = 0.02$).

2.4.3. Oxidative stress

Mussels in the fluctuating pH + copper treatment had significantly higher SOD activity compared to all other treatments (1.4 ± 0.23 SOD units mg^{-1} protein; Tukey's post-hoc $P = 0.017$; Figure 5A), double that of mussels in the static pH treatment (0.7 ± 0.1 SOD

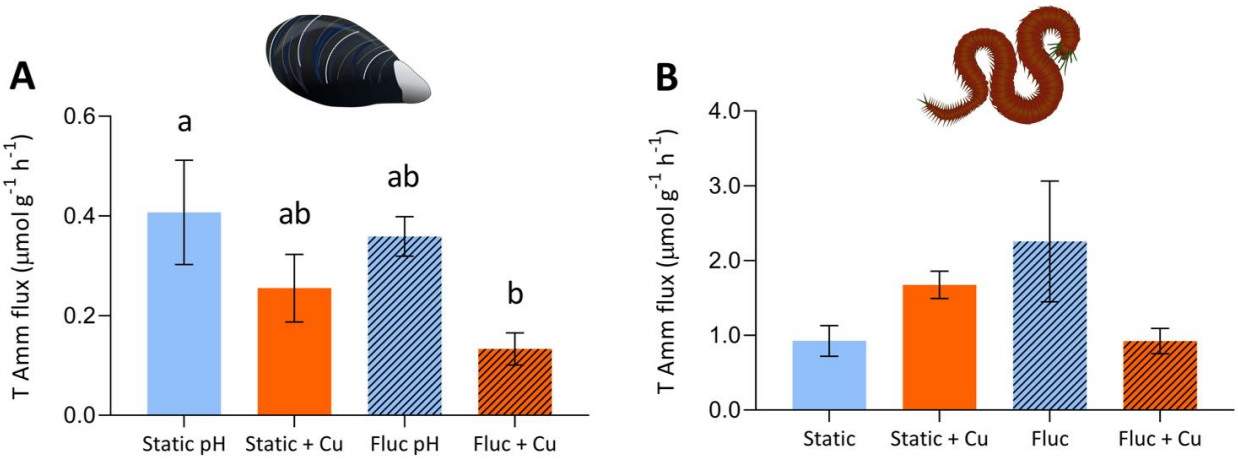

Figure 4. Ammonia excretion rate (±SEM) in *Mytilus edulis* (A) and *Alitta virens* (B) after 14 days exposure to a static or fluctuating pH regime with or without the addition of copper. Note differing scales. Bars denoted by different letters represent significant differences ($P < 0.05$).

units mg^{-1} protein), leading to a significant interaction term between pH and copper (two-way ANOVA for pH ∗ copper, $F_{1,47}=11.1$, $P=0.002$). Similar levels of SOD activity to the static pH treatment occurred for both static pH + copper and fluctuating pH, with 0.41 ± 0.11 and 0.63 ± 0.28 SOD units mg^{-1} protein respectively. Static pH+copper and fluctuating pH treatments induced increased LPO compared to the static pH treatment value of 1.56 ± 0.05 MDA nM mg^{-1} protein (static pH+copper 2.00± 0.17; fluctuating pH 2.05 ±0.26; Figure 5B). In contrast, LPO in the fluctuating pH+copper treatment was almost identical to the static pH treatment at 1.57 ± 0.15 MDA nM mg^{-1} protein; this is reflected in the significant interaction term for the two stressors (two-way ANOVA for pH ∗ copper, $F_{1,47} = 4.55$, $P = 0.04$).

Ragworms in the static pH treatment had similar levels of SOD activity to mussels under the same conditions (0.65 ± 0.041 SOD units mg^{-1} protein; Figure 5D). This doubled to 1.42 ± 0.21 SOD units mg^{-1} in the static pH + copper treatment, reflected in the significant effect of copper (two-way ANOVA for copper, $F_{1,47} = 12.29$, $P = 0.001$; Tukey's post-hoc, $P = 0.007$). Exposure to fluctuating pH alone had no significant effect on SOD activity (0.84±0.1 SOD units mg^{-1} protein; Tukey's post-hoc $P = 0.95$), and activity in the fluctuating pH + copper treatment was 1.15±0.17 SOD

units mg^{-1} protein, which was not significantly different to either the static pH or static pH + copper treatment. Despite similar levels of SOD activity, ragworm baseline LPO was more than 10 times that of mussels, ranging from 21 to 23.5 MDA $nMmg^{-1}$ protein across all treatments with no significant treatment effects (Figure 5E).

2.4.4. DNA damage

Mussel DNA damage in the static pH treatment was 4.7 ± 0.5 % (Figure 5C). There were significant effects of both copper and pH on DNA damage (two-way ANOVA for copper, $F_{1,47} = 7.09$, $P = 0.01$; for pH, $F_{1,47}=6.42$, $P=0.02$). These reflect a significant increase in DNA damage to 9.5 ± 1.1 % between the static pH and fluctuating pH + copper treatments (Tukey's post-hoc, $P = 0.005$), although the interaction term was not significant (two-way ANOVA for pH $*$ copper, $F_{1,47} = 0.05$, $P = 0.82$). There were non-significant increases in DNA damage in the static pH + copper and fluctuating pH treatments, which were 6.9 ± 0.7 and 7.5 ± 1.3 % respectively.

For ragworms, DNA damage increased significantly from 7.5 ± 0.8% in the static pH treatment to 11.6 ± 1.4 % with the addition of copper (two-way ANOVA copper, $F_{1,47} = 8.35$, $P = 0.006$; for pH, $F_{1,47} = 0.04$, $P = 0.85$; Tukey's post-hoc, $P = 0.02$; Figure 5F). Whilst there was an increase in the fluctuating pH + copper treatment to 10.5 ± 0.8 %, this was not significant and was lower than the damage induced by copper under static pH conditions (Tukey's post-hoc, $P=0.86$). There was also a slight increase in the fluctuating pH treatment to 8.9 ± 0.7 %, but this was not significant (Tukey's post-hoc $P = 0.7$).

2.5. Discussion

Our data reveal that fluctuations in seawater pCO_2 and pH, as naturally occur in coastal waters, alter the responses of two marine invertebrates to the common coastal contaminant copper, when compared to exposure to copper in seawater with static pCO_2/pH conditions that are more traditionally used in ecotoxicology exposure studies. This change in copper response induced by the fluctuating seawater carbonate chemistry also fundamentally differed between the two test species, the blue mussel *Mytilus edulis* and the ragworm *Alitta virens*, in terms of both their acid-base physiology and their copper toxicity responses. In mussels, exposure to copper during

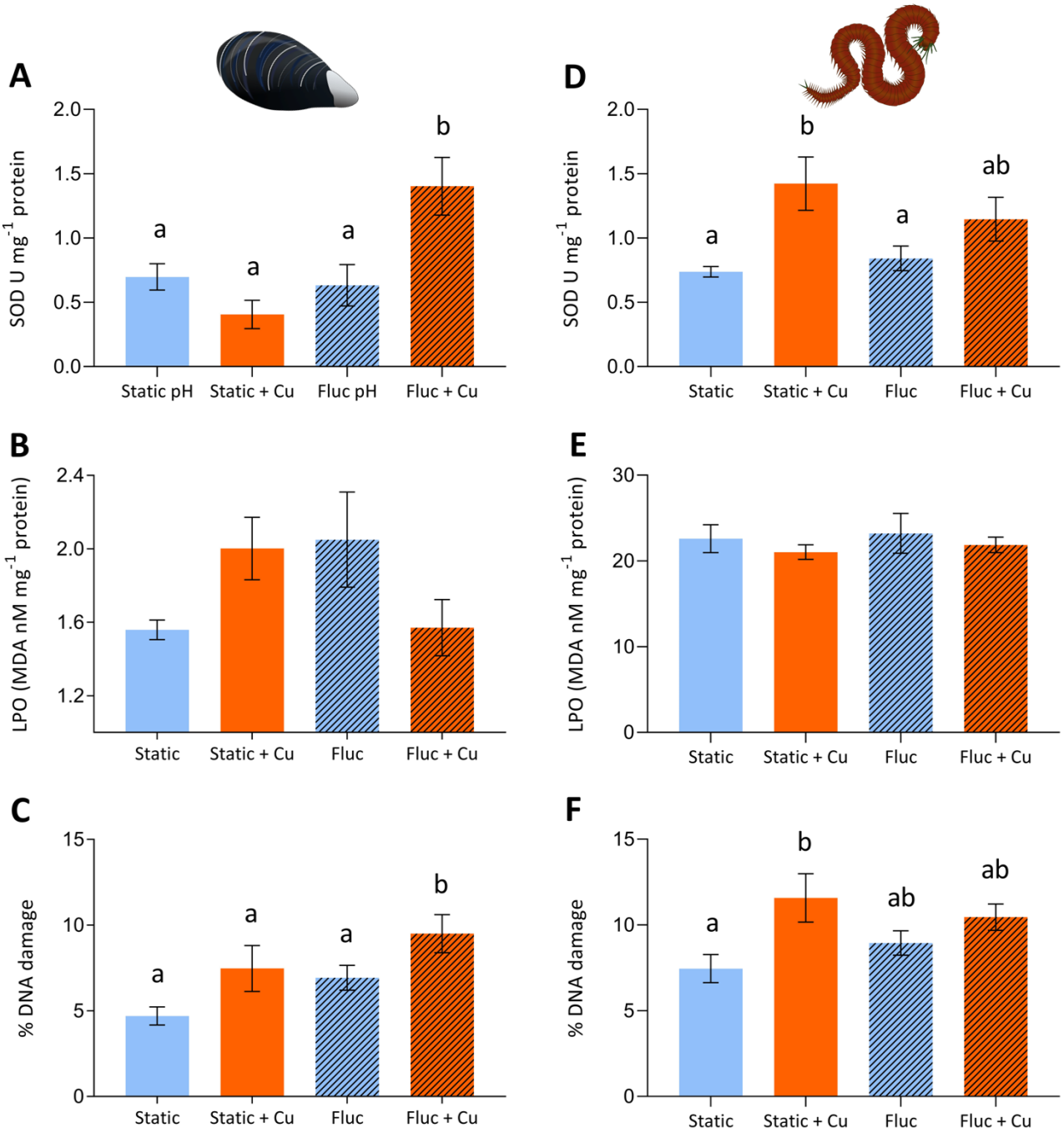

Figure 5. Oxidative stress indicators and DNA damage (±SEM) in *Mytilus edulis* (A, B, C) and *Alitta virens* (D, E, F) after 14 days exposure to a static or fluctuating pH regime with or without the addition of copper. Activity of the antioxidant enzyme superoxide dismutase (SOD) (A, D); lipid peroxidation (LPO) measured as malondialdehyde (MDA) concentration (B, E); DNA damage expressed as percentage of single-strand breaks in haemocytes/coelomocytes (C, F). Note differing scales. Bars denoted by different letters represent significant differences ($P < 0.05$).

a fluctuating pH regime drove two-fold increases in superoxide dismutase (SOD) activity and DNA damage compared to exposure to copper in the static pH scenario. In ragworms, however, the opposite was observed: fluctuating seawater pH appeared to mitigate the copper-induced increases in both SOD activity and DNA damage that were observed under static pH conditions leading to slight reductions in both copper-sensitive toxicity end points under the fluctuating conditions.

The differential copper toxicity responses of mussels and ragworms might be explained by their divergent acid-base physiologies: for ragworms, the internal alkalosis in response to the fluctuating pH regime may have reduced the proportion of copper present in the more toxic free ion form, whereas the haemolymph acidosis in mussels may have had the opposite effect. Free copper ions also readily complex with bicarbonate ions in solution (Stiff, 1971); the increase in coelomic fluid bicarbonate ions observed in ragworms may therefore act to reduce the proportion of free ionic copper, further mitigating its effects. No such bicarbonate increase was observed for mussels. Previous research concerning interactions between ocean acidification (OA) and copper toxicity found that in the sea urchin *Paracentrotus lividus* increased bicarbonate within their coelomic fluid, as part of their acid-base regulatory response to OA, corresponded to a smaller increase in copper toxicity under OA than was observed for mussels. Mussels lack this acid-base regulatory ability, as our data also show, and experienced a much greater increase in copper toxicity when exposed under OA conditions (Lewis et al., 2016). Our findings add to the growing evidence that stronger acid-base regulators, in this case ragworms, exhibit reduced or no increase in copper toxicity at reduced seawater pH compared to species that are less able to acid-base regulate and therefore exhibit internal acidosis. The changes in copper toxicity that occur under different seawater chemistries are therefore not solely mediated by the metal speciation changes in the surrounding seawater, but are also influenced by the organism's internal physiology. Physiology has previously been suggested to determine the interaction between copper and other environmental stressors in fish (Grosell et al., 2007). Götze et al. (2014) similarly found that the accumulation of copper in tissues appeared to be mediated more by physiological control over uptake and elimination than by the speciation behaviour of copper in seawater. Our data demonstrate that this holds true for fluctuating pH/pCO_2 conditions, with distinct responses in different species.

Acid-base parameters were markedly different between the two species. For mussels, there was no change in haemolymph pCO_2 in the fluctuating pH + copper treatment compared to the static pH treatment. This is in contrast to the effect of OA, which increases haemolymph pCO_2 both alone and in combination with copper (Lewis et al., 2016). As mussels were sampled at the point of the fluctuating cycle when carbonate chemistry in all treatments was similar, this suggests that haemolymph pCO_2 rapidly followed changes in the external pCO_2, even in the presence of copper which can disrupt outward CO_2 diffusion via inhibition of carbonic anhydrase (Duckworth et al., 2017). This may be an adaptation for coping with periods of emersion in intertidal habitats (Mangan et al., 2019). Mussel haemolymph pH also fluctuates with the surrounding seawater (Mangan et al., 2019, 2017), suggesting that, whilst not measured here, haemolymph pH may drop further throughout the fluctuating cycle as pCO_2 increases, potentially leading to greater interactions with copper toxicity due to the associated increase in free copper ions.

A similar increase in mussel haemolymph pCO_2 in response to sublethal copper exposure has been observed previously during a more conventional ecotoxicology exposure scenario (i.e. static seawater conditions of ambient pH 8.1; Lewis et al., 2016) which could be attributed to disruption of carbonic anhydrase (Duckworth et al., 2017). Alternatively, or in addition, mussels may have spent more time with valves closed to reduce copper uptake, resulting in reduced ventilation rate and hence CO_2 build-up (de Zwaan and Wijsman, 1976); this is corroborated by the significantly greater variance in haemolymph pCO_2 values in our data which could indicate differences in the time mussel valves were open prior to sampling. The mean decline in haemolymph pH of ~0.2 units in the static pH + copper treatment may also be due to the disruption of Na+/K+-ATPases (Viarengo et al., 1996), transporters which are also involved in acid-base homeostasis (Melzner et al., 2009). Our findings align with several studies reporting that mussels lack the ability to recover from acid-base disturbances by increasing haemolymph bicarbonate in either static OA or fluctuating regimes (Gazeau et al., 2013; Lewis et al., 2016;Mangan et al., 2017), potentially making them vulnerable to future OA (Melzner et al., 2009).

The ragworm acid-base regulatory response contrasted greatly with that of mussels. There was no significant change in ragworm coelomic fluid pCO_2 between any

treatments: as *A. virens* is a soft-bodied organism which relies on simple gaseous diffusion across the integument for CO_2 excretion (Gomme, 1984), this suggests that, as for the majority of aquatic animals, internal pCO_2 should closely follow seawater pCO_2 which was the same in all treatments at the time of sampling. Although coelomic fluid pCO_2 was similar in both the static and fluctuating pH treatments at the point of sampling, coelomic fluid bicarbonate concentrations in ragworms in the fluctuating treatments were double that of ragworms in the static treatments at this point, driving a strong coelomic fluid alkalosis. This increased bicarbonate within the coelomic fluid suggests a strong degree of acid-base regulation for this species, as this mechanism is employed by a range of taxa as an efficient response to changing seawater pCO_2 (Melzner et al., 2009). There appears to be a lag between the change in pCO_2, which occurs relatively quickly via diffusion (Gomme, 1984), and extracellular bicarbonate, which takes longer to alter as a result of transepithelial ion transport processes (Krieg et al., 2014).

A similar decline in ragworm coelomic fluid pCO_2 in response to sublethal copper exposure was reported in a previous study (Nielson et al., 2019); it is unclear what drove this response in either case as copper can inhibit carbonic anhydrase activity (Duckworth et al., 2017), and therefore an increase in internal pCO_2 driven by reduced excretion would be expected. The increase in ragworm bicarbonate in the static pH + copper treatment here was accompanied by a slight increase in bicarbonate leading to a mild alkalosis, in opposition to the acidosis seen in mussels. The differing responses of mussels and ragworms are highlighted in pH-bicarbonate diagrams (Davenport, 1974) (Figure 3). Whilst mussels underwent a respiratory acidosis driven by slight increases in haemolymph pCO_2, ragworms underwent a metabolic alkalosis, driven by increased coelomic fluid bicarbonate.

Mussels and ragworms also differed in other physiological responses. In mussels, ammonia excretion occurs across respiratory epithelia via Rhesus proteins, facilitated by the motion of respiratory cilia (Thomsen et al., 2016). These may have been inhibited by copper, which blocks similar proteins and inhibits ammonia excretion in trout (Lim et al., 2015), leading to the reduced rate of excretion observed in both copper treatments here. Mussels are also suggested to close their valves to reduce pollutant uptake which would prevent ammonia excretion (de Zwaan and Wijsman,

1976), similarly to CO_2 excretion as described above. The expected greater concentration of the free Cu2+ ion could have led to the greater decline in ammonia excretion rate in the fluctuating pH+copper treatment compared to the static pH+copper treatment. Metabolic depression has been observed in mussels during short-term OA (Michaelidis et al., 2005), and may be an adaptation to short periods of hypercapnia in intertidal habitats, potentially leading to the reduced ammonia excretion rate seen in the fluctuating pH treatments. The effect of starvation on this metabolic response should also be considered: Thomsen et al. (2013) observed that abundant food allowed *M. edulis* to tolerate high seawater pCO_2, therefore the responses observed here may be further influenced by food availability during the high pCO_2/low pH periods of fluctuating cycles in coastal habitats. As ammonia excretion (as NH4 +) is a form of proton removal in marine invertebrates, and hence crucial in blood pH homeostasis (Weihrauch and Allen, 2018), a reduction in excretion rate could have contributed to the decline in mussel haemolymph pH in the fluctuating pH + copper treatment in the absence of a significant pCO_2 increase.

The response of ragworms is again in contrast to that of mussels: mussel ammonia excretion reduced in all treatments, whilst in ragworms ammonia excretion increased in response to both fluctuating pH and static pH + copper. Reduced seawater pH enhances ammonia excretion by increasing the concentration gradient between internal fluids and seawater (Randall and Tsui, 2006); this may have caused the increase in ragworm ammonia excretion seen during the fluctuating pH treatment, as mean pH was lower. Although copper can inhibit ammonia excretion, *A. virens* is suggested to be efficient at regulating copper uptake and excretion (Pini et al., 2015; Watson et al., 2018); these processes involve high protein turnover in many taxa (Prohaska, 2008) and hence may have increased ammonia excretion. The contrasting lack of increase in ammonia excretion in the fluctuating pH + copper treatment, compared to both the static pH + copper and fluctuating pH treatments, could be related to the greater proportion of bioavailable copper, as Watson et al. (2018) suggest that higher copper concentrations may overwhelm *A. virens* copper uptake/detoxification processes.

The two species also exhibited opposing oxidative stress responses to the combination of fluctuating pH and copper. In this treatment, a lower mean seawater

pH would have led to a greater proportion of the copper free ion Cu2+, which cycles between redox states and generates reactive oxygen species (ROS) (Pham et al., 2013). The doubling of SOD activity for mussels in the fluctuating pH+copper treatment compared to all other treatments suggests a strong antioxidant response, which acts to prevent any increased ROS from initiating LPO. Elevating antioxidant activities can have high energetic costs (Lesser et al., 2001), and therefore may not be sustainable over longer term exposures, or may result in trade-offs in energy allocation. This could explain the lack of increase in SOD activity in the static pH+copper and fluctuating pH treatments, which suggests that there was a threshold of oxidative stress below which SOD activity was not stimulated, despite some LPO occurring.

In contrast, fluctuating seawater pH conditions appear to have mitigated the copper-induced increase in SOD activity for ragworms. This may have been driven by the increased coelomic fluid bicarbonate and internal alkalosis reducing the bioavailability of copper (Stiff, 1971), reducing the requirement for upregulation of SOD. Increased SOD activity in response to copper exposure appeared to protect against the expected LPO for ragworms, as for mussels: both copper treatments induced some increase in SOD activity, whilst there was no effect of any treatment on LPO. A previous study using the clam *Mercenaria mercenaria* also found that elevated seawater pCO_2 conditions reduced the levels of ROS induced by copper, suggesting that this was due to hypercapnia-induced upregulation of antioxidant defences (Ivanina et al., 2013). A similar process may have induced the elevated SOD and subsequent prevention of further LPO for mussels in the fluctuating pH + copper treatment here, although it is unknown why this would not have occurred for mussels in response to copper in a previous study mimicking an OA scenario (Lewis et al., 2016). Ragworms have previously been observed to increase SOD activity in response to OA (Nielson et al., 2019), but we observed no effect of fluctuating pH/pCO_2 on SOD activity in the present study. The physiological effects of OA and a fluctuating pCO_2/pH regime therefore appear to differ for these species, and both should be considered when assessing responses to coastal contaminants in a future ocean.

In mussels, exposure to copper under fluctuating seawater pCO_2/pH conditions also induced a two-fold increase in DNA damage, compared to that induced by copper under static pH conditions. This mirrors observations from OA (static conditions of pH

7.7) and copper studies for a number of marine invertebrate species and life-stages (Campbell et al., 2014; Lewis et al., 2016), suggesting that the lower mean seawater pH in our fluctuating treatment could be the cause of the increased damage compared to static pH+copper, likely via an increased proportion of copper in free ion form. Altered copper speciation may have caused damage directly, via increased generation of ROS as suggested by the increased SOD activity, increased binding of copper to DNA (Linder, 2012), or indirectly, via inhibition of zinc-finger DNA repair enzymes (Hartwig et al., 2002).

Conversely to mussels, DNA damage induced by copper under fluctuating pH conditions in ragworms was slightly lower than under static pH conditions, following a similar pattern to SOD activity. This slight reduction is in contrast to the increase observed in OA-copper conditions by Nielson et al. (2019), and does not reflect the expected increase in toxicity driven by the increase in free ionic copper. Nielson et al. (2019) also observed a reduction in bicarbonate in response to OA and copper, whereas here, the combination of coelomic fluid alkalosis and increased bicarbonate driven by fluctuating pH may have mitigated copper toxicity for ragworms. Fluctuating pH in coastal habitats may therefore prove protective against OA-induced changes in contaminant toxicity for some species.

Both species experienced an increase in DNA damage but no change in lipid peroxidation (LPO) during the fluctuating pH + copper treatment compared to static pH alone. These differences could be explained by their different locations within the organism: lipids are primarily located within cells and cell membranes, whereas DNA is within the nucleus, and copper is transported, bound, sequestered and excreted differentially between these compartments (Prohaska, 2008). Copper binds readily to DNA, and excessive quantities can complex with bases leading to their excision (Linder, 2012), and DNA repair mechanisms are also vulnerable to copper (Hartwig et al., 2002); therefore, damage may occur even when oxidative stress is mitigated by the increased antioxidant enzyme activity observed in both species here. Intracellular acid-base status is also more closely regulated than extracellular in marine invertebrates (Lindinger et al., 1984; Portner et al., 2000). It is possible that differences in acid-base homeostasis and hence in copper chemistry between these compartments, and between species, may also have influenced copper chemistry.

