Physiological and ecotoxicological interactions of copper and ocean acidification in the polychaete worms *Hediste diversicolor* and *Alitta virens*.

Submitted by Clara Nielson to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biological Sciences, February 2020.

I certify that all material in this dissertation which is not my own work has been identified and that any material that has previously been submitted and approved for the award of a degree by this or any other University has been acknowledged.

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Abstract

For coastal aquatic habitats the change in seawater pH occurring as a result of ocean acidification has the potential to alter the speciation and toxicity of the many contaminants that remain in high concentrations in coastal systems. Of particular concern are metals, such as copper, whose speciation is pH sensitive within the OA range. A meta-analysis of studies to date investigating OA-contaminant interactions using marine invertebrates reveals that 72% of the 44 studies conducted have indeed focused on metals such as copper, with only a few studies looking at polycyclic aromatic hydrocarbons (PAH) and pharmaceuticals. No clear trends in the pH-effect size on contaminant toxicity for either species or contaminant group were present however, suggesting species specific physiological responses may influence this interaction as well as contaminant chemistry. A relatively understudied group were the polychaetes, a key functional group for many coastal sediments. Sediments act as a sink for contaminants where they can accumulate to high concentrations. Hence there is high potential for polychaetes to experience elevated metal exposures under reduced seawater pH as OA progresses.

To address this knowledge gap, the responses of two common coastal polychaetes, *Alitta virens* and *Hediste diversicolor*, were studied under three different experimental scenarios (both water-borne and sediment based) focusing on the physiological and toxicological responses under combined exposures to ocean acidification and copper. Water-borne exposures of *Alitta virens* to 0.25 μM copper under ambient seawater (pH 8.10) showed a significant increase in DNA damage, along with a rise in both SOD activity and lipid peroxidation. However, when exposed to copper under OA conditions (pH 7.70) there was no further increase in DNA damage and a significant decrease in SOD activity was observed alongside a fall in lipid peroxidation suggesting that OA looks to buffer the toxicity responses to this species. This is in contrast to previous studies using mussels and sea urchins, where copper toxicity responses were significantly higher when exposed under OA conditions.

To assess whether local adaptations to high levels of copper contamination influences this OA-copper interaction, a population comparison using a metal resistance population of the harbour ragworm, *Hediste diversicolor* and a nearby non-resistant

population was then conducted. Exposures were run using copper concentrations that elicit comparable toxicity responses, using 0.50 uM copper for the resistant population, compared to 0.25 uM for the non-resistant population, reflecting the two-fold differences in LC50 values for these population. These experiments reveal a significant increase by 19.70% in metabolic rate effect size (the combined stressor when compared to the control) in the resistant population compared to a decrease by 24.02% the non-resistant population, along with differences in ammonia excretion rate and the O:N ratio, thus revealing an energetic cost of this genetic resistance when faced with the combined stressors of OA and copper. These data are in line with the emerging energy limited tolerance to stressors' hypothesis which states that tolerance to stress can be energy limited, with bioenergetics playing a central role in the tolerance to environmental stress.

Finally, a more environmentally realistic exposure scenario was conducted using *Alitta virens* to test the influence of sediment and tidal cycles on worm acid-base and oxidative stress responses. Field measurements of sediment pH revealed that the pH_{NBS} range over a tidal cycle varies from 6.97 to 7.87, indicating that polychaetes are already experiencing pH's lower than the predictions for near future open oceans. In aquarium exposures, with overlying water of pH_{NBS} 8.10, sediment pH_{NBS} remained within the range of 7.45 to 7.31, when the overlying water was manipulated to OA conditions (pH_{NBS} 7.70) sediment pH_{NBS} was within the same range as the ambient treatment. The lack of change in sediment pH, despite a 0.40 unit drop in seawater pH, removed any comparative differences in physiological and toxicity end points in the worms between treatments. Tidal emersion induced a slight reduction in sediment pH, with a significant copper effect on sediment pH causing a further decrease in pH levels. Interestingly emersion resulted in a significant OA-copper interaction for coelomic fluid bicarbonate, which increased over the emersion period, however, there was no emersion driven acidosis within coelomic fluid.