Our results demonstrate that exposure to sub-lethal concentrations of copper in seawater with a fluctuating pCO_2 and pH regime leads to different physiological and toxicity outcomes than observed when animals are exposed under static pCO_2/pH conditions. Importantly, organism physiology influenced this outcome, with different responses between our two test species likely linked to their acid-base physiology. Although the majority of laboratory ecotoxicology studies are performed under static seawater carbonate chemistry conditions, coastal species naturally experience fluctuating conditions in situ. Coastal waters generally contain the highest concentrations of anthropogenic contaminants due to their proximity to land, and organisms here will therefore be exposed to these alongside characteristic fluctuations in carbonate chemistry. As OA continues to drive natural fluctuations in carbonate chemistry to greater extremes, understanding the relationship between contaminant toxicity and acid-base physiology will be central to predicting the severity of future impacts of these two global stressors to marine species.

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2.7. Supporting information

Species	Parameter	Factors/interaction		
		pH	Copper	pH*Copper
Mytilus edulis	Haemolymph pCO_2	$F_{1,47}$ = 2.092, P = 0.156	**$F_{1,47}$ = 5.934, P = 0.0195***	$F_{1,47}$ = 2.653, P = 0.1114
	Haemolymph HCO_3^-	$F_{1,47}$ = 0.01325, P = 0.9089	$F_{1,47}$ = 1.351, P = 0.2517	$F_{1,47}$ = 0.0951, P = 0.7594
	Haemolymph pH	$F_{1,47}$ = 0.2044, P = 0.6535	$F_{1,47}$ = 3.396, P = 0.0723	$F_{1,47}$ = 1.241, P = 0.2714
	Ammonia excretion	$F_{1,47}$ = 1.519, P = 0.2271	**$F_{1,47}$ = 7.503, P = 0.0101***	$F_{1,47}$ = 0.2858, P = 0.5968
	DNA damage	**$F_{1,47}$ = 6.416, P = 0.0151***	**$F_{1,47}$ = 7.093, P = 0.0109***	$F_{1,47}$ = 0.0527, P = 0.8196
	SOD	**$F_{1,47}$ = 8.567, P = 0.0055****	$F_{1,47}$ = 2.255, P = 0.1405	**$F_{1,47}$ = 11.1, P = 0.0018****
	TBARS	$F_{1,47}$ = 0.09741, P = 0.7566	$F_{1,47}$ = 0.0094, P = 0.923	$F_{1,47}$ **= 4.551, P = 0.0393***
Alitta virens	Coelomic fluid pCO_2	$F_{1,47}$ = 0.08496, P = 0.7723	$F_{1,47}$ = 2.058, P = 0.1598	$F_{1,47}$ = 0.1299, P = 0.7206
	Coelomic fluid HCO_3^-	**$F_{1,47}$ = 17.73, P = 0.0002*****	$F_{1,47}$ = 1.444, P = 0.2373	$F_{1,47}$ = 1.224, P = 0.2758
	Coelomic fluid pH	**$F_{1,47}$ = 8.248, P = 0.0065****	$F_{1,47}$ = 0.7414, P = 0.3943	$F_{1,47}$ = 1.512, P = 0.226
	Ammonia excretion	$F_{1,47}$ = 0.4796, P = 0.4933	$F_{1,47}$ = 0.485, P = 0.4909	**$F_{1,47}$ = 6.177, P = 0.018***
	DNA damage	$F_{1,47}$ = 0.03686, P = 0.8487	**$F_{1,47}$ = 8.352, P = 0.0062****	$F_{1,47}$ = 1.808, P = 0.1864
	SOD	$F_{1,47}$ = 0.3704, P = 0.5462	**$F_{1,47}$ = 12.29, P = 0.0011****	$F_{1,47}$ = 1.819, P = 0.185
	TBARS	$F_{1,47}$ = 0.2, P = 0.6571	$F_{1,47}$ = 0.8062, P = 0.3745	$F_{1,47}$ = 0.0048, P = 0.9451

Table S1. Effects of fluctuating pH, copper exposure and their interaction on physiological parameters for mussels and worms analysed by two-way ANOVA. Significant effects ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***) are highlighted in bold.

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Chapter 3. Ocean acidification differentially alters the effects of pharmaceutical drugs on sperm swimming and fertilisation success in two marine invertebrates

Contributions

Conceptualisation of the study, method development, experimental design, field and laboratory work, data analysis and writing were led by Alice Wilson McNeal with the following contributions:

- Method development: Ceri Lewis, Cameron Hird, Kathryn E. Smith, Katie Somerville-Hall
- Laboratory work: Cameron Hird, Ceri Lewis, Katie Somerville-Hall
- Data analysis: Kathryn E. Smith, Katie Somerville-Hall
- Project supervision: Ceri Lewis, Rod W. Wilson

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3.1. Abstract

To contribute to future generations, the sperm of marine invertebrates must swim to and fertilise an egg in seawater, exposing them to a host of environmental stressors. This includes ocean acidification (OA) which is estimated to reduce mean surface ocean pH by approximately 0.4 units within 80 years. This may impact the ionisation of pH-sensitive marine contaminants, altering their biological effects. We hypothesise that the effects of the acidic pharmaceutical drug tolcapone will increase in OA conditions, whilst the effects of the basic pharmaceutical drug fluoxetine will decrease, based on their expected ionisation. We analysed the influence of OA on the effects of each pharmaceutical to the early life stages of two invertebrates, the urchin Lytechinus pictus and the lugworm Arenicola marina. OA increased measurements of sperm swimming (curvilinear velocity, straight-line velocity and average path velocity) twofold for L. pictus but decreased these parameters by half for A. marina. Pharmaceuticals also differentially altered the magnitude of these effects: fluoxetine decreased velocity parameters by more than half in OA conditions for both species, whilst tolcapone increased these measurements for L. pictus but decreased them for A. marina. Changes to fertilisation success also differed between species, resulting in differential effects of OA and pharmaceuticals on this key measurement of reproductive output. In these comparatively simple life stages, changes to the toxicity of ionisable pharmaceuticals were not as hypothesised based solely on their acid dissociation chemistry, and species differences also played a role in determining pharmaceutical effects in OA conditions.

3.2. Introduction

Gametes and embryos are a fundamental part of the marine invertebrate life cycle. For many species, eggs and sperm are released directly into the water column via broadcast spawning (Levitan, 1998a) and must complete early life processes such as locating eggs and fertilisation whilst directly exposed to seawater conditions. In order for successful fertilisation to occur, sperm must be effective swimmers, but the parameters defining effectiveness vary between species, populations and environmental conditions. For example, trade-offs between sperm swimming velocity and sperm longevity occur between males of many species (Levitan, 2000), as the energy required for both is limited, and each is more valuable in different conditions. The generally accepted paradigm is that faster sperm swimming speeds equate to higher fertilisation success, with males producing faster sperm having a greater chance of fertilising eggs: this occurs for the urchin *Strongylocentrotus purpuratus* (Levitan, 1993) when spawning in high population densities requiring successful sperm to reach eggs quickly (Levitan, 1998b). However, in certain scenarios longevity is more important in determining fertilisation outcomes. For example, the urchin *Strongylocentrotus droebachiensis* produces longer-lived but slower swimming sperm when living at lower population densities where sperm may have to swim for some time to locate an egg (Levitan, 1998b, 1993). These sperm traits can also be driven by parental effects and can influence offspring health: male ascidians *Styela plicata* produce more motile, longer-lived sperm when kept at higher densities (Crean and Marshall, 2008), and offspring sired by longer-lived sperm exhibit higher performance (Crean et al., 2012). As population densities and seawater conditions, such as pH and temperature, can vary between spawning events, many males produce sperm encompassing a range of swimming traits to increase chances of fertilisation in heterogenous environments (Crean and Marshall, 2008; Smith et al., 2019).

In the context of global and local environmental change, the early life stages of broadcast spawning marine invertebrates are directly exposed to seawater stressors such as warming, hypoxia, acidification and pollution (Aitken et al., 2004). Whilst adult marine invertebrates possess a range of protective and defensive mechanisms against the negative impacts of ocean change, gametes lack many of these mitigation strategies, potentially increasing their susceptibility to marine stressors (Kroeker et al.,

2010; Marshall, 2006; Xie et al., 2005). Sperm are generally considered to lack the main repair mechanisms for cellular and DNA damage, as well as antioxidant defence capabilities (Aitken et al., 2004), and are widely considered to be particularly susceptible to oxidative stress (Nissen and Kreysel, 1983; Quijano et al., 2016). These are common impacts of marine stressors, in particular marine contaminants such as metals, pesticides and pharmaceutical drugs (Lesser, 2006; Lewis and Galloway, 2008; Mearns et al., 2019). A range of biological effects have been documented for early life history stages exposed to contaminants, for example the metal copper, which can reduce fertilisation success (Campbell et al., 2014); multiple antidepressant drugs, which can exhibit spermicidal effects (Kumar et al., 2006); and fungicide azoles, which can impact fertilisation and early development (Pennati et al., 2006).

In addition, a number of processes involved in sperm swimming and fertilisation are pH-dependent (Nishigaki et al., 2014). These processes may therefore be impacted by ocean acidification (OA) as ocean pH continues to decline from the current global mean of 8.15 to approximately 7.7 by the year 2100, driven by increasing atmospheric CO_2 concentrations (IPCC, 2021). This has prompted a number of investigations into whether this change in ocean carbonate chemistry will have negative implications for broadcast spawning invertebrates. Whilst the majority of studies have observed reductions in sperm swimming and fertilisation success under OA conditions (Morita et al., 2010; Nakamura and Morita, 2012; Schlegel et al., 2015, 2014, 2012; Uthicke et al., 2013; Vihtakari et al., 2013), others report no effects (Havenhand and Schlegel, 2009; Sung et al., 2014), and some have observed increases in both parameters (Caldwell et al., 2011; Smith et al., 2019). These variable responses may be linked to ecological and/or physiological mechanisms: for example, there are thought to be species differences in the optimum pH ranges for the multitude of enzymes involved in processes such as activation and fertilisation (Beltrán et al., 2007), and organisms living in coastal environments with variable pCO_2 and pH may be adapted to a greater range of carbonate chemistry parameters (Kapsenberg and Cyronak, 2019).

For coastal species, gametes will be spawned into waters experiencing both contamination and changing carbonate chemistry, therefore understanding interactions between these stressors will be important in assessing future population health for broadcast spawning organisms. A small number of studies have investigated

the effects of a combination of OA and contaminants on marine invertebrate early life stages, with several identifying interactive effects between these stressors (Caetano et al., 2021; Campbell et al., 2014; Lewis et al., 2013). One potential cause of such interactive effects is the effect of seawater pH on contaminant speciation or ionisation. Some contaminants exist in multiple chemical forms when dissolved in water, and for certain compounds the relative proportions of these chemical forms are partially dependent on aqueous pH (Rendal et al., 2011; Stockdale et al., 2016). This includes the metal copper, for which the proportion of the highly bioavailable ion Cu^{2+} increases between pH 8.1 and pH 7.7 in seawater, producing interactive toxic effects for early life stages of the polychaetes *Arenicola marina* (Campbell et al., 2014) and *Pomatoceros lamarckii* (Lewis et al., 2013).

It is also an established principle in freshwater that the bioaccumulation potential and subsequently toxicity of an ionisable organic compound is partially dependent on its acid dissociation constant (pK_a): acidic compounds become less ionised, and hence better able to penetrate biological membranes (Camenisch et al., 1996), when pH decreases towards their pK_a, whereas bases become more ionised as pH decreases below their pK_a (Rendal et al., 2011; Simon and Beevers, 1952). This theory is yet to be investigated in a marine context, but suggests that the effects of an OA-induced pH reduction on ionisable acids and bases may be predictable based on their pK_a. Although a limited number of non-metal ionisable compounds have been investigated in OA conditions, increased effects in an OA scenario have been observed for the acidic pharmaceutical diclofenac (Munari et al., 2020, 2019) and sulfamethoxazole (Serra-Compte et al., 2018), whilst decreased bioconcentration has been observed for the weakly basic compounds venlafaxine, carbamazepine and citalopram (Serra-Compte et al., 2018). However, it is not well understood how the effects of ionisable contaminants to marine invertebrate early life stages might be altered by an OA-induced decline in pH.

Although limited in number, investigations into the internal pH of the sperm of broadcast spawning invertebrates demonstrate that this can decline in tandem with seawater pH in OA conditions in multiple species (Esposito et al., 2020). Sperm also have high surface-area to volume ratios, meaning that they may be at greater risk of uptake of increased concentrations of CO_2, H^+ ions and contaminants via passive

diffusion (Melzner et al., 2020, 2009), and their reduced cytoplasm is thought to reduce their pH-buffering capacity (Campbell et al., 2016). This makes them ideal study organisms for assessing the direction and magnitude of OA-induced changes to the effects of ionisable contaminants, as changes to both acid-base chemistry and the ionisation of contaminants in seawater should also occur in their internal environments. For the present study, we selected sperm from two marine invertebrate species with differing reproductive ecologies to assess whether OA and contaminants would have similar impacts on fertilisation success for species for which different aspects of sperm physiology are key to fertilisation success. For the painted urchin *Lytechinus pictus*, low-to-medium swimming speed is a key determinant of fertilisation success as this allows sperm more time to search for their small eggs, but OA disrupts this connection resulting in faster swimming speeds being linked to greater fertilisation success (Smith et al., 2019). Longevity is also key for the lugworm *Arenicola marina* as sperm are released at low tide and await the incoming tide to be washed into female burrows hours later (Williams et al., 1997), but they demonstrate faster swimming and a higher percentage of motile sperm than other broadcast spawning invertebrates, suggesting that these are also important in fertilisation success for this species (Campbell et al., 2014). As well as potential direct impacts on fertilisation processes, OA and contaminants could further affect fertilisation success by altering sperm swimming speed and motility.

We also selected two ionisable pharmaceutical drugs hypothesised to be differentially affected by OA to explore whether the direction of OA effect could be predicted based on their pK_a values. Tolcapone, a catechol-o-methyltransferase inhibitor used in the treatment of Parkinson's disease, is an acidic compound with pK_a 4.5 (Nissinen, 2010). Fluoxetine, a selective serotonin re-uptake inhibiting antidepressant, is a basic compound with pK_a 10.1 (Nakamura et al., 2008). Both pharmaceuticals interfere with neurotransmitters involved in sperm motility, fertilisation and early development in marine invertebrates (Alavi et al., 2014; Buznikov et al., 2007; Nelson and Cariello, 1989). Both also induce oxidative stress in various species and cell types (Di Poi et al., 2016; Gonzalez-Rey and Bebianno, 2013; Grünig et al., 2017; Maser et al., 2017; Tafazoli et al., 2005) to which early life stages are thought to be particularly susceptible (Aitken et al., 2004; Quijano et al., 2016), and fluoxetine is spermicidal to human sperm (Kumar et al., 2006).

We exposed gametes from each species to each of these pharmaceuticals at ambient pH (current mean surface ocean pH 8.15) and in OA conditions (future predicted mean surface ocean pH 7.7). We test the hypothesis that tolcapone will impair sperm swimming in both species due to its ability to induce oxidative stress, and that these effects will be exacerbated in OA conditions as a weak acid with pK_a 4.5 should become less ionised between ambient pH and OA (H_1). We additionally test the hypothesis that fluoxetine will impair sperm swimming in both species through oxidative stress and spermicidal activity, but that these effects will be reduced in OA conditions as a weak base with pK_a 10.1 should become more ionised between ambient pH and OA (H_2). As sperm swimming is linked to successful fertilisation for both species, the percentage of successfully fertilised eggs was also measured in order to quantify the fitness impacts of interactions between OA and contaminants, as this ultimately determines the health of the future population.

3.3. Methods

3.3.1. Animal and gamete collection

Painted urchins (*Lytechinus pictus*) were collected from Dunamnus Seafoods Ltd., Ireland, and transported under controlled conditions to the University of Exeter, UK. Urchins were maintained in 300 L recirculating artificial seawater (ASW) holding tanks (35.0 ± 0.5 PSU Tropic Marin© salts, pH 8.15 ± 0.03, 15.0 ± 0.5˚C), and fed *ad libitum* with macroalgae (*Laminaria* spp.), collected from Teignmouth, UK, for several months to stimulate gravid status; this was confirmed prior to experiments in September 2019 via weekly attempted spawning of groups of individuals which were subsequently relocated to separate stock tanks to avoid the detection of spawning cues by other urchins. Lugworms (*Arenicola marina*) were collected from Mothecombe beach, Devon, UK (50°31″23 N, −3°94″58 W) three times between October and November 2020, their natural spawning season, to assess maturity prior to experiments. Lugworms were maintained in 16 L holding tanks with sand (20 cm depth) collected from the same location, with full ASW water changes performed daily.

For urchins, spawning was induced in males (n = 10) and females (n = 10) via injection with 0.5 ml of 0.5 M potassium chloride into the perivisceral coelomic cavity through

the peristomal plating, stimulating the gonadal wall to contract and release gametes from the aboral gonophores (Levitan, 2000). Females were placed, inverted, on falcon tubes containing 50 ml ASW, allowing oocytes to collect in the bottom. Sperm were collected “dry” from males, to prevent premature activation, and stored on ice in 1.5 ml microcentrifuge tubes until use.

For lugworms, females (n = 8) were injected with 1 ml prostomial homogenate (equivalent to 1 per individual), obtained from other gravid females, and kept overnight in individual crystallising dishes containing 180 ml ASW to prevent adults from drying out but minimise the volume of water for eggs to be spawned into (Campbell et al., 2014). Eggs were collected the next morning, transferred to falcon tubes in fresh ASW and kept on ice. Male lugworms (n = 10) were injected with 1 ml 8,11,14-eicosatrienoic acid (Merck) into the coelomic cavity to induce spawning (Pacey and Bentley, 1992). Sperm were collected “dry” as they were released from nephromixia approximately 1 hour later and stored on ice in 1.5 ml microcentrifuge tubes until use.

Eggs were quantified in both species by counting a mean number of settled oocytes from three 1.5 µl subsamples. The dilution factor for sperm to be used in fertilisation experiments was calculated by performing serial dilutions in ASW, fixing samples with 50 % ethanol, and counting in triplicate using a haemocytometer. All gametes were stored on ice and used within 10 hours of collection, with swimming parameters of sperm in control conditions assessed throughout this time period to ensure that there was no deterioration in sperm quality.

3.3.2. Experimental design

Doses of each contaminant were selected which would allow for the quantification of both increases and decreases in sperm functional parameters under OA conditions, enabling us to compare how effects changed, as opposed to simply comparing the effects of the compounds. Following pilot work using *A. marina*, tolcapone and fluoxetine were tested at 6.4 µg/L and 2.6 µg/L respectively as doses which produced similar effects on sperm swimming parameters but were below the maximum recorded effect (Figure S1).

Sperm functional parameters and fertilisation success were assessed for every male in each of six treatments: control seawater (with no pharmaceutical added) at ambient pH (pH 8.12 ± 0.01); control seawater in OA conditions (pH 7.71 ± 0.02); tolcapone at ambient pH; tolcapone in OA conditions; fluoxetine at ambient pH; and fluoxetine in OA conditions. Seawater pH corresponding to the IPCC RCP 8.5 scenario was targeted rather than pCO_2 levels (IPCC, 2014) as pH is expected to be the dominant driver of changes to the ionisation of contaminants (Rendal et al., 2011). Seawater conditions for each treatment are described in further detail in Table 1. For both sperm functional analyses and fertilisation assays, each male was treated as a replicate (n = 10 for both species), whilst eggs from multiple females were pooled for fertilisation experiments (n = 10 for urchins, n = 8 for lugworms).

3.3.3. Seawater chemistry

Tolcapone and fluoxetine hydrochloride were obtained from Merck in solid form and dissolved in ASW (35 ± 0.5 PSU Tropic Marin© salts) to the appropriate dose. OA conditions were obtained by aerating ASW with an air/CO_2 mix via mass flow controllers (AALBORG® Mass Flow Controller GFC17). Temperature, salinity and pH_{NBS} were measured in each treatment immediately prior to experiments and every 1.5 hours throughout the experimental period (YSI® 30 Salinity, Conductivity and Temperature System; HANNA® HI-98191 Professional Waterproof meter with HI-72911B electrode) and are presented in Table 1.

3.3.4. Sperm functional analysis

Sub-samples of sperm from each male were exposed to each of the above treatments. 7 µl of dry ejaculate was added to 1 ml treatment water, gently agitated then sealed and incubated (at 15 ˚C for urchins and 12 ˚C for lugworms to reflect natural spawning conditions) for 10 minutes; previous work has confirmed that pH does not change within a similar volume of seawater when sealed for this period of time (Campbell et al., 2016). A 3 µL sub-sample of this sperm-seawater mixture was transferred to 4-well slide chambers in duplicate, as a technical replicate, and motility immediately analysed using a Microptic Sperm Class Analyser (Microm, UK) with a Nikon Eclipse 50i negative phase contrast microscope at 100 x magnification, with a Peltier stage cooled to match incubation temperature. Percentage of motile sperm, curvilinear

Treatment	Temp. (˚C)	pH_{NBS}	Salinity (PSU)	Pharmaceutical (nominal, µg/L)
Control seawater – ambient pH	15.2 (± 0.1)	8.12 (± 0.01)	35.0 (± 0.09)	N/A
Control seawater – OA	15.0 (± 0.1)	7.71 (± 0.01)	34.9 (± 0.1)	N/A
Tolcapone – ambient pH	14.9 (± 0.1)	8.10 (± 0.002)	35.1 (± 0.1)	6.3
Tolcapone – OA	14.9 (± 0.1)	7.73 (± 0.01)	34.8 (± 0.1)	6.3
Fluoxetine – ambient pH	15.1 (± 0.1)	8.11 (± 0.01)	35.1 (± 0.1)	2.5
Fluoxetine – OA	15.0 (± 0.1)	7.71 (± 0.01)	34.9 (± 0.08)	2.5
Control seawater – ambient pH	11.9 (± 0.1)	8.12 (± 0.003)	35.1 (± 0.09)	N/A
Control seawater – OA	12.0 (± 0.1)	7.72 (± 0.01)	34.8 (± 0.08)	N/A
Tolcapone – ambient pH	11.7 (± 0.2)	8.11 (± 0.01)	35.2 (± 0.1)	6.3
Tolcapone – OA	11.8 (± 0.1)	7.71 (± 0.01)	34.9 (± 0.07)	6.3
Fluoxetine – ambient pH	11.9 (± 0.1)	8.10 (± 0.01)	34.8 (± 0.1)	2.5
Fluoxetine – OA	12.0 (± 0.1)	7.70 (± 0.02)	35.0 (± 0.1)	2.5

Table 1. Seawater chemistry (mean ± SEM) and pharmaceutical concentrations (nominal) for the six experimental treatments for *Lytechinus pictus* (top, white) and *Arenicola marina* (bottom, grey).

velocity (VCL), average path velocity (VAP) and straight-line velocity (VSL), were recorded for a minimum of 500 sperm per slide chamber, with sperm individually tracked for 0.5 s and recorded at 100 frames s^{-1}. Immotile sperm (VCL < 10 µM s^{-1} and VAP < 3.2 µM s^{-1}) were excluded from subsequent motility analyses as per Campbell et al. (2014), in order to calculate mean motility parameters only for motile sperm. Functional parameters were assessed in sperm in control seawater at ambient pH at both the start and end of the experimental period to confirm that there was no deterioration in sperm quality.

3.3.5. Fertilisation success

Fertilisations were performed following Campbell et al. (2014). Sperm from individual males was added to eggs pooled from multiple females, to provide an identical fertilisation environment for each male, minimise the influence of gamete compatibility, and replicate natural conditions where multiple adults would spawn concurrently and male fitness is dependent on their ability to fertilise eggs from multiple females. The

number of eggs ml^{-1} seawater for each female were counted and volume equivalents of the same number of eggs were pooled in a 15 ml falcon tube which was agitated immediately before each subsample was taken to ensure eggs were evenly distributed within seawater. Volume equivalents of 1,000 eggs per ml were added to treatment water (as above) in 6-well plates. Sperm from each male was diluted in corresponding treatment water, gently agitated for 20 seconds, then added to wells in triplicate to give a final dilution of approximately 100,000 sperm ml^{-1} for *L. pictus*, and 10,000 sperm ml^{-1} for *A. marina*. These concentrations give an approximate fertilisation success rate of 50 % under control conditions (Smith et al., 2019; Williams et al., 1997), allowing for both increased and decreased success to be quantified. Wells were agitated to mix gametes and incubated for 10 minutes (at 15 ˚C for urchins and 12 ˚C for lugworms to reflect seasonal spawning conditions), then rinsed gently with treatment water to remove free sperm and prevent further fertilisations. Eggs were incubated in treatment water for 18 hours for fertilised eggs to begin development, then fixed with 4 % paraformaldehyde. Wells containing only eggs were also incubated to ensure no cross-contamination of sperm occurred. The presence or absence of the fertilisation membrane and/or first cleavage was counted in 100 eggs per well. All counts were performed using a compound microscope at 40x magnification (Zeiss Primo Star iLED). Fertilisations were carried out in control seawater at ambient pH at both the start and end of the experimental period to confirm that there was no deterioration in gamete quality.

3.3.6. Statistical analysis

Data were analysed in GraphPad Prism 9.0, with log transformations applied to any data that violated the homogeneity of variance assumption according to the Brown-Forsythe test or the normality assumption according to the Shapiro-Wilk test. The effects of tolcapone and fluoxetine on sperm swimming parameters and fertilisation success in each species at ambient pH were analysed by one-way analyses of variance (ANOVA) with multiple comparisons performed using Tukey's post-hoc. To determine the effect of OA on sperm swimming parameters and fertilisation success for all three treatments (control seawater, tolcapone and fluoxetine), the percentage change between the effects of pharmaceuticals for each male at ambient pH and OA was calculated. Following this, the effect of pharmaceuticals on this percentage

change was determined using one-way ANOVAs (separately for each species), with Tukey's post-hoc for multiple comparisons. Results are expressed as mean ± SEM. Significance is assumed at $P \leq 0.05$ for all tests. Full statistical results are presented in Table 2 and Table 3.

3.4. Results and discussion

3.4.1. Effects of tolcapone and fluoxetine at ambient pH

In control seawater at ambient pH, all measurements of sperm velocity (VCL, VSL and VAP) and the percentage of motile sperm were comparable to previous findings for *Lytechinus pictus* (Smith et al., 2019; Figure 1A-D). Mean measurements of velocity were 53.3 ± 4.4 µM s^{-1} for VCL, 21.6 ± 2.1 µM s^{-1} for VSL and 30.8 ± 2.7 µM s^{-1} for VAP. This species utilises slow sperm swimming speeds which are suggested to enable greater longevity and hence more time to locate and fertilise its small eggs (Levitan, 2000; Smith et al., 2019). Fertilisation success was 53.6 ± 2.1 % for *L. pictus* in control seawater at ambient pH (Figure 2), which is again similar to previously reported results for this species at the targeted sperm and egg densities (Smith et al., 2019). These representative findings demonstrate that the method of spawning did not cause urchins to release immature gametes, which can occur for some species (Ward et al., 2005).

Mean sperm swimming speed and percentage motility for *Arenicola marina* were greater those of *L. pictus* in all measured parameters (Figure 1A-D). The range of 50.2 to 118.7 µM s^{-1} for VCL (Figure 1A), and percentage of motile sperm of 83.5 to 98.8 % (Figure 1D) are comparable to previous findings for this species (Campbell et al., 2014). Both these parameters are suggested to be important in sperm competitiveness in *A. marina* (Campbell et al., 2014): whilst longevity is important in fertilisation success for *A. marina* sperm, which can still fertilise eggs several days after spawning, fertilisation success markedly declines over time once sperm are diluted in seawater (Williams and Bentley, 2002). This is likely an adaptation for their spawning ecology, as sperm spend many hours in highly concentrated "pools" at low tide, and are washed into female burrows with the incoming tide (Williams et al., 1997), therefore retaining energy until diluted in seawater followed by fast swimming to be the first to reach eggs may provide a competitive advantage. Hence, for the present study where fertilisations

	Lytechinus pictus				*Arenicola marina*			
	One-way ANOVA	**Tukey's post-hoc (C v T)**	**Tukey's post-hoc (C v F)**	**Tukey's post-hoc (T v F)**	**One-way ANOVA**	**Tukey's post-hoc (C v T)**	**Tukey's post-hoc (C v F)**	**Tukey's post-hoc (T v F)**
Curvilinear velocity (VCL)	**$F_{2,27}$ = 9.9 P = 0.0006*** **	P = 0.08	**P = 0.01** **	**P = 0.0006*** **	$F_{2,27}$ = 2.1 P = 0.1	P = 0.3	P = 0.2	P = 0.9
Straight line velocity (VSL)	**$F_{2,27}$ = 7.9 P = 0.002** **	P = 0.1	**P = 0.03***	**P = 0.002** **	**$F_{2,27}$ = 4.1 P = 0.03***	P = 0.08	**P = 0.04***	P = 0.9
Average path velocity (VAP)	**$F_{2,27}$ = 13.1 P = 0.0001*** **	P = 0.06	**P = 0.004** **	**P < 0.0001**** **	**$F_{2,27}$ = 3.6 P = 0.04***	P = 0.1	**P = 0.05***	P = 0.9
Percentage of motile sperm	**$F_{2,27}$ = 9.2 P = 0.0009*** **	P = 0.2	**P = 0.01** **	**P = 0.0009*** **	$F_{2,27}$ = 1.1 P = 0.3	P = 0.9	P = 0.3	P = 0.6
Fertilisation success	**$F_{2,27}$ = 101.3 P < 0.0001**** **	**P < 0.0001**** **	**P < 0.0001**** **	**P < 0.0001**** **	**$F_{2,27}$ = 5.9 P = 0.009** **	P = 0.64	**P = 0.009** **	P = 0.06

Table 2. Results of one-way analyses of variance (ANOVA) and post-hoc tests for the effects of control seawater (C) versus the pharmaceuticals tolcapone (T) and fluoxetine (F) on sperm swimming parameters and fertilisation success in *Lytechinus pictus* and *Arenicola marina*. * indicates a significant effect at P ≤ 0.05; ** at P ≤ 0.01; *** at P ≤ 0.001; **** at P ≤ 0.0001.

were performed on the order of minutes, we would expect fast swimming and/or high total motility to be linked to fertilisation success for *A. marina*. This was similar to fertilisation success for *L. pictus* at 48.2 ± 5.1 % in control seawater at ambient pH (Figure 2) and is close to the expected 50 % success rate for *A. marina* given the targeted egg and sperm density (Williams et al., 1997).

The effects of each pharmaceutical on sperm swimming and fertilisation success were first analysed in ambient pH conditions to provide a point of comparison for the results in OA conditions. Tolcapone had opposing directions of effect on the sperm of the two test species, with clear trends of increased speeds for *A. marina* and decreased speeds for *L. pictus* in all measured sperm velocity parameters. However, whilst the directions of these sperm swimming responses were consistent within a species these parameters were not statistically significantly different from those in the control seawater treatments due to high variability between individual males (Table 2). This is not unexpected as the mean sperm swimming responses of individuals from the same

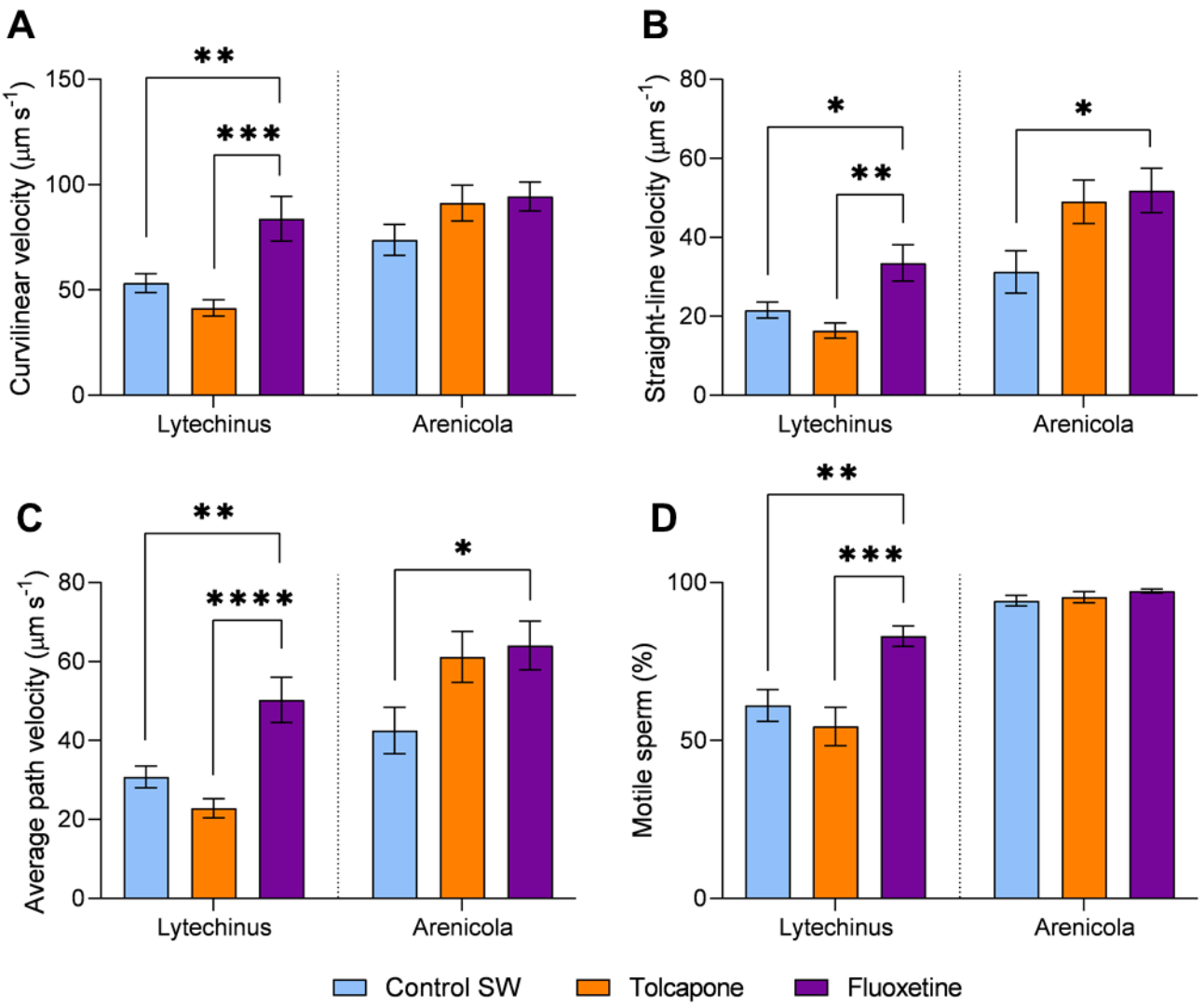

Figure 1. Effects of the pharmaceuticals tolcapone (6.3 µg/L) and fluoxetine (2.5 µg/L) versus control seawater (SW) on sperm swimming parameters (mean ± SEM) in the sea urchin *Lytechinus pictus* and the lugworm *Arenicola marina*. (A) Curvilinear velocity; (B) Straight-line velocity; (C) Average path velocity; (D) Percentage of motile sperm. Swimming parameters were recorded in a minimum of 500 sperm per replicate male (n = 10) for each treatment using computer aided sperm analysis. * indicates a significant difference between selected groups at P ≤ 0.05; ** at P ≤ 0.01; *** at P ≤ 0.001; **** at P ≤ 0.0001.

species commonly vary in their responses to environmental stressors (Campbell et al., 2016; Schlegel et al., 2014, 2012; Smith et al., 2019), and this may be more common in species inhabiting environmentally heterogenous habitats, such as coastal regions (Kwiatkowski et al., 2020; Torres et al., 2021). For *L. pictus*, all sperm swimming

parameters declined following exposure to tolcapone at ambient pH, with non-significant decreases of between 22.1 % and 25.9 % (Figure 1A-C). Tolcapone causes mitochondrial uncoupling and oxidative stress in mitochondria of various cell types via generation of reactive oxygen species (Grünig et al., 2017; Maser et al., 2017; Tafazoli et al., 2005), which may have induced the observed decreases in sperm swimming parameters as *L. pictus* sperm rely on mitochondrial energy for motility (Christen et al., 1983). In contrast, all measurements of sperm velocity increased for *A. marina* when exposed to tolcapone, with the greatest change observed in VSL which increased by 56.7 % (Figure 1A-C).

These differences in the effects of tolcapone may be due to differences in how the sperm of these two species utilise mitochondrial energy: sea urchin sperm motility is provided by their single mitochondrion which respires maximally (Christen et al., 1983), therefore even partial mitochondrial uncoupling will have a significant effect on ATP production in this species. Sea urchin sperm also rely solely on energy generated from endogenous lipids (Dorsten et al., 1997), therefore lipid peroxidation, to which sperm are thought to be susceptible in the presence of reactive oxygen species (Aitken et al., 2004; Nissen and Kreysel, 1983), could affect their only source of fuel. *A. marina* sperm possess several mitochondria and utilise internal stores of both carbohydrates and fat (Kaldis et al., 1997), therefore the effects of both uncoupling and lipid peroxidation may not impact this species to the same extent as *L. pictus*. It is not clear why tolcapone led to increases in sperm swimming parameters for *A. marina*, although Pacey et al. (1994) observed that the addition of cadmium chloride almost eliminated natural periods of reduced motility in this species, indicating that seawater contaminants have the potential to increase motility for this species. The percentage of motile sperm increased by less than 1 % for *A. marina* in response to tolcapone (Figure 1D), however as this was already 94.2 % in control conditions this may have been approaching a physiological maximum as this species already has high total motility compared to other broadcast spawning marine invertebrates (Esposito et al., 2020; Smith et al., 2019).

The reductions in velocity and motility measurements for *L. pictus* parallel a significant decline in fertilisation success of 25.6 % between control seawater and tolcapone treatments (one-way ANOVA, $F_{2,27} = 101.3$, $P < 0.0001$; Tukey's post-hoc $P < 0.0001$;

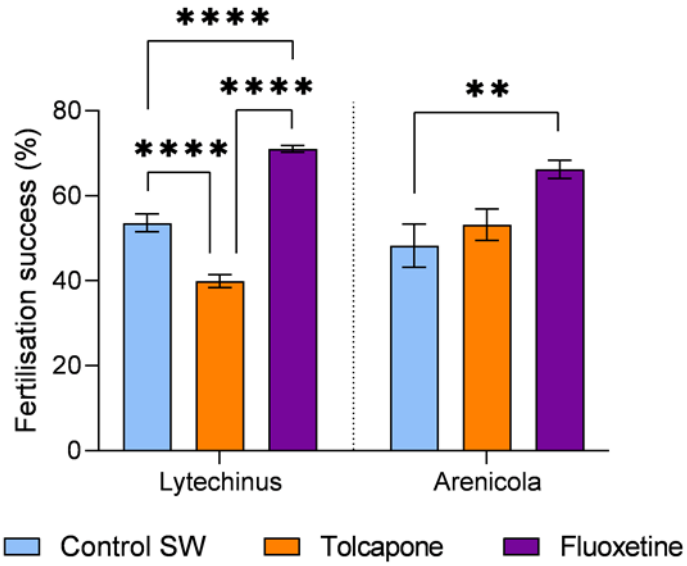

Figure 2. Effects of the pharmaceuticals tolcapone (6.3 µg/L) and fluoxetine (2.5 µg/L) versus control seawater (SW) on the percentage of successfully fertilised eggs (mean ± SEM) in the sea urchin *Lytechinus pictus* and the lugworm *Arenicola marina*. Sperm from each male (n = 10) were exposed to eggs from multiple females (n = 10 for urchins, n = 8 for lugworms) in each treatment, to simulate natural conditions where fertilisation success is dependent on the ability to fertilise eggs from multiple females. ** indicates a significant difference between selected groups at P ≤ 0.01; *** at P ≤ 0.001; **** at P ≤ 0.0001.

Figure 2). The similar magnitude of these concurrent changes suggests that the reduction in fertilisation success is due to the effect of tolcapone on sperm rather than any further effects of tolcapone on fertilisation or developmental processes. Whilst relatively slow swimming speeds, and therefore greater longevity (Levitan, 2000), are thought to be beneficial for *L. pictus* sperm as this enables a greater period during which sperm remain viable for them to search for their small eggs (Smith et al., 2019), there will be a point at which reductions in swimming speed will negatively impacts fertilisation rates. This could particularly impact lower density populations if gametes are quickly diluted as slower sperm may be unable to reach eggs. In contrast, there was no effect of tolcapone exposure on fertilisation success for *A. marina* (Tukey's post-hoc P = 0.64; Figure 2), suggesting that the non-significant increases in mean sperm velocity were not sufficient to affect mean fertilisation success. Alternatively, or in addition, oxidative stress induced by tolcapone may have directly impacted oocyte

quality (Lardies et al., 2008; Moran and McAlister, 2009), thereby reducing overall fertilisation success for *L. pictus* and mitigating the increase in fertilisation success for *A. marina* that might be expected to occur in the presence of faster sperm.

Fluoxetine had markedly different impacts on sperm motility to tolcapone for *L. pictus*, leading to significant increases in all swimming parameters at ambient pH (Table 2; Figure 1A-D). The greatest increase was observed for VAP which rose from 30.8 ± 2.7 to 50.3 ± 5.7 µM s^{-1} (one-way ANOVA, $F_{2,27}$ = 13.1, P < 0.0001; Tukey's post-hoc P = 0.004). The effect of fluoxetine was also significantly greater than that of tolcapone for all parameters (Table 2). Given the oxidative stress effects previously reported for both pharmaceuticals (Di Poi et al., 2016; Gonzalez-Rey and Bebianno, 2013; Maser et al., 2017; Tafazoli et al., 2005), which sperm are thought to be susceptible to (Aitken et al., 2004), it is surprising that exposure to these pharmaceuticals resulted in opposing directions of effect for *L. pictus* particularly due to its reliance on lipid stores as described above. Increases between 27.8 and 65.8 % also occurred for *A. marina* when sperm were exposed to fluoxetine (Figure 1A-C), although only these were only significant for VSL (one-way ANOVA, $F_{2,27}$ = 4.1, P = 0.03; Tukey's post-hoc P = 0.04) and VAP (one-way ANOVA, $F_{2,27}$ = 3.6, P = 0.04; Tukey's post-hoc, P = 0.05).

It is not clear why fluoxetine induced increases in all measured reproductive parameters for both species. Serotonin activates and sustains motility in the sperm of broadcast spawning marine bivalves (Alavi et al., 2014), therefore as a serotonin receptor antagonist fluoxetine might be expected to reduce this positive action as occurs in mammalian sperm (Fujinoki, 2011; Kumar et al., 2006). Both species also demonstrated significant increases in fertilisation success in response to fluoxetine at ambient pH (Table 2), with a 32.6 % increase for *L. pictus* and 37.3 % for *A. marina* (Figure 2). Fluoxetine exposure has been demonstrated to enhance the percentage of fertilised eggs in the urchin *Heliocidaris crassipina* (Lo et al., 2021), although the precise mechanism is not known. The increase for *A. marina* may be tied to the increases in sperm velocity which is thought to be important for this species (Campbell et al., 2014). As slower swimming speeds are considered beneficial for maximising fertilisation success in *L. pictus* at ambient pH (Smith et al., 2019), the increased fertilisation success may instead be a result of the increase in the percentage of motile sperm which underwent a near-identical increase of 35.8 % compared to the higher

increases in mean velocity parameters (Figure 1D). Our findings demonstrate that fluoxetine impacts the sperm of these two marine invertebrate species differentially to *in vitro* vertebrate sperm studies, suggesting that results from vertebrate species may not be applicable to broadcast spawning invertebrates in a contaminated ocean.

3.4.2. Ocean acidification alters the effects of tolcapone and fluoxetine

OA altered the effects of both tolcapone and fluoxetine on sperm swimming and fertilisation success, but these alterations differed in both direction and magnitude between species. OA increased all sperm swimming parameters in all three treatments (control seawater, tolcapone and fluoxetine) for *L. pictus*; conversely, OA caused all swimming parameters to decline for *A. marina* (Figure 3A-D). These effects were also far greater in magnitude for *L. pictus* than *A. marina* in both control seawater and tolcapone, but similar in magnitude for fluoxetine although in opposing directions. Although OA appeared to be the key determinant of the direction of effect for both species, tolcapone and fluoxetine both altered the magnitude of this effect, but not consistently in ways hypothesised in H_1 and H_2.

In control seawater, OA led to large increases in VCL, VSL and VAP for *L. pictus*, with the greatest increase observed for VSL which rose by 162.7 ± 25.0 % (Figure 3A-C). The percentage of motile sperm also increased by approximately half (Figure 3D). This species has previously been observed to increase swimming speed and the number of motile sperm in OA conditions (Smith et al., 2019), but this is in contrast to the majority of studied broadcast spawning invertebrates for which sperm swimming is decreased under OA conditions (Morita et al., 2010; Nakamura and Morita, 2012; Schlegel et al., 2015, 2014, 2012; Uthicke et al., 2013; Vihtakari et al., 2013). More in-keeping with other broadcast spawning invertebrates, OA had smaller but negative effects on swimming speed for *A. marina* with all measurements of velocity declining by approximately one-third (Figure 3A-C), although the percentage of motile sperm was minimally affected, declining by 3.7 % (Figure 3D). This also reflects previous findings for this species (Campbell et al., 2014).