Overall this work further points to contaminant-OA interactions being species specific driven, in part driven by animal physiology. It also highlights the importance of environmentally relevant exposures with sediment dwelling organisms experiencing lower pH levels than the overlying seawater which could potentially affect metal speciation and could lead to OA-contaminant interactions occurring very differently in

this environment. These are important considerations for ecotoxicology studies in the face of global ocean changes.

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Chapter 1

An introduction to Polychaete worms in an acidifying and polluted ocean.

1.1. General introduction

Our oceans are changing at an unprecedented rate leaving marine organisms exposed to a whole suite of ecosystem changes, including rising ocean temperatures, acidifying waters and oxygen loss (Rogers and Laffoley 2013, Hoegh-Guldberg et al. 2007), causing substantial changes in the physical, chemical and biological environment (Gruber 2011). Warming, freshening and associated stratification are driving ocean deoxygenation, which is enhanced in coastal areas by upwellings of hypoxic deep water (Bijma et al. 2013). Coastal environments, especially estuaries, are important natural environments providing a wide range of habitats to many species, including breeding and feeding grounds (Ronnback 1999, Lopez-Mendilaharsu et al. 2005). They also have significant social and economic value (Martinez et al. 2007, Costanza 1999). However, due to high human populations (Vitousek et al. 1997), these waters are also exposed to high levels of pollution.

Polychaetes are an important group not traditionally used to study ocean acidification (OA) and are a keystone species often living in contaminated sediments in coastal environments. The potential for elevated CO_2 to negatively impact a wide variety of marine organisms and biological processes is well documented (Kroeker et al. 2010, Kroeker et al. 2013, Vargas et al. 2017, Sunday et al. 2017). However, the impacts of elevated CO_2 on soft sediment ecosystems and the biota that comprise them remain less understood (Godbold and Solan 2013, Laverock et al. 2013). Elevated levels of CO_2 and reduced pH are known to alter the speciation of many of the contaminants, including copper, in coastal waters and sediments with changes in pH changing the ionized fraction of chemicals and altering the uptake in organisms (Karlsson et al. 2017).

This introductory chapter will review the issues that OA and copper pollution may cause to this organism with the aim of looking at these two stressors in combination with each other as we know that OA is happening against a background of other changes. The understanding of how different stressors may interact with each other to current and future climate conditions is vital in enhancing our knowledge of the consequent effects this may have on marine organisms. This will help us understand better what future ecosystems may look like and how best to protect different species.

1.2. Global ocean acidification

Ocean acidification is now widely regarded as one of the major threats to marine organisms globally (Dupont and Portner 2013, Doney et al. 2009, Gattuso et al. 2015). Carbon dioxide levels in the atmosphere now regularly exceed 410 parts per million (ppm), reaching a record high in May 2019 of 415.70 ppm at the Mauna Loa Observatory, and are predicted to rise further to between 720 to over 1000 ppm by the end of the century or shortly into the start of the next century without serious mitigation to reduce global emissions (Pachauri and Meyer 2014). For the last 800,000 years atmospheric CO_2 has been fluctuating between 180 and 280 ppm (Tripati et al., 2009) and present concentrations are the highest seen during these last 800,000 years and probably during the last 20 million years (Raven et al. 2005, Canadell et al. 2007). Since the industrial revolution atmospheric CO_2 has risen from 280 ppm, with 330 x 10^9 metric tons of carbon being released into the atmosphere via fossil fuel and cement emissions from 1850 to 2006 (Canadell et al. 2007).