Sperm are not believed to be able to regulate their internal pH in OA conditions, which decreases in tandem with seawater pH (Esposito et al., 2020). Therefore, the effects

	Lytechinus pictus				*Arenicola marina*			
	One-way ANOVA	Tukey's post-hoc Δ_{OA}-C v Δ_{OA}-T)	Tukey's post-hoc (Δ_{OA}-C v Δ_{OA}-F)	Tukey's post-hoc (Δ_{OA}-T v Δ_{OA}-F)	One-way ANOVA	Tukey's post-hoc (Δ_{OA}-C v Δ_{OA}-T)	Tukey's post-hoc (Δ_{OA}-C v Δ_{OA}-F)	Tukey's post-hoc (Δ_{OA}-T v Δ_{OA}-F)
Curvilinear velocity (VCL)	**$F_{2,27}$ = 17.9 P < 0.0001******	**P = 0.01****	**P = 0.04***	**P < 0.0001******	**$F_{2,27}$ = 5.8 P = 0.01****	**P = 0.02***	**P = 0.02***	P > 0.99
Straight line velocity (VSL)	**$F_{2,27}$ = 14.1 P < 0.0001******	**P = 0.02***	P = 0.06	**P < 0.0001******	**$F_{2,27}$ = 4.6 P = 0.02***	**P = 0.04***	**P = 0.04***	P > 0.99
Average path velocity (VAP)	**$F_{2,27}$ = 16.7 P < 0.0001******	**P = 0.05***	**P = 0.007****	**P < 0.0001******	**$F_{2,27}$ = 4.9 P = 0.02***	**P = 0.04***	**P = 0.03***	P > 0.99
Percentage of motile sperm	**$F_{2,27}$ = 6.2 P = 0.006****	P = 0.6	**P = 0.05***	**P = 0.006****	$F_{2,27}$ = 0.9 P = 0.4	P = 0.5	P = 0.5	P > 0.99
Fertilisation success	**$F_{2,27}$ = 27.9 P < 0.0001******	**P < 0.0001******	P = 0.7	**P < 0.0001******	$F_{2,27}$ = 0.2 P = 0.8	P = 0.9	P = 0.9	P = 0.8

Table 3. Results of one-way analyses of variance (ANOVA) and post-hoc tests for the effect of OA (Δ_{OA}) on sperm swimming parameters and fertilisation success in control seawater (C) versus the pharmaceuticals tolcapone (T) and fluoxetine (F) in *Lytechinus pictus* and *Arenicola marina*. * indicates a significant effect at P ≤ 0.05; ** at P ≤ 0.01; *** at P ≤ 0.001; **** at P ≤ 0.0001.

observed here may be due to species differences in the optimum pH of the many pH-dependent processes involved in sperm swimming (Beltrán et al., 2007; Nomura and Vacquier, 2006; Vacquier et al., 2014). Increases in sperm motility and swimming speed under OA conditions in the urchin *Psammechinus miliaris* were suggested to be due to the low pH conditions in the paleo-ocean under which sea urchin reproductive physiology evolved (~pH 7.0; Caldwell et al., 2011); for example, urchin sperm-activating peptides, pH-induced activation and proteins produced by eggs are functional well below the target of pH 7.7 used in the present study (Caldwell et al., 2011; Hirohashi and Vacquier, 2002). In contrast, motility in *A. marina* is only triggered above pH 7.6, suggested to be due to sperm remaining immotile until seawater from the incoming tide washes them into female burrows (Pacey et al., 1994b). There were minimal impacts on the percentage of motile sperm for *A. marina* (Figure 3D), indicating that pH 7.7 was indeed sufficient to sustain motility, but optimum pH for swimming speed may be higher for this species to better reflect seawater from an incoming tide (Blackford et al., 2017; Duarte et al., 2013). This highlights the

importance of differences in physiology and ecology in determining responses to marine stressors.

Most broadcast spawning marine invertebrates produce sperm encompassing a range of traits (Campbell et al., 2016; Levitan, 2000; Smith et al., 2019), therefore whilst mean swimming velocity may change there are often still sperm able to fertilise eggs. Hence, it is perhaps not surprising that mean fertilisation success was not altered in the majority of OA treatments (Table 3; Figure 4) in spite of the changes in sperm swimming parameters. Additionally, both species inhabit coastal environments where carbonate chemistry is highly variable (Duarte et al., 2013; Torres et al., 2021) and may therefore produce sperm able to successfully fertilise in a range of pH conditions.

Changes to the effects of tolcapone induced by OA were not as hypothesised. As an acid with pK_a 4.5, according to H_1 the effects of tolcapone exposure would be increased in an OA scenario compared to ambient pH. In fact, OA reversed the direction of effect of tolcapone for both species in all sperm swimming parameters. *L. pictus* exposed to tolcapone suffered slight declines in sperm swimming measurements at ambient pH; these became significant increases between OA-control seawater and OA-tolcapone treatments (Table 3), with the greatest increase observed for VAP which rose by almost half again compared to OA in control seawater (one-way ANOVA, $F_{2,27}$ = 16.7, P < 0.0001; Tukey's post-hoc P = 0.05; Figure 3A-D). In contrast, *A. marina* demonstrated slight increases in all velocity parameters between ambient pH-control seawater and ambient pH-tolcapone treatments; between OA-control seawater and OA-tolcapone treatments all parameters underwent significant additional decreases, with VCL demonstrating the greatest decrease (one-way ANOVA, $F_{2,27}$ = 5.8, P = 0.01; Tukey's post-hoc, P = 0.02; Figure 3A-C). These changes mirrored the direction of the effect of OA in control seawater for both species, but all were greater in magnitude than this effect of OA alone which indicates an interaction between OA and tolcapone for both species. A reversal in the direction of the effect of tolcapone is unlikely to be due to an OA-induced increase in tolcapone effects based on the pH-pK_a relationship according to H_1. The influence of OA on tolcapone effects also clearly differed between species, indicating that, even in these relatively simple early life-stages, differences between species can result in different outcomes.

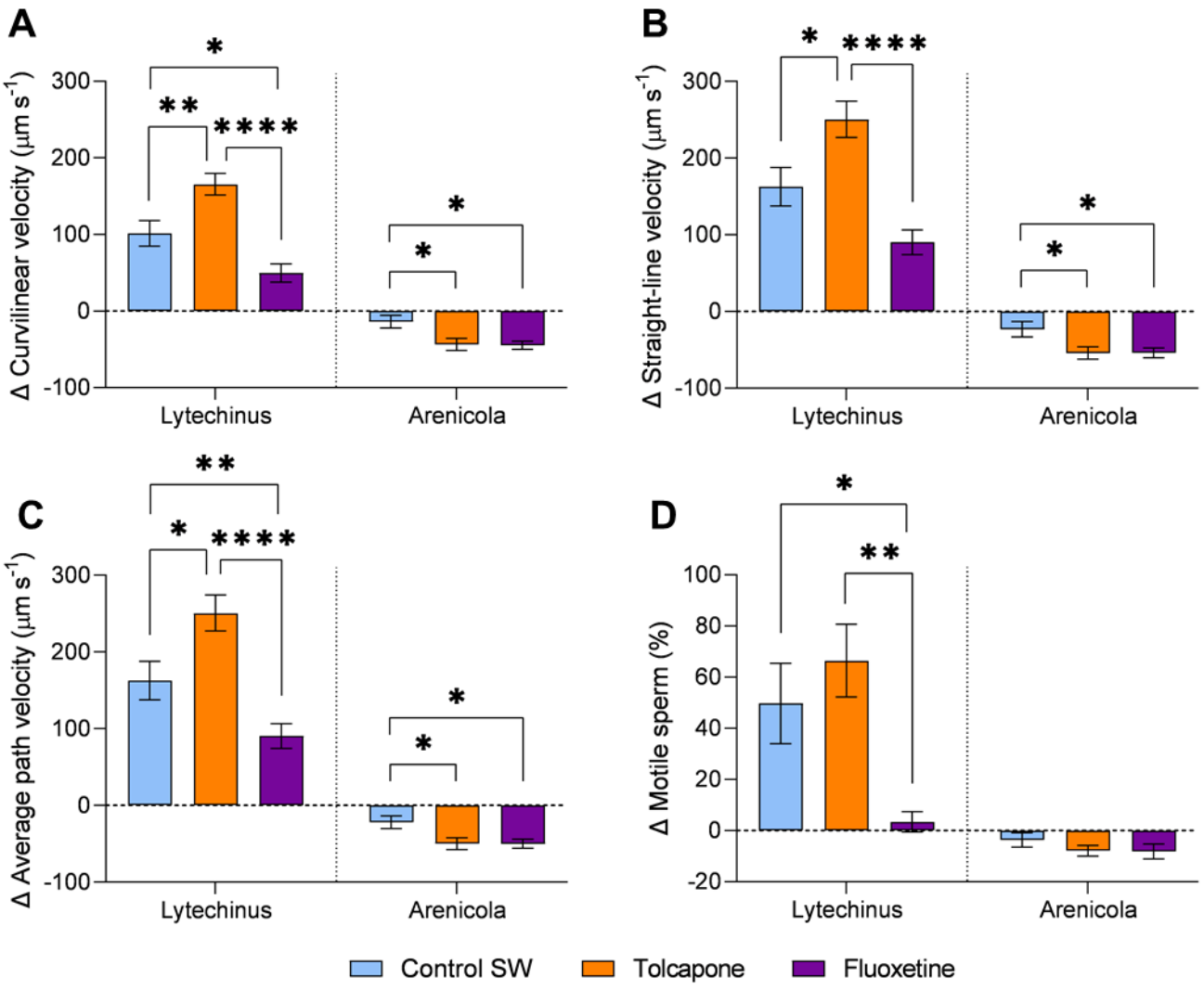

Figure 3. Effect of ocean acidification on responses in control seawater (SW) and to the pharmaceuticals tolcapone (6.3 µg/L) and fluoxetine (2.5 µg/L) in the sea urchin *Lytechinus pictus* and the lugworm *Arenicola marina*. Data are presented as the percentage difference between the response to no drug, tolcapone or fluoxetine at pH 7.7 and pH 8.1 (mean ± SEM). (A) Change in curvilinear velocity; (B) Change in straight-line velocity; (C) Change in average path velocity; (D) Change in percentage of motile sperm. Swimming parameters were recorded in a minimum of 500 sperm per replicate male (n = 10) for each treatment using computer aided sperm analysis. * indicates a significant difference between selected groups at P ≤ 0.05; ** at P ≤ 0.01; *** at P ≤ 0.001; **** at P ≤ 0.0001.

Conversely, whilst sperm swimming parameters also increased for *L. pictus* between ambient pH-fluoxetine and OA-fluoxetine treatments (Figure 3A-D), these increases were significantly lower than the increase induced by OA in control seawater. The

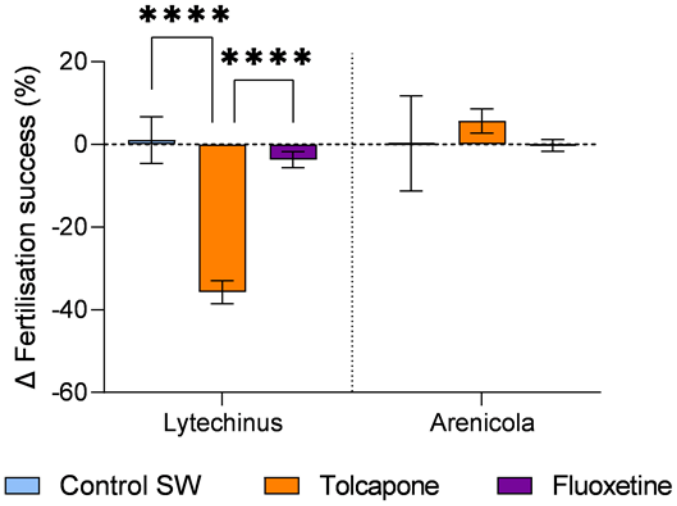

Figure 4. Effect of ocean acidification on the percentage of successfully fertilised eggs in the sea urchin *Lytechinus pictus* and the lugworm *Arenicola marina* in control seawater (SW) and when exposed to the pharmaceuticals tolcapone (6.3 µg/L) and fluoxetine (2.5 µg/L). Data are presented as the percentage difference between the response to no drug, tolcapone or fluoxetine at pH 7.7 and pH 8.1 (mean ± SEM). Sperm from each male (n = 10) were exposed to eggs from multiple females (n = 10 for urchins, n = 8 for lugworms) in each treatment, to simulate natural conditions where fertilisation success is dependent on the ability to fertilise eggs from multiple females. **** indicates a significant difference between selected groups at P ≤ 0.0001.

greatest decline of more than half occurred for VAP (one-way ANOVA, $F_{2,27}$ = 13.1, P = 0.0001; Tukey's post-hoc P = 0.01). These increases were also significantly lower than the increases induced by tolcapone exposure (Table 3). Similarly, despite fluoxetine increasing sperm swimming speed for *A. marina* at ambient pH, these parameters decreased in the OA-fluoxetine treatment (Figure 3A-C). These decreases were again greater than the effect of OA in control seawater, most notably for VCL which declined almost threefold (one-way ANOVA, $F_{2,27}$ = 5.8, P = 0.01; Tukey's post-hoc P = 0.02), and were similar to the effect of OA on tolcapone (Table 3). These findings support the hypothesis that fluoxetine will become more ionised and hence less biologically available between ambient pH and OA (H_2). It is worth noting that this theory did not hold true for tolcapone, and only a single basic contaminant was investigated here, but a reduction in pH has previously been shown to reduce biological uptake and impacts of fluoxetine in freshwater studies (Karlsson et al., 2017;

Nakamura et al., 2008). A recent study also observed that OA mitigated the effect of fluoxetine on urchin larval DNA damage and development in tandem with a modelled increase in ionisation (Lo et al., 2021). As both species experienced these reduced fluoxetine effects, it is worth considering that other species, life-stages and/or other basic ionisable compounds may show similarly reduced effects in OA conditions; this has been demonstrated during a pH reduction in freshwater conditions for other basic pharmaceuticals in multiple species (Alsop and Wilson, 2019; Neuwoehner and Escher, 2011). OA still appeared to be the dominant driver for the direction of effect here for both species, thus the differences in physiology and ecology as described above were likely key to the opposing effects between species, even with the addition of fluoxetine.

Tolcapone exposure was the only treatment to alter mean fertilisation success in OA conditions, and only for *L. pictus* (one-way ANOVA, $F_{2,27} = 27.9$, $P < 0.0001$; Tukey's post-hoc, $P < 0.0001$; Figure 4). At ambient pH, tolcapone caused both slower swimming and lower fertilisation success for *L. pictus*, whilst in OA conditions swimming speed increased but fertilisation success decreased further. Although OA did increase the effect of tolcapone on mean fertilisation success as hypothesised, the fact that this only occurred in this species and for this endpoint suggests that this effect is unlikely to have been driven by altered ionisation of tolcapone at pH 7.7 (H_1) as this would have resulted in similar effects for both species and more measured parameters. The increase in sperm swimming speeds may have been detrimental to the ability of *L. pictus* to locate eggs, as suggested elsewhere (Smith et al., 2019), given the well-established trade-offs between speed and longevity in sperm (Levitan, 2000). In combination with the reduced fertilisation success and slower mean swimming speeds induced by tolcapone at ambient pH, this suggests that swimming speeds outside of a narrow range may impact egg encounter rates for *L. pictus*. This indicates that the spawning ecology of *L. pictus* is key to reproductive success even in the presence of both OA and contaminants.

For *A. marina*, the decreases in sperm swimming speed induced by OA also did not correspond to reduced fertilisation success in either pharmaceutical treatment (Table 3; Figure 4). The only sperm parameter which did not change significantly between ambient and OA treatments for this species was the percentage of motile sperm

(Figure 3D), which reflects previous findings that this aspect of swimming is robust to OA (Campbell et al., 2014). This suggests that total motility may be a more important factor in fertilisation success than speed in *A. marina* in OA conditions, which is evidenced by the high percentage of motile sperm in all treatments compared to *L. pictus* and other broadcast spawning species (Esposito et al., 2020). This key aspect of sperm physiology, driven by the reproductive ecology of *A. marina*, appeared to be a greater determinant of mean fertilisation success than either stressor here. As fluoxetine increased fertilisation success at ambient pH, the lack of any effect of OA on fertilisation success for fluoxetine means that this parameter did still increase between OA-control seawater and OA-fluoxetine treatments, indicating that contaminants can still affect reproductive outcomes for this species even if there are no additional effects induced with the addition of OA.

Although OA and contaminants did not affect fertilisation success in the majority of treatments (Figure 4), whilst not assessed here, either or both of these stressors may have altered which sperm were most successful in fertilising eggs by altering the relative levels of importance between sperm traits. This includes swimming speed but also longevity, egg penetration ability or chemotaxis, as occurs for a range of environmental stressors (Crean and Immler, 2021). This can alter the contribution of different sperm, and hence males, to a future population which has previously been observed in OA conditions for multiple species including *L. pictus* (Campbell et al., 2016; Smith et al., 2019); this means that the effects of stressors on future populations can extend beyond the mean changes assessed in the majority of single and multi-stressor ecotoxicology studies.

OA appeared to be the dominant driver of the direction of effects on sperm swimming parameters and fertilisation success, although both tolcapone and fluoxetine influenced the magnitude of effect for both species. The differing effects of OA between species correspond to differences in physiology and spawning ecology, and the hypothesised pH-induced changes to the ionisation of tolcapone (H_1) and fluoxetine (H_2) either did not occur or did not override the effect of OA, even where the effects of fluoxetine appeared to be reduced. Taken together, these results indicate that such physiological and ecological differences, in combination with OA, were key drivers of mean fertilisation success for both species in all OA treatments, exerting a

greater influence on reproductive outcomes than the pharmaceuticals. Even for these relatively simple life-stages, it is not enough to apply theorised changes in contaminant effects and these changes must instead be considered in the context of OA, reproductive ecology and sperm physiology.

3.5. Supplementary information

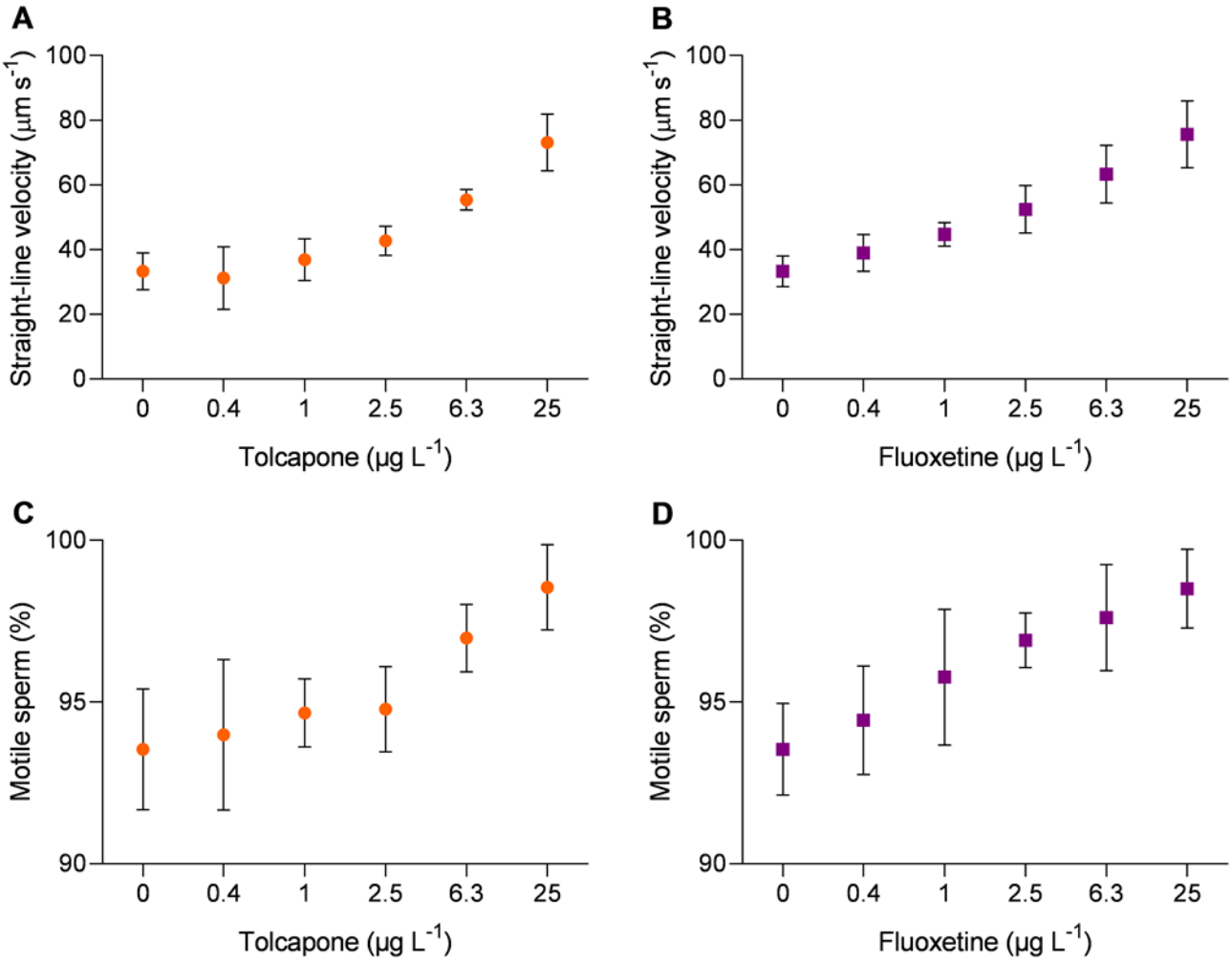

Figure S1. Data from pilot experiments using sperm from *Arenicola marina* (n = 4) to determine appropriate doses of tolcapone and fluoxetine for Chapter 3 experiments. (A) Straight-line velocity on exposure to tolcapone; (B) Straight-line velocity on exposure to fluoxetine; (C) Percentage of motile sperm on exposure to tolcapone; (D) Percentage of motile sperm on exposure to fluoxetine. Doses on the X axis are nominal concentrations: 10 mg of each compound was dissolved in artificial seawater with serial dilutions performed to obtain each concentration. Sperm functional analyses were performed as described in Chapter 3.

3.6. References

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Chapter 4. Ocean acidification impacts on the toxicity of pharmaceutical drugs to marine invertebrates do not follow ionisation-based patterns

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4.1. Abstract

Ocean acidification (OA) is driving a decline in global ocean pH, which can alter the toxicity of ionisable marine contaminants. Established freshwater models predict that the toxicity of acidic contaminants should increase, and the toxicity of basic contaminants decrease as pH declines, but this has rarely been assessed in marine invertebrates. We test this prediction by exposing two marine invertebrates, with differing ability to maintain acid-base homeostasis in OA conditions, to the acidic pharmaceutical tolcapone and the basic pharmaceutical fluoxetine under ambient (8.1) and OA (7.7) pH scenarios. OA-induced toxicity changes were not as hypothesised: OA increased the oxidative stress induced by tolcapone to the urchin *Paracentrotus lividus* by 50 %, whilst the mussel *Mytilus edulis* instead experienced altered metabolic parameters. OA increased fluoxetine-induced antioxidant activity in *P. lividus* by 183 % and decreased ammonia excretion rate by 57 %, whilst antioxidant activity decreased by 73 % in *M. edulis* alongside an 81 % increase in oxygen uptake. These differing effects did not correspond to differences in OA-induced alterations in extracellular fluid acid-base status between species. These data indicate that the effects of these ionisable pharmaceuticals to these marine invertebrates in OA conditions cannot be predicted based on expected changes to their ionisation.

4.2. Introduction

Ocean acidification (OA) is altering ocean carbonate chemistry. The dissolution of increasing atmospheric carbon dioxide (CO_2) is driving a global ocean pH decline, with a mean reduction of 0.3-0.4 units to approximately 7.7 predicted in the surface ocean by the year 2100 (IPCC, 2021). Coastal regions are expected to experience even lower pH extremes as OA exacerbates natural fluctuations in carbonate chemistry (Torres et al., 2021). CO_2 dissolved in seawater can diffuse into animal tissues, increasing H+ ion concentrations and thereby decreasing pH. Mechanisms used by different species to compensate for these changes include passive buffering of internal fluids, altered ion transport, and metabolic suppression (Melzner et al., 2020). The ability to accumulate extracellular bicarbonate as a pH buffer is thought to be a key determinant in resilience to OA, with animals lacking this ability frequently experiencing greater acid-base disturbances (Melzner et al., 2009). Uncompensated internal acidification can alter biochemical processes, reduce capacity for oxygen transport, and reduce calcification ability in calcifying organisms (Fabry et al., 2008). The ability of different species to maintain internal acid-base homeostasis in OA conditions varies widely, with some organisms undergoing complete compensation to maintain internal pH, whilst others are unable to buffer against any changes in seawater pH (Melzner et al., 2020, 2009).

Marine organisms are experiencing OA in combination with a multitude of ocean contaminants: historic contaminants, such as trace metals and oil from spills, and contaminants of emerging concern (Nyström, 2019; Tang et al., 2019). The latter includes pharmaceutical products, as they are designed to work at low doses (Fabbri and Franzellitti, 2016) and the structures and processes they target are frequently conserved in aquatic organisms (Gunnarsson et al., 2008). Physiological impacts of pharmaceuticals are wide-ranging and include metabolic rate (Falfushynska et al., 2019), growth and reproductive potential (Peters and Granek, 2016) and behaviour (Bossus et al., 2014). These can be induced either by the direct impact of pharmaceuticals on conserved drug targets or by additional mechanisms, such as oxidative stress via generation of reactive oxygen species and/or overwhelming of antioxidant defences (Lesser, 2006; Mearns et al., 2019). Many contaminants are thought to be pH-sensitive within the OA-relevant pH range, including certain ionisable

organic pharmaceutical compounds, due to the influence of ionisation state on the ability of a compound to penetrate biological membranes (Karlsson et al., 2017; Sun et al., 2020). Molecules of ionisable compounds can exist in either a neutral or ionised state, with the ratio of these depending on whether the compound is an acid or a base, the acid dissociation constant (pK_a) of the compound, and the pH of the media it is dissolved in. Neutral molecules are less polar, and hence better able to permeate fatty tissues and membranes than their ionised equivalents (Camenisch et al., 1996). In general, acidic compounds become more neutral as pH declines, and basic compounds become less so; therefore, uptake and toxicity of acids might be expected to increase at a lower pH, whereas uptake and toxicity may decrease for bases (Rendal et al., 2011).

Whilst there are well-established models describing the influence of aquatic pH on the toxicity of ionisable compounds in freshwater environments, such as biotic ligand models (Paquin et al., 2000), the influence of an OA-induced pH reduction on contaminants of this type is not well understood. Rendal et al. (2011) predicted an effect of ionisation within a freshwater pH range of 6 to 9 for acids with pK_a 3 and above, and bases with pK_a 12 and below. This pH range includes current mean open ocean pH of 8.1 and the end-of-century prediction of pH 7.7 (IPCC, 2021), and the majority of the 100 most used pharmaceuticals in the EU fall within these pK_a values (Patel et al., 2019). In addition, there has been little research concerning the influence of animal acid-base status, which can be altered by OA, on the toxicity of ionisable compounds. Differences in pH between seawater, extracellular and intracellular environments can lead to differential movement and partitioning of ionisable compounds between biological compartments (Rendal et al., 2011); this effect is sometimes used in pharmaceutical design to target specific biological compartments (Trapp et al., 2008). Alterations in internal pH induced by OA may therefore alter internal partitioning and hence toxic effects of pharmaceuticals, particularly if they affect their movement into intracellular compartments where key biological processes take place. It has also been demonstrated that pharmaceutical effects can be enhanced or altered during co-exposure with OA (Freitas et al., 2016; Maulvault et al., 2019; Munari et al., 2020). It is therefore crucial to understand how pharmaceutical toxicity will be affected in OA conditions, both by alterations in seawater pH and by concurrent alterations in the internal pH of marine organisms.

Given this pK_a-pH relationship, here we test the hypothesis that, in an OA scenario with reduced seawater pH, contaminants that behave as weak acids with pK_a lower than OA-associated ocean pH will produce greater toxicity effects to marine organisms, whilst contaminants that behave as weak bases with pK_a higher than current ambient ocean pH will induce lesser toxicity effects (H_1). To test these hypotheses we selected one acidic and one basic pharmaceutical as test compounds. The catechol-o-methyltransferase inhibitor tolcapone, which alters the physiological breakdown of the neurotransmitter dopamine, is a weakly acidic compound with pK_a 4.5 (Nissinen, 2010). Hence, under H_1, we should observe an increase in its biological effects when organisms are exposed to it under an OA scenario as seawater pH is decreased. In contrast, the anti-depressant fluoxetine which increases the bioavailability of the neurotransmitter serotonin, behaves as a weak base with pK_a 10.1 (Nakamura et al., 2008). Hence, under H_1, we should observe a decrease in its biological effects under OA conditions. As internal pH for marine invertebrates can differ to the external seawater pH depending on the acid-base regulation abilities of the species, we additionally test a second hypothesis (H_2): that any OA effect on the toxicity of acids will be greater in species for which extracellular pH is reduced in OA conditions, due to a greater proportion of unionised molecules which are better able to cross cell membranes and hence increase intracellular concentrations. Conversely, any OA effect on the toxicity of bases will be lesser in these species than for those which maintain a higher extracellular fluid pH, due to a lower proportion of unionised molecules when extracellular fluid is at a lower pH.

To test these hypotheses, we exposed two marine invertebrates with differing acid-base regulatory abilities to two pharmaceutical drugs in present day and future OA conditions. The mussel *Mytilus edulis* is considered a “poor” acid-base regulator due to its inability to accumulate extracellular bicarbonate in OA conditions, leading to a decline in extracellular pH (Zittier et al., 2018). The urchin *Paracentrotus lividus* is considered a “strong” acid-base regulator as it effectively maintains extracellular pH in OA conditions via accumulation of bicarbonate (Lewis et al., 2016). A range of biological endpoints were selected that can be measured in these species and that have previously been described as sensitive to our test pharmaceuticals and/or to OA exposures. Fluoxetine has been previously demonstrated to affect *Mytilus* species,

producing neurotoxic effects on acetylcholinesterase activity (Gonzalez-Rey and Bebianno, 2013) and increased oxidative stress (Franzellitti et al., 2014), and this pharmaceutical also affects development in larval sea urchins (Lo et al., 2021). There is limited research on the responses of invertebrates to tolcapone, but common responses in a range of organisms include oxidative stress (Grünig et al., 2017; Maser et al., 2017), altered cell metabolism (Tafazoli et al., 2005) and impacts on highly conserved dopaminergic pathways (Bonifácio et al., 2007). Serotonin and dopamine affect important physiological functions in *M. edulis*, such as muscular action (Gies, 1986) and responses to stress (Cao et al., 2007), and these pharmaceuticals could therefore alter metabolic rate. Little research has focused on these pharmaceuticals in adult sea urchins, although some species are known to be sensitive to serotonin (Squires et al., 2010) and dopamine (Van Alstyne et al., 2006). We measured acid-base physiology of both species in addition to metabolic, oxidative stress and enzymatic endpoints when exposed to each pharmaceutical in ambient seawater conditions to establish a baseline for these effects. These endpoints were also measured for each species when exposed to each pharmaceutical in OA conditions, to address our hypotheses by exploring the effects of both reduced seawater pH and acid-base homeostasis on the toxicity of pharmaceuticals.

4.3. Material and methods

4.3.1. Animal husbandry

Common mussels, *Mytilus edulis* (56-68 mm length, 100 in total), were collected from the Exe estuary, UK (50°62’19’’N, 3°44’35’’W). Purple urchins, *Paracentrotus lividus* (52-72 mm test diameter, 80 in total), were obtained from Roscoff Marine Station, France (48°43’35’’N, -3°59’18’’W), sourced from nearby wild populations. Stock animals were maintained in 300 L stock tanks with recirculating artificial seawater (Tropic Marin© salts 30 PSU, pH_{NBS} 8.14 ± 0.03, 15 ± 0.5˚C) and fed *ad libitum*: mussels with microalgae (Shellfish Diet 1800®, Instant Algae) and urchins with macroalgae collected from Teignmouth, UK (*Laminaria* spp., 50°32'19''N, 3°29'57''W). Good health status was confirmed in animals by assessing feeding, behaviour, colouration and response to physical stimuli.

4.3.2. Exposure conditions

Animals (n = 10) were exposed to one of six treatments in individual glass tanks (2 L for mussels, 3.5 L for urchins) for 10 days: ambient pH (current mean surface ocean pH 8.1); ambient pH-tolcapone (pH 8.1 + nominal 0.1 µg L^{-1} tolcapone); ambient pH-fluoxetine (pH 8.1 + nominal 0.1 µg L^{-1} fluoxetine); OA (predicted mean end-of-century surface ocean pH of 7.7); OA-tolcapone; or OA-fluoxetine. Animals were starved throughout exposure to avoid uptake of pharmaceuticals via food. Artificial seawater was aerated with either 100 % air to maintain pCO_2 and pH close to current ocean levels (442.4 ± 8.0 µatm pCO_2, pH 8.1 ± 0.01), or an air/CO_2 mix via mass flow controllers (AALBORG® Mass Flow Controller GFC17) to produce OA conditions based on end-of-century atmospheric CO_2 predictions (1333.0 ± 30.8 µatm pCO_2, pH 7.71 ± 0.01). Full seawater changes took place every 48 hours using water pre-gassed to target conditions, and 100 µl of tolcapone or fluoxetine stock solution (2 mg L^{-1} for mussels and 3.5 mg L^{-1} for urchins due to differing tank sizes) added to corresponding tanks. Salinity, pH and temperature were measured daily in four randomly selected tanks from each treatment (YSI® 30 Salinity, Conductivity and Temperature System; HANNA® HI-98191 Professional Waterproof pH meter with HI-72911B electrode). Seawater samples (12 ml) were taken from four randomly selected tanks per treatment on days 2, 4, 6 and 8 for analysis of dissolved inorganic carbon (DIC) using a custom system as described in Lewis et al. (2013). Temperature, salinity, pH and DIC measurements were used to calculate full seawater carbonate chemistry using CO2SYS v2.1 (Pierrot et al., 2006), with $KHSO_4$ from Dickson (1990) and dissociation constant from Dickson and Millero (1987) (Table 1).

4.3.3. Oxygen uptake, ammonia excretion, and O/N

Oxygen uptake and ammonia excretion rates were measured on day 9 of the exposure, prior to the extraction of extracellular fluid samples on day 10, to avoid stress responses to handling or sampling. Animals were transferred to respirometry chambers with water matching their exposure conditions and allowed to settle for one hour before air flow was shut off and tanks sealed. Oxygen (mmHg) and compensatory temperature (˚C) were measured (FireSting®-O2 with OXROB10 oxygen probe and TBSUB21 temperature probe) and water samples taken for ammonia analysis at the start and end of a four-hour period. Animals were returned to experimental tanks for

Treatment	Temp. (˚C)	pH_{NBS}	Salinity (PSU)	TA (µmol/kg)	pCO_2 (µatm)	HCO_3^- (µmol/kg)	CO_3^{2-} (µmol/kg)	ΩCa	ΩAr	Pharmaceutical (nominal, µg/L)
Ambient pH	15.8 (± 0.1)	8.16 (± 0.01)	30.0 (± 0.1)	2330.3 (± 14.1)	436.6 (± 6.7)	1975.9 (± 11.2)	146.3 (± 2.4)	3.6 (± 0.06)	2.3 (± 0.04)	N/A
OA	15.9 (± 0.1)	7.73 (± 0.01)	30.0 (± 0.1)	2297.3 (± 11.4)	1289.9 (± 18.4)	2153.8 (± 10.1)	59.0 (± 1.1)	1.5 (± 0.03)	0.9 (± 0.02)	N/A
Ambient pH-Tolcapone	16.0 (± 0.1)	8.16 (± 0.01)	30.0 (± 0.1)	2293.1 (± 17.8)	432.8 (± 12.2)	1943.2 (± 15.8)	144.0 (± 3.4)	3.6 (± 0.08)	2.3 (± 0.05)	0.1
OA-Tolcapone	15.9 (± 0.1)	7.73 (± 0.01)	30.0 (± 0.1)	2264.8 (± 26.5)	1270.8 (± 21.1)	2122.8 (± 23.7)	58.3 (± 1.7)	1.4 (± 0.04)	0.9 (± 0.03)	0.1
Ambient pH-Fluoxetine	15.9 (± 0.1)	8.16 (± 0.01)	29.9 (± 0.1)	2291.7 (± 21.1)	424.3 (± 8.1)	1938.4 (± 14.6)	145.5 (± 3.9)	3.6 (± 0.09)	2.3 (± 0.06)	0.1
OA-Fluoxetine	16.0 (± 0.1)	7.73 (± 0.01)	30.0 (± 0.1)	2277.5 (± 24.2)	1272.1 (± 21.8)	2133.3 (± 21.3)	59.2 (± 1.7)	1.5 (± 0.04)	0.9 (± 0.03)	0.1
Ambient pH	16.2 (± 0.1)	8.12 (± 0.01)	30.1 (± 0.1)	2230.0 (± 16.6)	462.4 (± 7.8)	1924.5 (± 14.6)	135.0 (± 1.6)	3.1 (± 0.04)	2.0 (± 0.02)	N/A
OA	15.9 (± 0.1)	7.69 (± 0.01)	30.1 (± 0.2)	2221.9 (± 23.1)	1469.7 (± 42.3)	2100.1 (± 20.9)	49.8 (± 1.7)	1.2 (± 0.04)	0.8 (± 0.02)	N/A
Ambient pH-Tolcapone	15.9 (± 0.1)	8.13 (± 0.01)	30.2 (± 0.1)	2238.4 (± 9.3)	452.6 (± 7.5)	1913.7 (± 9.6)	132.9 (± 1.3)	3.3 (± 0.03)	2.1 (± 0.03)	0.1
OA-Tolcapone	15.9 (± 0.1)	7.68 (± 0.01)	29.9 (± 0.2)	2175.0 (± 21.1)	1350.6 (± 34.0)	2049.4 (± 19.0)	51.2 (± 1.4)	1.3 (± 0.04)	0.8 (± 0.03)	0.1
Ambient pH-Fluoxetine	15.8 (± 0.1)	8.13 (± 0.004)	30.2 (± 0.1)	2240.1 (± 19.4)	445.4 (± 5.9)	1911.7 (± 16.5)	134.5 (± 1.8)	3.3 (± 0.04)	2.1 (± 0.02)	0.1
OA-Fluoxetine	15.9 (± 0.1)	7.69 (± 0.02)	30.1 (± 0.2)	2180.9 (± 17.7)	1345.1 (± 47.2)	2052.9 (± 23.8)	52.2 (± 2.0)	1.3 (± 0.05)	0.8 (± 0.02)	0.1

Table 1. Seawater carbonate chemistry (mean ± SEM) and pharmaceutical concentrations (nominal) for the six experimental treatments for *Mytilus edulis* (top, white) and *Paracentrotus lividus* (bottom, grey) calculated using CO2SYS (Pierrot, 2006).

24 hours for recovery before extracellular fluid samples were taken as described below, after which animals were flash-frozen in liquid nitrogen and weighed. Oxygen uptake rate is expressed as μmol O_2 g^{-1} h^{-1}.

Water samples were analysed for total ammonia using a colourimetric ammonia detection assay based on that described by Li et al. (2005). Sodium salicylate (1.44 M), sodium nitroprusside (5.5 mM), sodium dichloroisocyanurate (0.031 M) and potassium hydroxide (2.7 M) were added to water samples in a 96-well plate and read at 660 nm using a Tecan Infinite® M200 PRO plate reader. Following Cooper and Wilson (2008), ammonia excretion rates are expressed as total ammonia flux (μmol) g^{-1} $hour^{-1}$. Oxygen to nitrogen ratios (O/N) were determined using oxygen uptake and ammonia excretion rates as described by Taboada et al. (1998).

4.3.4. Acid-base physiology

Extracellular fluid samples (haemolymph for mussels and coelomic fluid for urchins) were taken at the end of the 10-day exposure period. Approximately 1.5 mL of fluid was drawn from the posterior adductor muscle of mussels, or coelomic cavity of urchins, using a chilled 21 G needle and 1 mL syringe. The pH_{NBS} was measured immediately (HANNA® HI-98191 Professional Waterproof pH/ORP/ISE meter with HI-1330B glass body refillable semi-micro electrode and HI-7662 temperature probe), and 50 μL was added to a glass haematocrit tube in duplicate for measurement of total CO_2 (Corning 965 Total CO_2 Analyser). A modified Henderson-Hasselbach equation was used to calculate acid-base parameters, using values derived from Truchot (1976) for the solubility constant for CO_2 and first dissociation constant (p*K*) for carbonic acid.

4.3.5. Oxidative stress

Activity of the antioxidant enzyme superoxide dismutase (SOD) in extracellular fluid was quantified using the Nitrotetrazolium blue (NBT) reduction assay as described elsewhere (Parry and Pipe, 2004). Xanthine oxidase was used to generate free radicals and the resultant rate of colour change of NBT was used to determine the activity of SOD. The thiobarbituric reactive substances assay (TBARS) was used to assess lipid peroxidation via the quantification of malondialdehyde (MDA), a by-product of oxidative stress to lipids, as described elsewhere (Camejo et al., 1999).

Protein content, quantified using the Braford assay, was used to normalise results (Bradford, 1976). All assays were run in triplicate in 96-well plates and analysed using a Tecan Infinite® M200 PRO plate reader.

4.3.6. Acetylcholinesterase

Acetylcholinesterase (AChE) activity was determined via the hydrolysis of acetylthiocholine iodide. Haemolymph or coelomic fluid samples were centrifuged at 3000 rpm for five minutes and 50 µL added to microplate wells in triplicate, followed by 150 µL of 5,5'-Dithiobis-(2-Nitrobenzoic Acid) (DTNB) at 270 µM. Samples were read kinetically at 405 nm for five minutes using a Tecan Infinite® M200 PRO plate reader. Immediately following this, 50 µL of 3mM acetylthiocholine iodide was added to wells and samples were again read kinetically at 405 nm for five minutes. AChE activity was standardised to protein content using the Bradford assay (Bradford, 1976), and is expressed as µmol substrate hydrolysed min^{-1} mg^{-1} protein.

4.3.7. Statistical analysis

Data that violated the assumptions of normality, assessed via the Shapiro-Wilk test, or homogeneity of variance, assessed via the Brown-Forsythe test, were log-transformed prior to analysis. Initial comparisons between control and pharmaceutical treatments at ambient pH were performed using student's t-tests in GraphPad Prism 9.0 and are presented as mean ± standard error. Data for tolcapone and fluoxetine at ambient pH and in OA conditions were analysed separately using two-way analysis of variance (ANOVA) with fixed factors "OA" and "species" to explore the effect of OA on responses to each pharmaceutical separately, rather than comparing the effects of the pharmaceuticals. Multiple comparisons were assessed using Tukey's post-hoc. Results are presented as mean percentage difference between ambient pH and OA treatments with 95 % confidence intervals. Full results of statistical analyses are presented in Tables S1 and S2.

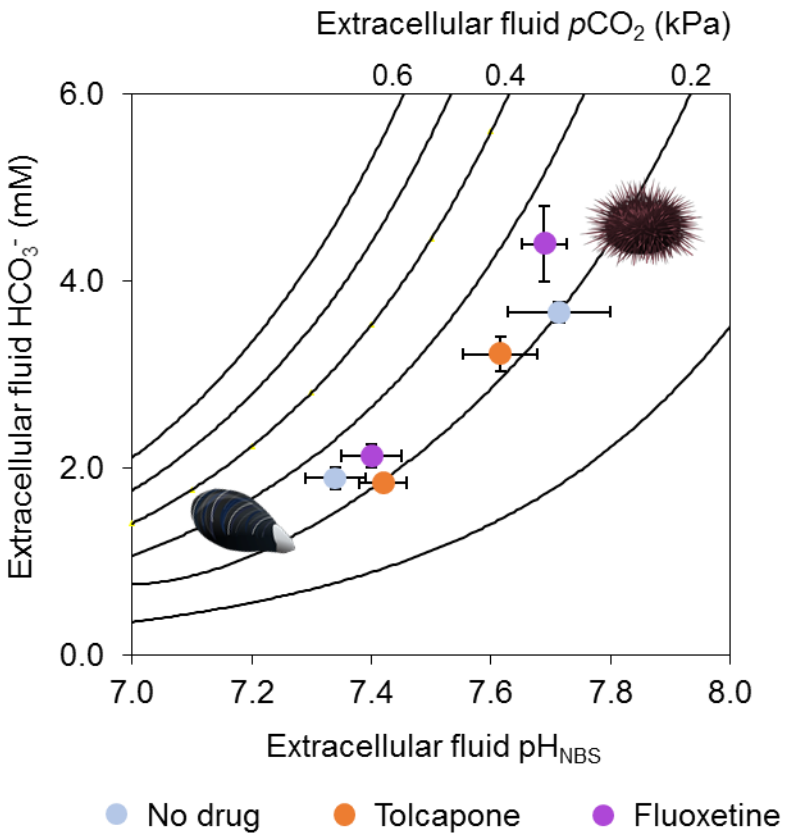

Figure 1. Acid-base diagram illustrating the relationships between extracellular fluid pCO_2, bicarbonate and pH (mean ± SEM) in *Mytilus edulis* (bottom left) and *Paracentrotus lividus* (top right), calculated from measured HCO_3^-, pH and TCO_2 according to Truchot (1976). Lines represent isopleths of equal pCO_2 (mmHg).

4.4. Results and discussion

4.4.1. Effects of pharmaceutical exposure under ambient seawater conditions (pH 8.1)

Acid-base parameters are reported first since these determine the internal (extracellular) pH conditions for the test species under which the pharmaceutical exposures are experienced. These are presented as an acid-base diagram in Figure 1 (Davenport, 1974). Under ambient seawater pH conditions (pH 8.1) with no pharmaceutical present, mussel extracellular fluid pCO_2 was 0.21 ± 0.02 kPa, bicarbonate concentration at 1.84 ± 0.08 mM and pH 7.42 ± 0.04. Despite comparable extracellular fluid pCO_2 at 0.16 ± 0.02, urchins maintained higher bicarbonate concentration and pH at 1.84 ± 0.08 mM and 7.71 ± 0.08 respectively. These data align with previous findings that *M. edulis* maintains a lower extracellular fluid pH and

bicarbonate concentration than *P. lividus* under ambient conditions (Lewis et al., 2016). These differences were maintained in the tolcapone and fluoxetine treatments (Figure 1). Extracellular fluid pCO_2 was slightly higher upon exposure to both tolcapone and fluoxetine, although this was not statistically significant for either species (Table S1). Neither pharmaceutical significantly affected mussel or urchin extracellular bicarbonate concentration or pH (Table S1). Whilst some OA-sensitive contaminants, such as the metal copper (Lewis et al., 2016), and the pharmaceutical carbamazepine (Freitas et al., 2016) can alter acid-base homeostasis, tolcapone and fluoxetine do not appear to have these effects for these species in ambient seawater conditions.