The world's oceans act as a major sink for anthropogenic CO_2 (Gattuso and Hansson 2011) and for the last 200 years have absorbed 25 - 30 % of all carbon dioxide released into the atmosphere, equating to 560 billion tons (Schnoor 2014); without this process the atmospheric level of CO_2 would be 55 % higher (Sabine et al. 2004). Between the years 1994 and 2007, the oceans globally have absorbed 30-38 Pg C of the 102-118 Pg C anthropogenic CO_2 produced, equating to 27-35% of total anthropogenic emissions (Gruber et al. 2019). This CO_2 uptake drives down seawater pH and alters the carbonate chemistry of seawater reducing the carbonate saturation state (Doney et al. 2009). Ocean acidification is a dominant driver of long term

changes in pH in the open ocean (Duarte et al. 2013). Since the industrial revolution the average global ocean pH has dropped by 0.1 units, equating to a 30 % increase in hydrogen protons (Raven et al. 2005) and since the 1980s the open ocean surface water pH is declining by 0.017-0.027 pH units per decade (Bindoff et al. 2019). Average open ocean pH is predicted to drop by another 0.287-0.291 by 2018-2100, according to the RCP 8.5 scenario from the IPCC special report on the ocean and cryosphere in a changing climate (Bindoff 2019), resulting in a 100 - 150 % increase in protons (Orr et al. 2005) and a global average pH of 7.73 (Bao et al., 2012) . The atmospheric levels of CO_2 have always varied over time, however, over the past century the rate of increase is unprecedented in the last 22,000 years (Pachauri and Meyer 2014) and it is this rapid change that is of such high concern, especially when looking at organism's ability to adapt in time. These chemical changes are practically irreversible on a time scale of centuries due to the inherently slow turnover of biogeochemical cycles in the oceans (Council 2010).

1.2.1. *The carbonate chemistry system*

The inorganic carbon system is one of the most important chemical equilibria in the ocean and it is this process that is mainly responsible for controlling the pH of open ocean seawater (Fabry et al. 2008). Inorganic carbon is present in 3 main forms: bicarbonate ions (HCO_3^-), carbonate ions (CO_3^{2-}) and aqueous carbon dioxide ($CO_{2(aq)}$), with the majority of carbon in the form of bicarbonate. A fourth form, carbonic acid (H_2CO_3), is also present in much smaller amounts (<0.3%) (Fabry et al. 2008, Wolf-Gladrow 2001).

Atmospheric CO_2 dissolves in seawater to form H_2CO_3 which dissociates quickly into HCO_3^- and H^+ (hydrogen ion). This can then dissociate further to CO_3^{2-} and $2H^+$. All reactions are reversible (Doney et al. 2009) and near equilibrium (Millero et al. 2002). The increase in atmospheric CO_2 and therefore aqueous CO_2 will lead to a net increase in H_2CO_3, HCO_3^-, and H^+ concentrations and a net decrease in CO_3^{2-} concentrations due to the increase in H^+ ions. The pH is a measure of the increase in the hydrogen ions (Doney et al. 2009, Fabry et al. 2008). However, it is worth noting that a doubling of CO_2 concentration in the atmosphere will not cause a doubling of

the total dissolved inorganic carbon, instead an increase of 10% will be seen (Wolf-Gladrow 2001).

$$CO_{2(atmosphere)} \rightleftharpoons CO_{2(aq)} + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \rightleftharpoons 2H^+ + CO_3^{2-}$$

The figure below, taken from the IPCC Special Report on the Ocean and Cryosphere in a changing climate (chapter 5), shows the predicted global changes in surface pH between 1900 - 2100 at RCP (representative concentration pathway) 8.5 and RCP 2.6 (Figure 1.1a) and the spatial patterns of change in surface pH predicted between 2018-2100 compared to 1850-1900 Figure 1.1b) (Bindoff et al. 2019). RCPs are scenarios that include time series of emissions and concentrations of the full suite of greenhouse gases, with each RCP providing one of many possible scenarios and usually refer to the portion of the concentration pathway extending up to 2100 (Moss et al. 2010, Moss et al. 2008). The four RCPs span the range of radiative forcing values of the year 2100, from 2.6 W/m^2 to 8.5 W/m^2 and it is virtually certain that surface ocean pH will decline by 0.036-0.042 or 0.237-0.29 pH units by 2018-2100, relative to 2006-2015, for RCP2.6 or RCP8.5 respectively (Bindoff et al. 2019).