Tolcapone exposure differentially affected some metabolic parameters measured for mussels and urchins. At ambient pH with no pharmaceutical added, oxygen consumption in mussels was 0.92 ± 0.11 and ammonia excretion rate was 0.08 ± 0.02 μmol g^{-1} h^{-1} (Figure S1A-B). Exposure to tolcapone had no significant effect on either of these parameters (Table S1). However, oxygen-nitrogen ratio (O/N), which indicates the relative proportions of proteins and lipids used as metabolic substrate (Taboada et al., 1998), decreased by more than half for mussels exposed to tolcapone (t-test, P = 0.05) from 23 ± 5.77 to 10.14 ± 1.88 (Figure 2A). This indicates an increase in the proportion of proteins used as metabolic substrate. Oxygen uptake rate for urchins was lower than mussels at 0.54 ± 0.05 μmol O_2 g^{-1} h^{-1}, although ammonia excretion rate was similar at 0.07 ± 0.01 μmol g^{-1} h^{-1} (Figure S1A-B), which resulted in O/N being approximately half that of mussels in ambient pH conditions. Exposure to tolcapone had no effect on any metabolic parameters for urchins (Table S1).

This reduced O/N for mussels exposed to tolcapone may be related to increased antioxidant activity which increases protein turnover rate (Lesser, 2006). Superoxide dismutase (SOD) activity was significantly increased by tolcapone exposure in mussels (t-test, P = 0.03), from 0.51 ± 0.14 to 1.07 ± 0.19 units mg^{-1} protein (Figure 2B). This aligns with previous findings that tolcapone generates superoxide radicals in cells (Grünig et al., 2017; Maser et al., 2017) which would trigger SOD activity in response, thereby increasing protein production and catabolism due to the reduced half-life of SOD at high concentrations and hence reducing O/N (Ighodaro and Akinloye, 2018). Urchin SOD activity increased threefold from 0.94 ± 0.28 to 3.18 ±

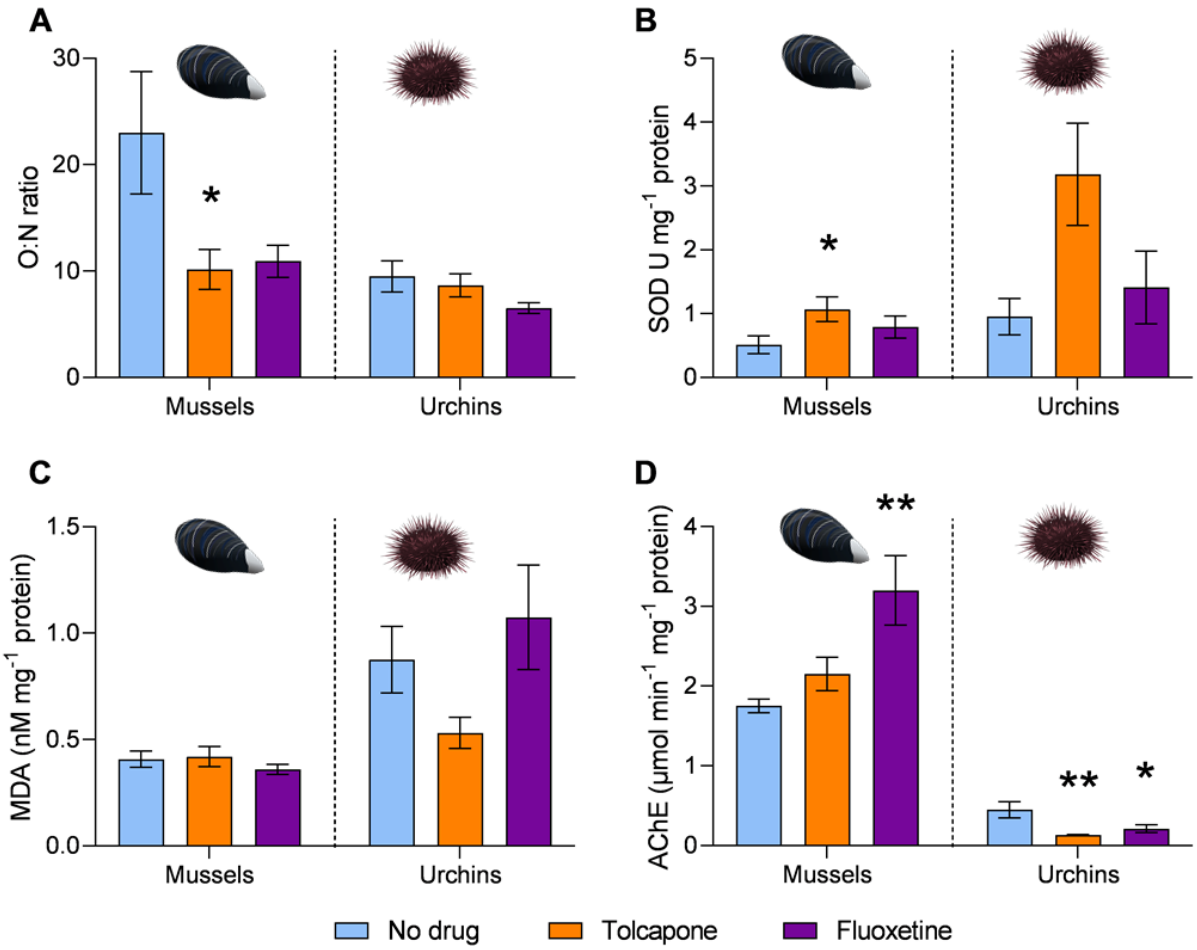

Figure 2. Toxicity and physiological biomarkers (mean ± SEM) in *Mytilus edulis* and *Paracentrotus lividus* following 10-day exposures to either tolcapone or fluoxetine at ambient pH 8.1. (A) Ratio of oxygen uptake to nitrogen excretion; (B) Superoxide dismutase activity; (C) Lipid peroxidation measured as malondialdehyde concentration; (D) Acetylcholinesterase activity. *, ** and *** represent significant differences at the $P < 0.05$, < 0.01 and < 0.001 level respectively.

0.80 units mg^{-1} protein in response to tolcapone (t-test, $P = 0.02$; Figure 2B), but was not accompanied by any metabolic changes; this could suggest a potential energy deficit for urchins over a longer term due to the energetic demands of antioxidant activity (Lesser, 2006). Lipid peroxidation (LPO) in ambient pH conditions was 0.41 ± 0.04 for mussels and 0.88 ± 0.16 for urchins, with no effect of tolcapone (t-test, for mussels $P = 0.83$; for urchins $P = 0.06$; Figure 2C). The lack of any increase in oxidative damage to lipids (LPO) for either species suggests that the increased SOD activity prevented oxidative stress by catalysing the dismutation of superoxide radicals (Ighodaro and Akinloye, 2018).

Exposure to fluoxetine had no significant effect on oxygen uptake for either species (Table S1; Figure S1A). Fluoxetine also had no effect on ammonia excretion rate for mussels (t-test, P = 0.93), but resulted in a significant increase in ammonia excretion for urchins to 0.13 ± 0.01 µmol g^{-1} h^{-1} (t-test, P = 0.0008; Figure S1B). Although exposure to fluoxetine led to decreases in O/N for both species, neither trend was significant (Table S1; Figure 2A). Fluoxetine exposure did not appear to elicit any oxidative stress responses in ambient conditions (Figures 2B and 2C), with no significant changes in SOD activity or LPO observed for either species (Table S1). Fluoxetine has been previously observed to trigger antioxidant activity in *Mytilus galloprovincialis* (Franzellitti et al., 2014), but this did not occur in all body tissues, such as the digestive gland, and therefore may not be a response in extracellular fluid as measured here. Additionally, the extent of fluoxetine's effects on oxidative stress parameters can vary non-monotonically with both dose and exposure time (Franzellitti et al., 2014; Gonzalez-Rey and Bebianno, 2013), therefore the present exposure concentration may not have triggered a response at the point of measurement.

At ambient seawater pH, acetylcholinesterase (AChE) activity for mussels was 1.75 ± 0.09 µmol min^{-1} mg^{-1} protein (Figure 2D). There was no significant effect of tolcapone on AChE activity for mussels (t-test, P = 0.11), but fluoxetine exposure induced a significant increase to 3.20 ± 0.43 µmol min^{-1} mg^{-1} protein (t-test, P = 0.006). An initial increase in AChE activity, followed by a decrease below control levels, was observed in *M. galloprovincialis* (Gonzalez-Rey and Bebianno, 2013) during exposure to a similar concentration of fluoxetine; this was suggested to be due to competition between serotonin and acetylcholine at synapses, or fluoxetine initially promoting AChE-associated apoptosis (Zhang et al., 2002). For urchins, AChE activity at ambient pH was 0.45 ± 0.10 µmol min^{-1} mg^{-1} protein (Figure 2D). In contrast to mussels, tolcapone exposure induced a significant decrease in AChE activity for urchins to 0.13 ± 0.01 µmol min^{-1} mg^{-1} protein (t-test, P = 0.009). Similarly, fluoxetine caused AChE activity to decline by half to 0.21 ± 0.05 µmol min^{-1} mg^{-1} protein (t-test, P = 0.04). This suggests that these neurotoxic compounds inhibited AChE activity for urchins, as occurs for other marine invertebrates (Dailianis et al., 2003; Gonzalez-Rey and Bebianno, 2013; Liu et al., 2018).

4.4.2. OA effects on physiology and toxicity endpoints

The effect size of OA on all endpoints (i.e. the percentage difference between endpoints measured in the OA and ambient pH treatments) was initially analysed between treatments with no pharmaceutical present, to establish whether effects were driven by OA alone or the combination of OA and pharmaceuticals. For mussels, the increase in seawater pCO_2 in OA conditions drove a concurrent significant increase in extracellular fluid pCO_2 of 172 % (two-way ANOVA for OA, $F_{1,39} = 27.5$, $P < 0.0001$; Tukey's post-hoc, $P = 0.001$; Figure 3). No corresponding change in bicarbonate concentration was observed in mussels (two-way ANOVA for OA, $F_{1,39} = 64.6$, $P < 0.0001$; Tukey's post-hoc $P = 0.5$); with a corresponding significant decline in extracellular fluid pH (two-way ANOVA for pH, $F_{1,39} = 7.2$, $P = 0.01$; Tukey's post-hoc $P = 0.04$). Urchins underwent a similar increase in extracellular fluid pCO_2 of 238 % (Tukey's post-hoc $P = 0.01$), and in contrast to mussels had a two-fold increase (presented in Figure 3 as a positive OA effect size of 100 %) in coelomic fluid bicarbonate concentration (Tukey's post-hoc, $P < 0.0001$), leading to a significant interaction term between OA and species (two-way ANOVA for OA*species, $F_{1,39} = 36.9$, $P < 0.0001$)). There was no corresponding decline in extracellular fluid pH, likely due to the buffering effect of extracellular fluid bicarbonate (Melzner et al., 2020) for urchins (Tukey's post-hoc, $P = 0.8$). These results align with previous findings that *M. edulis* is a poor acid-base regulator with limited ability to buffer against OA-induced extracellular pH decline, whereas *P. lividus* is able to accumulate bicarbonate to buffer against a reduction in coelomic fluid pH (Lewis et al., 2016).

With no pharmaceutical present, OA did not affect oxygen uptake or ammonia excretion rate for either species (Table S2; Figure 3). However, OA had a significant negative effect on mussel O/N, reducing this by 58 % (two-way ANOVA for pH, $F_{1,39} = 6.3$, $P = 0.02$; Tukey's post-hoc $P = 0.02$; Figure 3), indicating a switch in metabolic substrate towards a higher proportion of proteins. This could represent increased metabolism of proteins in order to maintain biomineralisation, which occurs for *M. edulis* above 1000 µatm pCO_2, reducing shell integrity and potentially growth (Fitzer et al., 2014). OA also had no significant effects on SOD activity or LPO for either species, (Table S2; Figure 3), which corresponds to previous findings that OA does

not induce oxidative stress in these species (Lewis et al., 2016). Similarly, OA did not affect AChE activity for mussels or urchins (Table S2; Figure 3).

4.4.3. Effect sizes of OA on tolcapone responses differed in magnitude and direction for mussels and urchins.

Calculating the relative effect of OA exposure on the physiology and toxicity endpoints measured in mussels and urchins exposed to tolcapone reveals key differences in both the magnitude and direction of the effect of OA on responses to tolcapone between these two test species. For tolcapone-exposed mussels and urchins, the effect of additional exposure to OA conditions on their acid-base parameters mirror the responses observed in the OA-no drug treatments (Figure 3), indicating that OA was the primary influence on acid-base physiology and thereby allowing us to discuss toxicity changes to pharmaceuticals in the context of internal acid-base change, as per our hypothesis (H_2). There were significant increases in extracellular fluid pCO_2 for both species (two-way ANOVA for OA, $F_{1,39} = 16.32$, $P < 0.001$; Tukey's post-hoc for mussels $P = 0.04$; for urchins $P = 0.03$), with a positive OA effect size of 54 % for mussels and 90 % for urchins (Figure 3). The increase in extracellular pCO_2 induced by OA was smaller, though not significantly, with tolcapone present than with no pharmaceutical for both species. Mussels are known to close their shells in response to contaminants (Fdil et al., 2006), potentially altering extracellular fluid pCO_2, but it is not clear how CO_2 uptake would be altered in urchins as they rely on a diffusion gradient for gas exchange and cannot alter CO_2 uptake (Collard et al., 2013).

The slightly lower pCO_2 in the tolcapone treatments did not appear to impact the effect of OA on other acid-base parameters for these species, which were close to identical to the effects induced by OA alone (Figure 3). There was no significant effect of OA on extracellular fluid bicarbonate concentration for mussels exposed to tolcapone (Table S2), but OA had a significant negative effect on extracellular fluid pH (two-way ANOVA for OA, $F_{1,39} = 0.92$, $P = 0.35$; Tukey's post-hoc $P = 0.047$). In contrast, OA induced a positive effect of 85 % on coelomic fluid bicarbonate for tolcapone-exposed urchins (two-way ANOVA for OA, $F_{1,39} = 34.31$, $P < 0.0001$; Tukey's post-hoc $P < 0.0001$), but there was no effect of OA on coelomic fluid pH (Tukey's post-hoc, $P = 0.41$). This produced a significant interaction between OA and species for extracellular

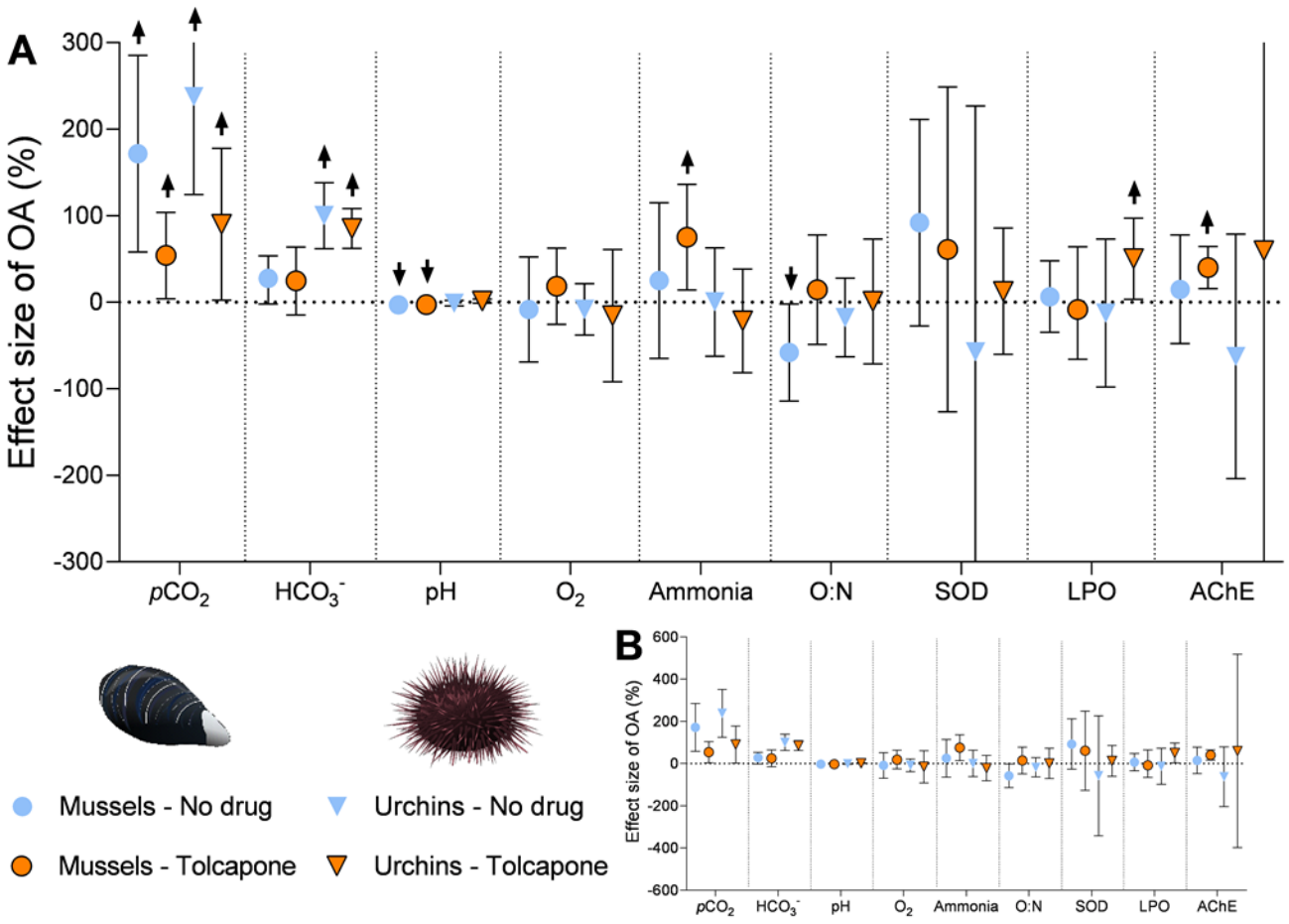

Figure 3. The effect size of OA (i.e. the percentage difference between responses in ambient (pH 8.1) and OA (pH 7.7) exposure conditions) on acid-base parameters, metabolic parameters and toxicity biomarkers for *Mytilus edulis* (circles) and *Paracentrotus lividus* (triangles) exposed to no pharmaceutical (blue) or to tolcapone (orange). Results are expressed as mean % change ± 95 % confidence intervals. (A) Arrows represent significant increases or decreases in each parameter between ambient and OA exposures, defined as whether confidence intervals cross zero; (B) panel shows full range of confidence intervals.

bicarbonate concentration (two-way ANOVA for OA*species, $F = 68.35$, $P < 0.0001$) and for extracellular fluid pH (two-way ANOVA for OA*species, $F_{1,39} = 9.52$, $P = 0.004$). According to H_2, greater toxicity effects may occur in mussels than urchins, as a reduction in extracellular pH will potentially increase permeability of tolcapone across cell membranes (Camenisch et al., 1996; Rendal et al., 2011).

As none of the significant effect sizes for metabolic or toxicological endpoints induced by OA in the tolcapone treatments were induced by OA alone (Figure 3), we can conclude that these were driven by the combination of OA and pharmaceuticals and therefore discuss these in the context of H_1. Additional exposure to OA did not alter oxygen uptake rate for mussels exposed to tolcapone (two-way ANOVA for OA, $F_{1,39}$ = 0.17, P = 0.68; Tukey's post-hoc P = 0.67). OA did have a significant effect on ammonia excretion rate for tolcapone-exposed mussels (two-way ANOVA for OA, $F_{1,39}$ = 3.38, P = 0.08; Tukey's post-hoc P = 0.01), with a mean positive effect size of 75 % (Figure 3). Despite this change in ammonia excretion, O/N in mussels exposed to tolcapone was not affected by OA (two-way ANOVA for OA, $F_{1,39}$ = 0.22, P = 0.64; Tukey's post-hoc, P = 0.92). This could indicate an attempt at maintaining a stable O/N ratio by increasing ammonia excretion rate in order to maintain normal metabolism of lipids and proteins, which occurs in other marine invertebrates (Hird et al., 2016).

For tolcapone-exposed urchins, OA had no significant effects on oxygen uptake or ammonia excretion rate (Table S2; Figure 3). Ammonia excretion is generally enhanced by reduced seawater pH as the concentration gradient between internal fluids and seawater increases (Randall and Tsui, 2006). It is therefore surprising that passive diffusion of ammonia did not enhance excretion rates for urchins when it did for mussels, possibly suggesting a reduced rate of protein turnover resulting in lower ammonia production. These differences led to a significant interaction term between OA and species for ammonia excretion rate (two-way ANOVA for OA*species, $F_{1,39}$ = 9.84, P = 0.004). As neither oxygen uptake nor ammonia excretion were significantly affected for urchins, it is not surprising that there was no effect of OA on O/N for tolcapone-exposed urchins (Tukey's post-hoc, P = 0.99; Figure 3).

This potentially reduced protein turnover rate for urchins compared to mussels is also supported by the different effects of OA on oxidative stress responses between species. In tolcapone-exposed mussels, OA did not significantly increase SOD activity or LPO (Table S2; Figure 3). Notably, there was a mean increase of 61 % in SOD activity for mussels, but this was not significant due to the high inter-individual variability, suggesting that strong antioxidant responses were only triggered in some individuals. Individual differences in SOD activity in polluted areas have previously

been observed in this species (Manduzio et al., 2004), and this also conforms to the "winners and losers" theory of global environmental change, where some individuals will be better able to cope due to natural physiological and genetic variation (Somero, 2010). As there was no mean change in LPO, this suggests that the SOD responses were triggered by greater effects of tolcapone in certain individuals, as otherwise we would have expected similar patterns in both mean and variation in LPO to that of SOD.

Conversely, although tolcapone-exposed urchins did not experience any additional increase in SOD activity when exposed to OA, LPO was increased by 50 % (two-way ANOVA for OA, $F_{1,39} = 3.29$, $P = 0.08$; Tukey's post-hoc $P = 0.03$; Figure 3). In ambient pH conditions, tolcapone caused a very strong increase in SOD activity for urchins which was effective in preventing LPO (Figure 2B-C). The lack of a further increase in SOD in OA conditions could indicate that further upregulation of SOD beyond this already threefold increase was not possible for urchins, which is supported by the suspected lack of an increase in protein turnover (Ighodaro and Akinloye, 2018). If SOD activity had been inhibited, for example by high concentrations of reactive oxygen species or by tolcapone itself as observed for other xenobiotics (Lewandowski et al., 2018), then we would still expect to see increased protein turnover as SOD would be upregulated but inactivated. There was a significant interaction term between OA and species for LPO (two-way ANOVA for OA*species, $F_{1,39} = 5.53$, $P = 0.02$), indicating significantly different effects of OA on the tolcapone response for each species, i.e. that urchins suffered a greater oxidative stress outcome. This did not conform to either hypothesis: greater bioavailability of tolcapone induced by lower pH (H_1) would have similarly impacted the direction of effect for both species, and the lower extracellular pH of mussels would have reduced the magnitude of this effect for this species (H_2). Therefore, some other biological differences between species must have driven these responses.

OA conditions may also have increased the effect of tolcapone on AChE activity, inducing a 40 % increase in for tolcapone-exposed mussels which was a greater increase than occurred in ambient pH conditions (two-way ANOVA for OA, $F_{1,39} = 10.47$, $P = 0.003$; Tukey's post-hoc $P = 0.0006$; Figure 3). Although not statistically significant, a similar mean percentage increase of 59 % occurred for urchins (Tukey's

post-hoc, P = 0.98). These may be a generalised stress responses, as AChE activity increases in response to a range of stressors such as temperature (Escartín and Porte, 1997) and anthropogenic noise (Vazzana et al., 2016), or a more complex response related to cross-talk between the interlinked cholinergic and dopaminergic systems (Gallo et al., 2016; Jones and Richards, 1993). As the greater increase occurred for urchins, this is again in opposition to our hypothesis that mussels would experience greater effects due to their reduced extracellular fluid pH (H_2).

4.4.4. Effect sizes of OA on fluoxetine responses differed in magnitude and direction for mussels and urchins.

As for tolcapone, the effect sizes of OA on responses to fluoxetine differ in magnitude and direction between mussels and urchins for several endpoints. According to H_1 & H_2, the effects of fluoxetine would decline in an OA scenario and to a greater extent for mussels compared to urchins. Similarly to the other OA treatments, OA exposure induced an increase in extracellular fluid pCO_2 in for both species when exposed to fluoxetine with mean positive effects of 67 % for mussels and 126 % for urchins (two-way ANOVA for OA, $F_{1,39} = 24.54$, $P < 0.0001$; Tukey's post-hoc for mussels, P = 0.02; for urchins, P = 0.002; Figure 4). As with tolcapone, these increases were smaller, but not significantly, than those induced by OA with no pharmaceutical present, again likely due to the slightly higher extracellular pCO_2 in both species exposed to fluoxetine at ambient pH (Figure 1).

As with the OA-only exposures, there was no effect of OA on haemolymph bicarbonate concentration for mussels exposed to fluoxetine (two-way ANOVA for OA, $F_{1,39} = 7.81$, P = 0.008; Tukey's post-hoc P = 0.93; Figure 4). Mussels also underwent a significant OA-induced haemolymph acidosis of 0.1 units, similar to the decrease in all other OA treatments (two-way ANOVA for OA, $F_{1,39} = 8.53$, P = 0.006; Tukey's post-hoc P = 0.03). Although OA had a significant positive effect of 30 % on urchin coelomic fluid bicarbonate concentration (Tukey's post-hoc P = 0.01), this was far lower than the increase stimulated by OA with no pharmaceutical or with tolcapone present despite comparable coelomic fluid pCO_2 (Figure 4). Serotonin is involved in osmoregulation in several marine invertebrates (Bonnefille et al., 2018; Liu et al., 2018) and altered serotonin bioavailability induced by fluoxetine (Franzellitti et al., 2014) could therefore

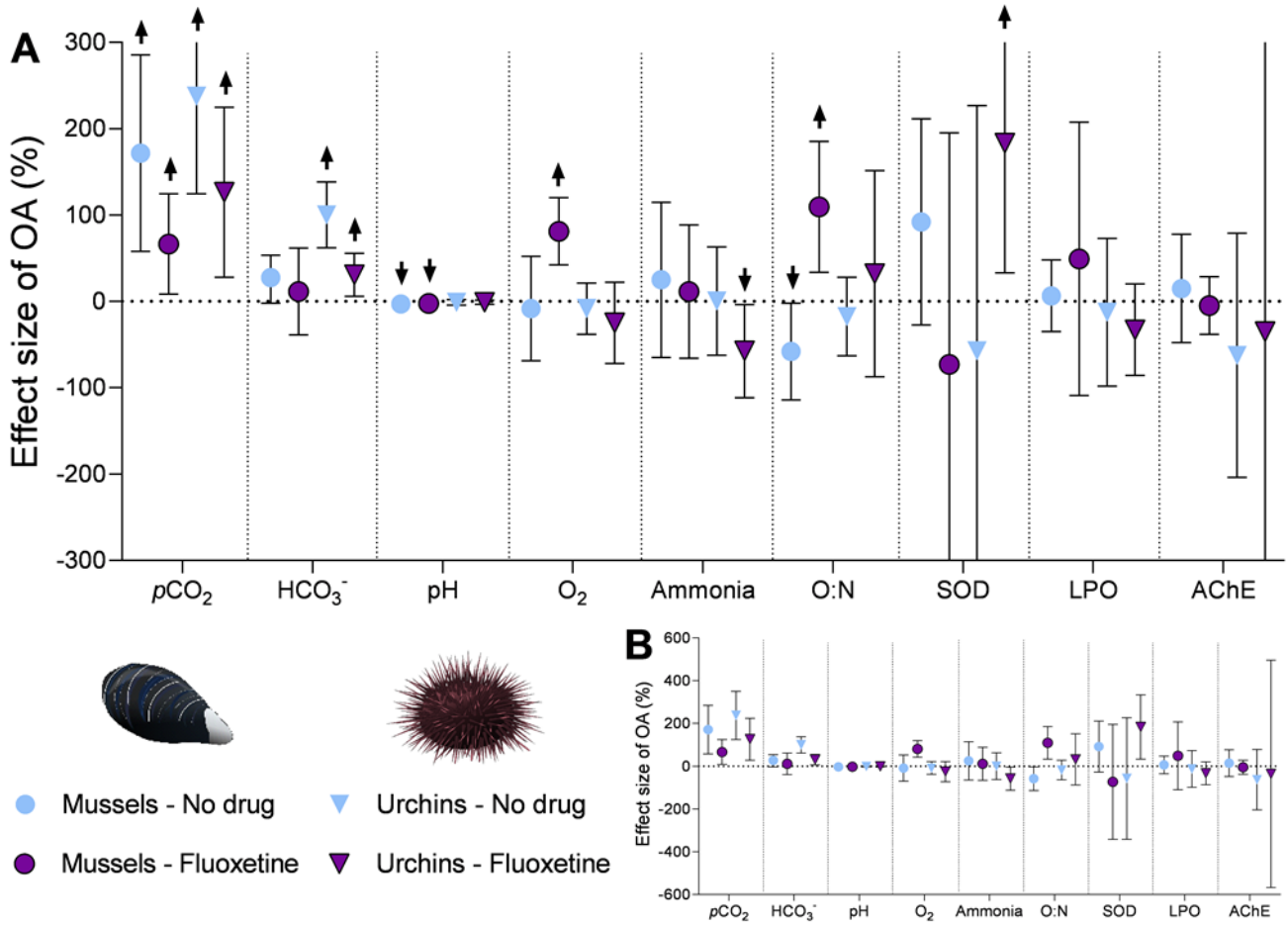

Figure 4. The effect size of OA (i.e. the percentage difference between responses in ambient (pH 8.1) and OA (pH 7.7) exposure conditions) on acid-base parameters, metabolic parameters and toxicity biomarkers for *Mytilus edulis* (circles) and *Paracentrotus lividus* (triangles) exposed to no pharmaceutical (blue) or to fluoxetine (purple). Results are expressed as mean % change ± 95 % confidence intervals. (A) Arrows represent significant increases or decreases in each parameter between ambient and OA exposures, defined as whether confidence intervals cross zero; (B) panel shows full range of confidence intervals.

alter the bicarbonate accumulation triggered by OA, e.g. via disruption of chloride-bicarbonate exchange proteins (Griffith, 2017; Ramesh et al., 2019). The lesser bicarbonate accumulation for urchins may be the cause of the non-significant but detectable coelomic fluid acidosis of 0.1 pH units in the OA-fluoxetine treatment (Tukey's post-hoc P = 0.60), where normally this species maintains extracellular pH in OA conditions (Lewis et al., 2016). This indicates that the combination of OA and contaminants could present additional risks to species ordinarily considered strong

acid-base regulators. This similar decline in extracellular fluid pH in both mussels and urchins also means that changes to the toxicity of fluoxetine cannot be discussed in the context of H_2, and we therefore hypothesise that similar toxicity changes will occur in both species here.

The OA-induced alterations to the effect of fluoxetine on metabolic parameters were more complex than theorised in H_1. OA had a significant positive effect of 81 % on oxygen uptake rate for fluoxetine-exposed mussels (two-way ANOVA for OA, $F_{1,39}$ = 10.30, P = 0.003; Tukey's post-hoc, P = 0.003; Figure 4). OA has been previously observed to increase oxygen uptake in this species (Munari et al., 2020; Thomsen and Melzner, 2010), and fluoxetine increases energy demand and hence oxygen uptake in the polychaete *Hediste diversicolor* (Hird et al., 2016); the combination of these stressors may have induced an additive effect which was not detectable in either single-stressor treatment. This increased response in OA conditions could not have been mediated by chemical changes to fluoxetine according to H_1, as this would have resulted in a lessened effect. Coupled with the reduction in haemolymph pCO_2 compared to the OA-no drug treatment, this could suggest an increase in ventilation rate, potentially increasing uptake of fluoxetine for mussels via increased exposure of gills to contaminated seawater. OA did not affect ammonia excretion rate in fluoxetine-exposed mussels (Table S2); combined with the increased oxygen uptake, this resulted in a 110 % increase in O/N for mussels (two-way ANOVA for OA, $F_{1,39}$ = 11.29, P = 0.002; Tukey's post-hoc, P = 0.003; Figure 4). This returned O/N to control (ambient pH with no pharmaceutical) levels of above 10 in the OA-fluoxetine treatment, indicating metabolism of normal proportions of proteins and lipids (Taboada et al., 1998). This could represent an attempt to harmonise oxygen uptake and ammonia excretion rates to maintain stable O/N (Hird et al., 2016).

Conversely, neither oxygen uptake rate nor O/N were affected by OA in fluoxetine-exposed urchins (Table S2; Figure 4). However, OA had a significant negative effect on ammonia excretion rate which decreased by 57 % (two-way ANOVA for OA, $F_{1,39}$ = 3.44, P = 0.07; Tukey's post-hoc, P = 0.03; Figure 4). This produced significant interaction terms between OA and species for all three parameters: oxygen uptake rate (two-way ANOVA for OA*species, $F_{1,39}$ = 26.39, P < 0.0001); ammonia excretion rate (two-way ANOVA for OA*species, $F_{1,39}$ = 5.71, P = 0.02); and O/N (two-way

ANOVA for OA*species, $F_{1,39}$ = 5.57, P = 0.03). These significantly different effects of OA on metabolic responses to fluoxetine between species, despite their similar acid-base responses, are in opposition to H_2 and indicate that other biological differences between species must have driven the different effects of OA on fluoxetine toxicity here.

The lack of an increase in metabolic rate for urchins between ambient pH-fluoxetine and OA-fluoxetine treatments is unexpected due to the strong energetically demanding antioxidant response (Ighodaro and Akinloye, 2018; Lesser, 2006) in the form of a 183 % increase in SOD activity (two-way ANOVA for OA, $F_{1,39}$ = 3.31, P = 0.08; Tukey's post-hoc, P = 0.01; Figure 4). Whilst this antioxidant activity appeared to be effective in preventing oxidative stress due to the lack of any effect on LPO (two-way ANOVA for OA, $F_{1,39}$ = 0.35, P = 0.56; Tukey's post-hoc P = 0.35; Figure 4), any resultant energy deficit could impact key processes such as growth over longer-term exposure.

It is not clear why urchins experienced such a strong antioxidant response in comparison to mussels exposed to fluoxetine, for which OA did not significantly affect SOD activity or LPO (Table S2). Similarly to mussels exposed to tolcapone in OA conditions, there was high inter-individual variability in SOD activity for fluoxetine-exposed mussels but here OA induced a decrease in mean SOD activity of 73 % (Figure 4). This could suggest that SOD activity was inhibited by fluoxetine as has been observed in other xenobiotics (Lewandowski et al., 2018), and likely contributed to the non-significant and again highly variable increase in LPO of 49 % observed for mussels (Figure 4). These differences produced a significant interaction term between OA and species (two-way ANOVA for OA*species, $F_{1,39}$ = 8.21, P = 0.008), indicating a significant difference between the effect of OA on SOD activity between species when exposed to fluoxetine. OA increases SOD activity in urchins, but not mussels, exposed to copper (Lewis et al., 2016), indicating that these species can differ in antioxidant responses to contaminants in OA conditions. Additionally, this demonstrates that H_2 is not applicable here as both species had similar acid-base responses but opposing oxidative stress responses. There was also no effect of OA on AChE activity for either species in fluoxetine treatments (Table S2; Figure 4). The lack of any additional effect of OA on toxicological biomarkers for mussels, and mostly

for urchins, when exposed to fluoxetine conforms to H_1, but as urchins experienced such a strong antioxidant response this hypothesis is clearly not applicable across the board.

4.4.5. OA differentially affected pharmaceutical toxicity for each species.

The overarching aim of these experiments was to test the hypothesis that the effects of tolcapone would be increased, and the effects of fluoxetine decreased, for both species in OA conditions due to the effect of a seawater pH reduction on the ionisation of these contaminants (H_1). Following ten-day exposures to these combined stressors, the present data does not follow this hypothesis for either pharmaceutical, with effects differing in both direction and magnitude between species and measured endpoints. This indicates that differences between our two test species play a key role in determining the final toxicity outcomes in OA conditions. We additionally hypothesised that species differences would be driven by their differing acid-base regulatory abilities in OA conditions (H_2), but our results did not support this, indicating that other biological mechanisms that differ between the two species were more important in determining OA-contaminant outcomes here. Although these data represent one acidic and one basic compound, and one “strong” and one “poor” acid-base regulator, taken together our findings indicate that changes to the toxicity of ionisable contaminants in future OA conditions are not predictable solely based on the pK_a-pH relationship or the sensitivity of organisms to OA. As the acidification of the oceans progresses, a greater understanding of the drivers of this relationship between seawater pH and the toxicity of ionisable compounds to marine species will be fundamental in understanding the risks of these global stressors in a future ocean.

4.5. Supplementary information

	Tolcapone		Fluoxetine	
	Mussels	**Urchins**	**Mussels**	**Urchins**
Extracellular fluid pCO_2	t_{18} = 2.05 P = 0.06	t_{18} = 1.67 P = 0.12	t_{18} = 1.43 P = 0.17	t_{18} = 1.36 P = 0.19
Extracellular fluid HCO_3^-	t_{18} = 0.32 P = 0.75	t_{18} = 2.07 P = 0.05	t_{18} = 1.99 P = 0.06	t_{18} = 1.72 P = 0.10
Extracellular fluid pH	t_{18} = 1.18 P = 0.26	t_{18} = 0.92 P = 0.37	t_{18} = 0.54 P = 0.60	t_{18} = 0.27 P = 0.79
Oxygen uptake rate	t_{18} = 0.41 P = 0.69	t_{18} = 0.72 P = 0.48	t_{18} = 0.81 P = 0.43	t_{18} = 0.90 P = 0.38
Ammonia excretion rate	t_{18} = 0.19 P = 0.85	t_{18} = 0.78 P = 0.44	t_{18} = 0.09 P = 0.93	**t_{18} = 4.15 P = 0.0008*****
Oxygen-nitrogen ratio	**t_{18} = 2.12 P = 0.049***	t_{18} = 0.47 P = 0.64	t_{18} = 2.02 P = 0.06	t_{18} = 1.94 P = 0.071
SOD activity	**t_{18} = 2.33 P = 0.32***	**t_{18} = 2.75 P = 0.015***	t_{18} = 1.27 P = 0.22	t_{18} = 0.71 P = 0.49
Lipid peroxidation	t_{18} = 0.21 P = 0.83	t_{18} = 1.99 P = 0.06	t_{18} = 1.03 P = 0.32	t_{18} = 0.68 P = 0.50
AChE activity	t_{18} = 1.69 P = 0.11	**t_{18} = 3.14 P = 0.0086****	**t_{18} = 3.28 P = 0.0055****	**t_{18} = 2.25 P = 0.041***

Table S1. Results of two-tailed unpaired student's t-tests for mussels and urchins exposed to tolcapone or fluoxetine versus seawater with no pharmaceutical present in ambient pH conditions. *, ** and *** represent significant effects at P < 0.05, P < 0.01 and P 0.001 respectively.

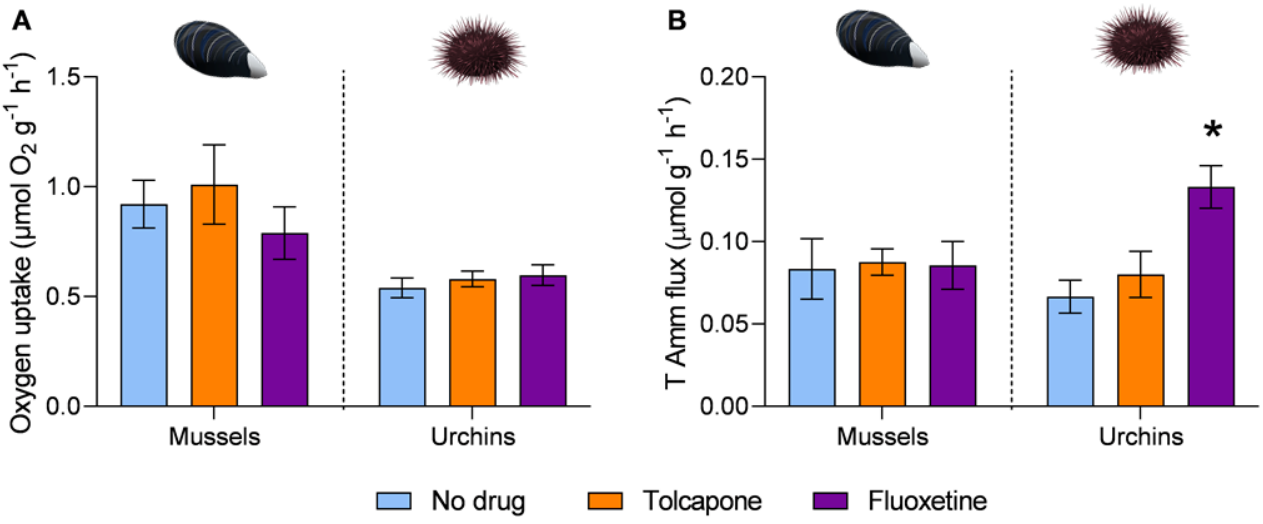

Figure S1. (A) Oxygen uptake rate and (B) Ammonia excretion rate (mean ± SEM) for *Mytilus edulis* and *Paracentrotus lividus* following 10-day exposures to either tolcapone or fluoxetine at ambient pH 8.1.

	No drug			Tolcapone			Fluoxetine		
	OA	**Species**	**OA*Species**	**OA**	**Species**	**OA*Species**	**OA**	**Species**	**OA*Species**
Extracellular fluid pCO_2	**$F_{1,39}$ = 27.51 P < 0.0001*****	$F_{1,39}$ = 1.39 P = 0.25	$F_{1,39}$ = 0.25 P = 0.62	**$F_{1,39}$ = 16.32 P = 0.0003*****	**$F_{1,39}$ = 8.83 P = 0.0056****	$F_{1,39}$ < 0.0001 P = 0.99	**$F_{1,39}$ = 24.54 P < 0.0001*****	$F_{1,39}$ = 3.49 P = 0.071	$F_{1,39}$ = 0.25 P = 0.62
Extracellular fluid HCO_3^-	**$F_{1,39}$ = 64.57 P < 0.0001*****	**$F_{1,39}$ = 171.1 P < 0.0001*****	**$F_{1,39}$ = 36.87 P < 0.0001*****	**$F_{1,39}$ = 68.25 P < 0.0001*****	**$F_{1,39}$ = 161.2 P < 0.0001*****	**$F_{1,39}$ = 34.31 P < 0.0001*****	**$F_{1,39}$ = 7.81 P = 0.008****	**$F_{1,39}$ = 98.42 P < 0.0001*****	$F_{1,39}$ = 3.77 P = 0.061
Extracellular fluid pH	**$F_{1,39}$ = 7.24 P = 0.011***	**$F_{1,39}$ = 36.84 P < 0.0001*****	$F_{1,39}$ = 1.81 P = 0.19	$F_{1,39}$ = 0.92 P = 0.35	**$F_{1,39}$ = 76.08 P < 0.0001*****	**$F_{1,39}$ = 9.52 P = 0.0043****	**$F_{1,39}$ = 8.53 P = 0.0063****	**$F_{1,39}$ = 67.3 P < 0.0001*****	$F_{1,39}$ = 1.40 P = 0.25
Oxygen uptake rate	$F_{1,39}$ = 0.57 P = 0.46	**$F_{1,39}$ = 21.11 P < 0.0001*****	$F_{1,39}$ = 0.04 P = 0.85	$F_{1,39}$ = 0.17 P = 0.68	**$F_{1,39}$ = 23.68 P < 0.0001*****	$F_{1,39}$ = 1.40 P = 0.24	**$F_{1,39}$ = 10.30 P = 0.0031****	**$F_{1,39}$ = 58.28 P < 0.0001*****	**$F_{1,39}$ = 26.39 P < 0.0001*****
Ammonia excretion rate	$F_{1,39}$ = 0.64 P = 0.42	**$F_{1,39}$ = 4.28 P = 0.046***	$F_{1,39}$ = 0.62 P = 0.44	$F_{1,39}$ = 3.38 P = 0.075	**$F_{1,39}$ = 13.73 P = 0.0008****	**$F_{1,39}$ = 9.84 P = 0.0037****	$F_{1,39}$ = 3.44 P = 0.073	$F_{1,39}$ = 0.063 P = 0.80	**$F_{1,39}$ = 5.71 P = 0.023***
Oxygen-nitrogen ratio	**$F_{1,39}$ = 6.26 P = 0.018***	**$F_{1,39}$ = 6.49 P = 0.016***	$F_{1,39}$ = 3.79 P = 0.06	$F_{1,39}$ = 0.22 P = 0.64	$F_{1,39}$ = 1.74 P = 0.20	$F_{1,39}$ = 0.18 P = 0.68	**$F_{1,39}$ = 11.29 P = 0.0021****	**$F_{1,39}$ = 20.00 P = 0.0001*****	**$F_{1,39}$ = 5.57 P = 0.025***
SOD activity	$F_{1,39}$ = 0.04 P = 0.85	$F_{1,39}$ = 0.12 P = 0.73	**$F_{1,39}$ = 6.62 P = 0.015***	$F_{1,39}$ = 0.88 P = 0.36	**$F_{1,39}$ = 12.38 P = 0.0014****	$F_{1,39}$ = 0.05 P = 0.83	$F_{1,39}$ = 3.31 P = 0.079	**$F_{1,39}$ = 15.90 P = 0.0004****	**$F_{1,39}$ = 8.21 P = 0.0075****
Lipid peroxidation	$F_{1,39}$ = 0.10 P = 0.76	**$F_{1,39}$ = 12.57 P = 0.0011****	$F_{1,39}$ = 0.30 P = 0.59	$F_{1,39}$ = 3.29 P = 0.078	**$F_{1,39}$ = 16.67 P = 0.0002****	**$F_{1,39}$ = 5.53 P = 0.024***	$F_{1,39}$ = 0.35 P = 0.56	**$F_{1,39}$ = 9.08 P = 0.0049****	$F_{1,39}$ = 3.16 P = 0.084
AChE activity	$F_{1,39}$ = 0.003 P = 0.95	**$F_{1,39}$ = 97.56 P < 0.0001*****	$F_{1,39}$ = 2.90 P = 0.10	**$F_{1,39}$ = 10.47 P = 0.0031****	**$F_{1,39}$ = 275.3 P < 0.0001*****	**$F_{1,39}$ = 7.30 P = 0.012***	$F_{1,39}$ = 0.16 P = 0.69	**$F_{1,39}$ = 108.9 P < 0.0001*****	$F_{1,39}$ = 0.018 P = 0.89

Table S2. Results of two-way analysis of variance with fixed factors “pH” and “species”. *, ** and *** represent significant effects at P < 0.05, P < 0.01 and P 0.001 respectively.