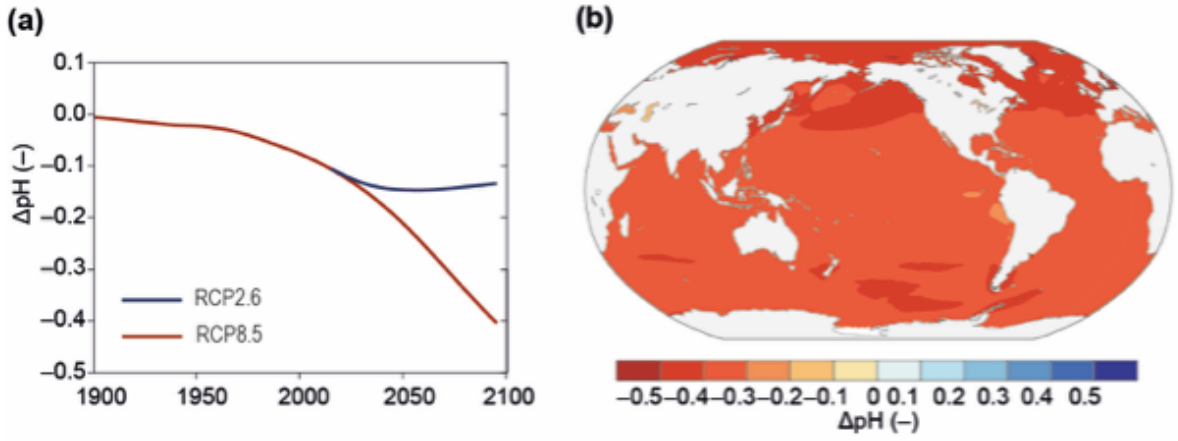


Figure 1.1. (a) The predicted global changes of surface seawater pH between 1900 and 2100 at two different RCPs (representative concentration pathways) , (b) the predicted spatial patterns of change in surface seawater pH between 2018 and 2100, compared to between 1850 and 1900. Taken from Bindoff et al. (2019).

Not all areas of the ocean will be similarly affected. The polar regions will be most strongly affected by ocean acidification, due to an increase in CO_2 solubility in colder waters and a lower carbonate saturation state due to fresh water input (Bates, Mathis and Cooper 2009, Yamamoto-Kawai et al. 2009). The solubility of CO_2 in waters close to freezing point is double that of tropical waters (Wolf-Gladrow and Rost 2014). These regions will also have the added stress of freshening by rivers and sea ice melting due to warming, which will reduce total alkalinity and increase the problem further (Yamamoto-Kawai et al. 2009). Seawater total alkalinity (TA) buffers changes in ocean pH due to the numerous acid-base pairs (Middelburg 2019). A reduction in TA will result in a reduction in pH adding to the already acidifying waters. It is the higher solubility in colder waters that means ocean acidification is not just limited to the upper layers of seawater. Cold water contains high levels of dissolved inorganic carbon which sinks to the deep ocean (Wolf-Gladrow and Rost 2014).

1.2.2 *Coastal ecosystems*

Many of the economically and ecologically important marine invertebrates that are considered to be sensitive to OA and that have been studied to date live in coastal and estuarine habitats. Coastal ecosystems are more complex than open oceans, being governed by interactions between processes on land, in the open ocean and the atmosphere (Aufdenkampe et al. 2011), making these habitats highly variable compared to the open ocean. These waters experience a much greater natural fluctuation of pH, temperature, salinity and oxygen on daily, tidal, seasonal and annual timescales with pH changes of 0.5-1.0 units being seen, exceeding the OA predictions for open ocean for the end of the century (Duarte et al. 2013, Frieder et al. 2012, Hofmann et al. 2011, Baumann et al. 2015, Blackford and Gilbert 2007). For example, measurements off the Californian coast at 7m depth show daily ranges in pH of 0.36 units with the average pH at 17m depth over the three months of measuring (November to February) being 7.87, representing a 37% increase in hydrogen ions (compared to near surface measurements) (Frieder et al. 2012). OA is currently influencing this trend by a 0.1 unit decline, the primary driver of pH change being enhanced primary production or respiration, (Duarte et al. 2013) with fresh water runoff also affecting the chemistry of these waters due to higher dissolved CO_2

concentrations and a lower pH than seawater. However, by the end of this century OA may become the dominant process reducing the pH levels in estuaries and coastal waters (Feely et al. 2010) and coastal ecosystems may regularly drop to well below pH 7.60 by the end of the century (Mangan et al. 2017, Melzner et al. 2013).