4.6. References

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Chapter 5. General discussion

The continuing acidification and contamination of the oceans are global issues, both considered to have “planetary boundaries” within which humanity must remain in order to prevent irreversible environmental change (Rockström et al., 2009). Despite this, their potential to interact in a globally significant way has only been considered in recent years (Boyd, 2011; Dupont and Pörtner, 2013; Zeng et al., 2015). With the publication of the latest report by the Intergovernmental Panel on Climate Change, it is clear that the oceans are already shifting towards a new carbonate chemistry regime (IPCC, 2021) and the conditions under which marine organisms experience contaminants may no longer be the same as the conditions targeted in traditional ecotoxicology studies. In this thesis, I have demonstrated that changes in ocean carbonate chemistry alter contaminant toxicity to a range of marine invertebrate species and life stages, but that these alterations do not always follow what might be predicted based on the acid-base chemistry of both contaminants and animal internal environments. Critically, changes to carbonate chemistry differentially altered the effects of the same contaminants between different species and life stages, which presents challenges for both academic and regulatory ecotoxicology.

5.1. Ocean carbonate chemistry is already changing

A month before the submission of this thesis, the Intergovernmental Panel on Climate Change (IPCC) released the Working Group I contribution to their Sixth Assessment report (IPCC, 2021; discussed in **Chapter 1**). As well as predicting a lower mean ocean pH for the year 2100 under the “worst case scenario” than the previous report, approximately 7.66 compared to 7.73 (IPCC, 2014), the report highlighted an important consideration for both research and regulatory work: the pH of the ocean has already declined so much, a decline that will continue for some time even if stringent CO_2 mitigations were to be brought in immediately, that the “control” conditions targeted in both academic and regulatory marine exposures may no longer represent the current ocean.

As discussed in **Chapter 1**, the majority of ocean acidification (OA) studies, including those incorporating contaminants, target a non-fluctuating pH 8.1-8.2 and/or atmospheric pCO_2 of ~400 µatm as their ambient or current ocean conditions (Doney et al., 2020; Kapsenberg and Cyronak, 2019; Wahl et al., 2016). This was problematic

even prior to the release of the new IPCC report due to local variability in carbonate chemistry (Duarte et al., 2013; Torres et al., 2021; **Chapter 2**). The 2021 IPCC report puts current mean surface ocean pH below 8.05, which is below the 2014 estimation of pH 8.1-8.15 (IPCC, 2014). As demonstrated by the experimental work of **Chapter 3** and **Chapter 4** of this thesis, and an increasing number of other published studies (e.g. Freitas et al., 2016; Lo et al., 2021; Maulvault et al., 2019; Munari et al., 2020, 2019) a pH decline of approximately 0.4 units can significantly alter the toxicity of pH-sensitive contaminants, sometimes producing effects which are not present at all at the higher pH. Whilst this is of course a far larger decline than 0.1 units, it is not outside the realms of possibility that a smaller pH difference could influence contaminant toxicity, particularly taking into account findings from freshwater models (Karlsson et al., 2017; Rendal et al., 2011; Sun et al., 2020) and the fact that pH is a logarithmic scale and this therefore represents an approximate 30 % increase in acidity. This also has implications for single-stressor studies of contaminants, which might under or overestimate effects compared to real ocean conditions. Multi-stressor OA studies might overestimate the future magnitude of the effect of OA on contaminant toxicity if targeting ambient and OA treatment conditions of pH 8.15 and 7.7, as opposed to targeting a smaller range of 8.05 and 7.66 according to the current mean and new lower “worst case scenario” mean surface ocean pH for the year 2021 (IPCC, 2021), although such a small difference in the pH range may have negligible effects. Additionally, the degree of ionisation of contaminants does not change uniformly with pH (Rendal et al., 2011; Sun et al., 2020), therefore this different range may produce different changes in toxicity.

For regulatory ecotoxicology studies, acceptable pH ranges are sometimes broader than the difference in pH between ambient and OA treatments. For example, several of the standard tests outlined in the internationally recognised Organisation for Economic Cooperation and Development (OECD) Guidelines for the Testing of Chemicals state that pH may be between 6.5 and 9.0 so long as it remains within a range of 0.5 units for the duration of the study (e.g. 21-Day Fish Assay) or do not mention pH at all even where tests are recommended to last for several weeks (e.g. Fish Early-life Stage Toxicity Test; OECD, 2021). In **Chapter 3** and **Chapter 4** I demonstrated significant effects on toxicity within a smaller pH range of 0.4 units. As well as the implications for toxicity within the same study if pH is allowed to fluctuate

(as demonstrated in **Chapter 2**), and the questionable environmental relevance of such pH ranges which may alone induce different biological responses, this calls into question the comparability of different studies where pH may be vastly different and their applicability to the marine environment where pH-sensitive contaminants are concerned.

Animal husbandry must also be considered here: regulatory testing is often conducted on laboratory cultures which have been maintained for many years, and whilst there are acceptable pH ranges for species in tests (OECD, 2021) the specific pH targets and ranges for stock animals are likely to vary between laboratories based on historical methodology and differences in equipment. In addition, other carbonate chemistry parameters such as pCO_2 and dissolved inorganic carbon are not required to be tested in the majority of standard toxicity tests (ISO, 2021; OECD, 2021; OSPAR Commission, 2006). Regulatory studies may therefore already be conducting toxicity tests in pseudo-OA conditions, or in conditions which no longer represent those of the current ocean. This has been previously suggested to occur in laboratories which source seawater and organisms directly from the ocean due to natural fluctuations in carbonate chemistry parameters (Galloway et al., 2020). Exposure to altered carbonate chemistry is also known to occur in aquaculture, with current guidelines proposing safe CO_2 levels up to 75 times higher than current atmospheric CO_2 concentrations and well beyond what might be expected by the year 2100 (Ellis et al., 2017). Some ecotoxicology studies source test organisms from aquaculture, therefore the pre-exposure conditions of organisms, and their ancestors (Schunter et al., 2018; Stiasny et al., 2018) should be considered when sourcing organisms and interpreting test results.

As discussed in **Chapter 1**, theories and models exist to estimate the potential toxicity changes to contaminants in different pH conditions in freshwater (Rendal et al., 2011; Smith et al., 2015). According to these models, if an acidic compound is tested at a high pH where it is predominantly ionised, it should exhibit lower toxicity than it would at a lower pH, thereby increasing the concentration which may be deemed acceptable for release to the environment. The same would occur for a basic compound tested at a low pH. This means that, for freshwater studies, one could theoretically examine the studies that were used in developing the safety data for the compound and use these

models to assess whether toxicity may be increased or decreased at a more realistic environmental pH. However, as demonstrated in **Chapter 3** and **Chapter 4**, the final outcomes for the species and pharmaceuticals investigated here do not clearly follow such patterns. Whilst to my knowledge there is no published data exploring the toxicity of tolcapone at more than one pH within one study, multiple models and studies of fluoxetine in freshwater have found it to reduce in bioaccumulation and toxicity as pH declines (Karlsson et al., 2017; Nakamura et al., 2008; Rendal et al., 2011). This was not the case in **Chapter 3** or **Chapter 4**, where toxicity outcomes were not consistently lower in OA conditions. If freshwater models cannot always be applied to the toxicity of ionisable contaminants in seawater, then for regulatory tests conducted in seawater we must ensure that these accurately reflect current and potentially future carbonate chemistry conditions, as well as tightening the acceptable pH ranges for studies of pH-sensitive compounds. This is particularly important for contaminants close to acceptable toxicity ranges, for which a change in pH might push them into unacceptable.

An additional consideration is the existence of fluctuating pCO_2/pH regimes, which I found in **Chapter 2** to alter the toxicity of the metal copper to two marine invertebrates. It cannot be determined from this data alone whether fluctuating pH would also alter the toxicity of other pH-sensitive contaminants. However, this does not seem unlikely given that the effects of contaminants can be altered over the pH ranges that occur in coastal habitats (Duarte et al., 2013) demonstrated in **Chapter 3, Chapter 4** and a number of other OA-contaminant interaction studies discussed in **Chapter 1** (e.g. Freitas et al., 2016; Maulvault et al., 2019b, 2019a; Munari et al., 2020, 2019). Additionally, there are already known to be differences in outcomes between static and fluctuating regimes (as reviewed by Kapsenberg and Cyronak, 2019) and the addition of another stressor could well be expected to further alter outcomes (Przeslawski et al., 2015). Further investigation into other pH-sensitive contaminants would therefore be worthwhile, particularly those which are already suspected to be common in coastal regions such as lead (Belivermiş et al., 2020) and other ionisable pharmaceuticals (Branchet et al., 2020). A larger question is whether such fluctuations should be incorporated into toxicity testing as standard, whether for research or regulatory purposes, given that this effect is so common to coastal habitats (Torres et al., 2021) where contamination is normally higher than the open ocean (Nyström,

2019). This is probably unrealistic given the differences in the frequency and patterns of variation between habitats (Duarte et al., 2013); the complexity of the experimental systems required to produce fluctuations; and the additional issues this would create for replicability between studies which is already a contentious issue in OA research (Clark et al., 2020a, 2020b; Munday et al., 2020). One way of accounting for carbonate chemistry fluctuations could be assessment of which species are most sensitive, based on methods described in **Chapter 1**, and then targeting these species for further assessment of the toxicity of contaminants in fluctuating conditions. This could then be applied to risk management for specific species and, by extension, habitats where they are key members of ecosystems.

Finally, the future impacts of OA on carbonate chemistry extremes in regions where it fluctuates must also be considered. As discussed in **Chapter 1**, OA is already driving pH in coastal habitats to lower extremes than the open ocean (Wootton et al., 2008) and is predicted to continue to do so (Kwiatkowski and Orr, 2018; Torres et al., 2021). This may drive organisms closer to "tipping points", particularly for those in coastal regions which often exist close to these (Tomanek and Helmuth, 2002; Widdicombe et al., 2011). It may therefore be prudent, in combination with the new IPCC predictions (IPCC, 2021), for researchers to target lower pH measurements as a "future OA" scenario when using species inhabiting coastal areas where future pH is likely to exceed a decline of 0.4 units. However, this must be balanced with the need for studies to be comparable with past work, and with one another for species which can be found in multiple habitats encompassing different low pH extremes; this includes all the species examined in this thesis which are common to various habitats around the United Kingdom and for some more widely.

5.2. Changes in the effects of pH-sensitive contaminants differed in direction and magnitude between species and life stages

As discussed above and in **Chapter 1**, established theories for metals and ionisable organic contaminants in freshwater predict that changes to bioaccumulation and toxicity occur consistently alongside pH changes according to speciation behaviour for metals and acid dissociation chemistry for ionisable organics (Karlsson et al., 2017; Rendal et al., 2011; Smith et al., 2015). Whilst such chemical changes may still occur

for contaminants in freshwater, in **Chapter 2**, **Chapter 3** and **Chapter 4** I demonstrated that consistent patterns of toxicity changes did not occur for multiple species and life stages, and that other factors appeared to be more important in determining outcomes for the endpoints measured here.

My findings throughout this thesis show opposing directions of interactions between altered carbonate chemistry and contaminants across different species and levels of biological organisation. In **Chapter 3**, OA appeared to be the dominant driver of the direction of effects with pharmaceuticals altering their magnitude, resulting in opposing directions of effect for *Lytechinus pictus* and *Arenicola marina* early life stages. Conversely, in **Chapter 4** OA had minimal effects on any measured endpoint apart from extracellular acid-base physiology; hence, the directions of effect appeared to be driven by the combined effect of OA and pharmaceuticals, rather than the effect of OA as a single stressor, resulting in different directions and magnitudes of effect between measured endpoints and between species. Similarly, in **Chapter 2**, fluctuating pH had few effects as a sole treatment past acid-base homeostasis, copper alone impacted several acid-base and toxicological biomarkers, but the combination of the two altered both the direction and magnitude of effects on different endpoints between species. Differences in the direction and magnitude of effects of OA on copper toxicity between species have been previously reported (Lewis et al., 2016; Nielson et al., 2019). Differences in the magnitude of effect of OA on toxicity between species and endpoints also occur for the pharmaceuticals diclofenac (Maulvault et al., 2018; Munari et al., 2019, 2018) and carbamazepine (Freitas et al., 2016; Serra-Compte et al., 2018), but effects did occur in the same directions. As described above, further investigation into pH-sensitive compounds in different species and life stages is needed to understand whether the differences in direction and magnitude of effects observed here are common to other contaminants.

In **Chapter 3**, OA appeared to be the main driver of effects for early life stages, whereas in **Chapter 2** and **Chapter 4**, interactions between altered carbonate chemistry and contaminants induced different effects to either stressor alone. As discussed above, pH change may be a greater driver of effects than contamination to gametes, which is corroborated by the importance of pH in sperm swimming and fertilisation (Nishigaki et al., 2014) and their inability to regulate internal pH in OA

conditions (Esposito et al., 2020). Adult marine invertebrates are generally able to regulate extracellular and/or intracellular pH (Melzner et al., 2020) which provides some protection to pH-dependent processes; even *M. edulis*, which is considered a "poor" acid-base regulator, is able to maintain intracellular pH in OA conditions (Zittier et al., 2018). Sperm are also suspected to be limited in their ability to defend and repair against damage, such as oxidative stress which is a common impact of marine contaminants, compared to adult organisms (Aitken et al., 2004; Nissen and Kreysel, 1983; Quijano et al., 2016). Despite this, we saw greater percentage changes in the effects of pharmaceuticals in ambient pH conditions in **Chapter 4** than in **Chapter 3**. Although strong conclusions cannot be drawn here due to the limited number of species tested and the different doses of pharmaceuticals used, understanding which life stages are more sensitive to each stressor and their interactions could aid decision making around which life stages should be prioritised for testing different stressors.

Key across all three chapters is that consistent patterns of direction or magnitude did not emerge between different species, life stages or even measured endpoints. This indicates that effects could not have been driven solely by pH-induced changes to the speciation or ionisation of the contaminants, as this would have resulted in similar changes to the effects of each contaminant between pH treatments. Particularly for fluoxetine and tolcapone, differing effects emerged across four species for both contaminants in **Chapter 3** and **Chapter 4**. In addition, the changes to the direction of effects of contaminants induced by altered carbonate chemistry indicates that the effects are not simply additive but represent some form of interaction between these two stressors (Preston et al., 2000). Although only two ionisable organic compounds were tested, it is clear that the theory that acidic compounds will increase in toxicity between pH 8.1 and pH 7.7 whilst basic compounds will decrease cannot be applied to all ocean contaminants, therefore theories and models derived from freshwater (e.g. Karlsson et al., 2017; Rendal et al., 2011) cannot necessarily be applied to the marine environment.

This raises the question of what could drive these differing effects if not chemical changes to the contaminant. One overarching hypothesis for this thesis was that differences in the ability of organisms to maintain acid-base homeostasis would be linked to the effect of altered carbonate chemistry on their responses to contaminants.

In **Chapter 2**, this was suggested to be the reason for the reduced toxicity of copper to *Alitta virens* compared to *Mytilus edulis* in fluctuating pH conditions, a theory which has been explored elsewhere for copper (Lewis et al., 2016; Nielson et al., 2019). However, in **Chapter 4** the limited acid-base regulatory ability of *M. edulis* compared to *Paracentrotus lividus* did not appear to influence the toxicity of ionisable pharmaceuticals, with the effects of OA on the toxicity of each pharmaceutical differing in direction and magnitude between species and even between endpoints within the same species. Additionally, despite the fluoxetine-induced alteration to acid-bae homeostasis in *P. lividus* the responses of these species to fluoxetine in OA conditions still differed. Different effects also occurred for the sperm of both *L. pictus* and *A. marina* in **Chapter 3**, despite both species being strongly suspected to possess limited acid-base regulatory abilities as with other sperm from broadcast spawning invertebrates (Esposito et al., 2020). Although each chapter presents a limited number of species and contaminants, taken together these results strongly suggest that the ability to maintain extracellular acid-base homeostasis was not the driver of the differential outcomes between species and life stages for ionisable organic contaminants.

A further possible driver of effects could be differences in uptake and accumulation of contaminants between species, as this was not measured here. However, previous studies have found consistent directions of change in fluoxetine uptake and accumulation induced by pH between species (Alsop and Wilson, 2019; Nakamura et al., 2008; Neuwoehner and Escher, 2011), as well as a similar general trend for acids like tolcapone (Rendal et al., 2011). Although these were all measured in freshwater, it is unlikely that uptake or accumulation would differ between species here as neither sperm nor *P. lividus* are thought to be able to regulate contaminant uptake more than other species, and whilst *Mytilus* species can close their shells to reduce contaminant uptake (Fdil et al., 2006) this was not reflected in metabolic responses. The metabolism and excretion of contaminants can be affected by OA (Maulvault et al., 2019a; Su et al., 2017), but the extent to which this differs for the same contaminant between species is not well understood.

Another potential biological driver could be differences in the ability to defend against and repair damage from oxidative stress, which can lead to a host of negative impacts

in marine species (Lesser, 2006). Oxidative stress and antioxidant activity vary between tissues (Gonzalez-Rey and Bebianno, 2013), individuals (Manduzio et al., 2004), species (Lewis et al., 2016) and even temporally (Di Poi et al., 2016), producing a huge variety of effects which could be considered both positive and negative to marine life (Lesser, 2006), therefore assigning this as a driver of further effects is extremely complex. Differences in metabolic substrate which could be damaged by oxidative stress was suggested to influence responses to contaminants in **Chapter 3**, and clear differences in oxidative stress responses emerged between species in **Chapter 4** which appeared to influence metabolic effects. Conversely, we suspected that acid-base physiology affected oxidative stress responses in **Chapter 2**. However, these suggestions are speculative as there are endless biological parameters not measured in each chapter which could drive species and life stage differences; the purpose of the endpoints selected was to provide a representative overview of organism-level impacts.

As well as the physiological differences between species which could potentially drive interactions, we must also consider the impacts of behaviour, ecology and wider ecosystem effects on interactions between OA and contaminants. Physiology does not exist in a vacuum and is interlinked with the evolutionary history and current behavioural and ecological context in which organisms live (Németh et al., 2013). In **Chapter 3**, the ecological and evolutionary factors behind physiological differences between species appeared to be important, with differential effects of pH on each species and different sperm swimming parameters linked to fertilisation success based on their spawning strategy. In **Chapter 2**, and in previous experiments involving *A. virens* (Nielson et al., 2019), it was suggested that the burrowing behaviour of ragworms may influence their acid-base response by enabling them to maintain a carbonate chemistry microclimate within the burrow, although it was not possible to measure the pH of burrows during this experiment. The shell-closing behaviour of *M. edulis* in response to seawater contaminants was also discussed in **Chapter 2** and **Chapter 4** (Fdil et al., 2006), although this was not believed to have occurred in the latter. Behavioural and ecological factors are known to be interlinked in determining responses to OA and to other stressors (Nagelkerken and Munday, 2016; Pollock et al., 2007), and could therefore also influence how species respond to contaminants in OA conditions.

For the studies described herein, behavioural and ecological factors were removed as much as possible in order to isolate biological and chemical factors in assessing changes to contaminant toxicity. For example, in **Chapter 4** animals were kept in individual tanks with no environmental enrichment. This fit the purposes of our study, but does not represent the ecological context in which organisms will experience these stressors which includes interactions with other individuals, food availability and predation. Similarly in **Chapter 3**, as these experiments were conducted at a single sperm density we cannot say for certain whether these effects translate to the marine environment where population densities will vary and likely influence which sperm traits are key for fertilisation success (Crean and Marshall, 2008; Levitan, 2000). Incorporating these additional factors, for example through mesocosm experiments, would be complex and unrealistic for the majority of laboratory academic and regulatory studies. A greater understanding of physiological and behavioural drivers of interactions on an individual level will need to be combined with knowledge of the ecological context of these drivers, potentially through models derived from experiments where large-scale mesocosms are not appropriate.

Even without knowing the precise drivers behind the differential effects of altered carbonate chemistry between species and life stages, it is clear that this presents an additional challenge for ecotoxicology from both a research and chemicals management perspective. It will not be enough to model toxicity changes to contaminants based on their pH-related speciation and ionisation behaviour, nor will it be enough to apply data from single species or life stages to the toxicity of marine contaminants in an acidified ocean. More specifically in a regulatory context, this demonstrates that multiple species at different life stages will need to be assessed in order to reliably determine the potential toxicity of chemicals where whole ecosystems are concerned. However, this needs to be considered in the context of the drive to reduce the number of animals used in science (Hutchinson et al., 2016; Prescott and Lidster, 2017). A balance will need to be struck between testing enough organisms to obtain a representative picture, whilst not using unnecessary numbers. Models are already available to estimate an appropriate number of individuals based on expected effect size (e.g. Faul et al., 2007), and future models may allow the choice of the most appropriate species and life stages as understanding of individual and population-level drivers behind responses to contaminants and altered carbonate chemistry deepens.

5.3. Remaining knowledge gaps

Building on my findings described here and throughout this thesis, I suggest some potential future research questions aimed at addressing key knowledge gaps.

- What chemical and physiological factors can we use to predict changes to the toxicity of contaminants in altered carbonate chemistry conditions?
    - Which species and life-stages are most sensitive to the effect of altered carbonate chemistry on marine contaminants?
    - Are toxicity changes tied to changes to the uptake of marine contaminants?
    - Does altered carbonate chemistry differentially alter the pH of different biological compartments?
    - How do these alterations affect the transport and accumulation of contaminants between biological compartments?
    - Can these factors be used to replace animal testing with models?
- How much have present-day carbonate chemistry alterations already affected the toxicity of pH-sensitive marine contaminants?
    - Can we use models to assess whether previous estimates of toxicity are accurate in academic and regulatory studies?
    - If studies must be repeated in different carbonate chemistry conditions, how can we ensure that future changes are accounted for?
- How will future ocean change further affect the toxicity of contaminants?
    - How will OA further alter patterns of carbonate chemistry fluctuations?
    - How will these alterations impact the toxicity of contaminants which are already affected by fluctuating carbonate chemistry?
    - How will additional stressors impact the combined effects of OA, carbonate chemistry fluctuations and marine contaminants, particularly those which may further alter contaminant chemistry e.g. temperature?

5.4. Conclusions

My research demonstrates that interactions between altered carbonate chemistry and marine contaminants produce differences in both the direction and magnitude of outcomes for a range of marine invertebrate species and life stages. The context of interactions between these stressors matters in determining outcomes for individuals, whether that context is individual physiology or population ecology. Additionally, the carbonate chemistry of the oceans is already changing, whether through long-term changes induced by increased atmospheric CO_2 or short-term fluctuations in many habitats, which may have impacted previous assessments of pH-sensitive chemical toxicity. Practices in both academic and regulatory science must be changed: to target appropriate pH ranges to standardise testing between different chemicals and improve environmental relevance; to incorporate the current and future carbonate chemistry of the oceans when developing guidelines for the safe use of such chemicals; and to conduct experiments using a range of species and life stages, but in appropriate numbers. Taken together, such changes will allow more accurate assessment of the toxicity of marine contaminants in a present-day and future ocean.

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