Many coastal areas also experience seasonal upwellings of CO_2-rich deep water. On the Pacific coast of North America these upwellings have resulted in a natural seasonal cycle in pH and seawater carbonate chemistry with pH levels dropping to 7.75 (pCO_2 = 850 µatm) in response to the strongest upwelling on the Oregon-Californian boarder (Feely et al. 2008). The impact of acidification has resulted in corrosive, aragonite (a carbonate mineral) dissolving water all the way to the surface (Feely et al. 2008). Further work off the coast of America has found similar variability and low pH in this natural upwelling zone. Over a 30 day period off the coast of California pH varied between 0.397-0.467 pH units over two different sites, with a low of 7.685 being recorded at Point Conception (Hofmann et al. 2011). Even larger fluctuations were seen in the Santa Barbara Channel, California, of 0.467 over a three-week period, with changes of up to 0.32 in a 24 hour period, where pH levels dropped to a low of 7.685 (804 µatm) (Yu et al. 2011).

In additional to upwellings, fluctuations in pH in coastal environments can occur on an annual, seasonal, weekly or diel time scale, mainly as a result of freshwater inputs, temperature variability, respiration and photosynthesis. These processes can act on a latitudinal, regional and habitat level scales, as summarised in Figure 1.2. (below), taken from Kapsenberg and Cyronak (2019), who highlight that each spatial scale exhibits different pH fluctuations, with a range of key processes affecting each one. Temperature can impact on seasonal changes with warming affecting temperate ecosystems, for example, the Mediterranean Sea is showing a 0.028 pH unit decrease per year (between 2007 and 2015), which equates to a larger decrease than is seen in open water pH (Kapsenberg et al. 2017). In polar systems, however, primary production is a big driver of pH fluctuations, with localised pH changes of up to 0.42 units seen in the Southern Ocean between summer and winter time (Kapsenberg et al. 2015b). In the Southern Ocean (McMurdo Sound), it is projected that by 2100 periods of deleterious winter time pH levels could potentially expand to 7-11 months (Kapsenberg et al. 2015b). The northern Channel Islands, California, also experienced

seasonal pH changes, with the pH dropping from 8.04 in May to 7.98 in October (Kapsenberg and Hofmann 2016). Further seasonal changes have also been recorded in the northern North Sea (continental shelf region) during monitoring taking place between 2004 and 2015 by Omar et al.,(2019). Here, pCO_2 levels peaked during late autumn/early winter at 360-400 µatm and reached a low of 280 µatm during spring due to phytoplankton blooms. Except during this bloom, pCO_2 for the rest of the year was controlled by thermodynamics, remineralization and mixing processes. This change in pCO_2, along with the effects of pH changes were the main drivers in the seasonal variation of aragonite saturation state. Maximum saturation levels (Ω>2.5) were recorded 1-2 months after maximum pH and minimum levels (=1.5) during winter. (Omar et al. 2019).

The fluctuations in pH can also be seen over shorter time scales; both event scale (weeks) and diel. For shallow coastal systems, primary production by phytoplankton blooms and wind stress can cause event-scale pH changes (Kapsenberg and Hofmann 2016). Seasonal upwellings, of cold, deep water, cause a decrease in pH. In the northern Channel Islands, California, this pH decrease was 0.12 units, lasting 26 days (Kapsenberg and Hofmann 2016). Phytoplankton blooms, seen in summer, cause an increase in pH levels. In the Southern Ocean, these blooms occur when 24 hour daylight is present (Kapsenberg et al. 2015a). Reasons for diel fluctuations include biological drivers and groundwater discharge (Cyronak et al. 2014). Photosynthesis during the day by species including kelp, coral and algae, results in an increase in pH. This is then followed by a decrease in pH at night due to respiration (Kapsenberg et al. 2015a). The effect of photosynthesis on pH can also be seen in tidal zones where coastal pools become isolated at low tide, with the timing of the tide being a key driver of pH change (Kwiatkowski et al. 2016). A study, by Kwaitkowski et al., (2016) on the Californian coast found that a low tide during daylight hours resulted in photosynthesis occurring to convert the dissolved CO_2 into sugars that are used as an energy source which effectively lowers or reverses the effects of OA. However, when low tide occurred during the night when there is no sunlight to support photosynthesis, plants, organisms and zooxanthellae respire which releases CO_2. Thus, night time low tides, increase the already elevated concentrations of CO_2 and magnify the effects of OA (Kwiatkowski et al. 2016). In these pools pH ranges reached their lowest point pre-sunrise of 7.22 (pCO_2 = 3276 µatm) and a maximum pH of 9.00

(pCO_2 = 10 µatm) mid-late afternoon resulting in a massive pH range for all exposed organisms (Kwiatkowski et al. 2016).

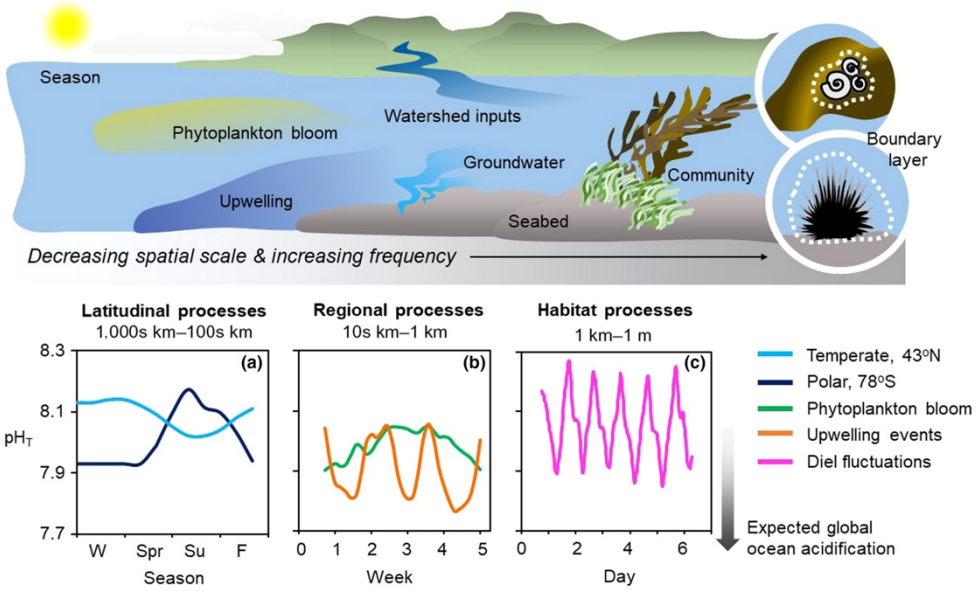


Figure 1.2. The pH changes (pH_T) over seasonal, weekly and daily timescales in a variety of coastal environments and the processes behind them. Taken from Kapsenberg and Cyronak (2019).

Aside from the effects of OA, coastal ecosystems, in particular estuaries, are likely to see further impacts from climate change. Sea level rise will increase the seawater intrusion and raise salinities in estuaries (Bindoff et al. 2019) and oxygen depleted dead zones are projected to increase due to the intensification of climate threats and eutrophication (Breitburg et al. 2018, Laurent et al. 2018). Eutrophication is also expected to increase due to human activities, coupled with an increase in precipitation (Sinha et al. 2017).

Only recently have studies started to account for variability in pH, with mixed results seen. Positive effects of a fluctuating pH, compared to a static pH, has been seen in corals (*Seriatopora caliendrum*), with coral recruits growing 6-19% larger (Dufault et al. 2012). The impact of low pH (7.51) on early stage development was decreased when this pH was fluctuated by 0.15 units in the mussels *Mytilus californianus* and *Mytilus galloprovincialis*, resulting in a quicker transition between larval stages and a

larger shell growth (Frieder et al. 2014). However, *Mytilus edulis* was energetically more stressed when exposed to fluctuating pH with significantly higher metabolic rates seen alongside increased antioxidant enzyme activities and lipid peroxidation (Mangan et al. 2017). Further impacts of pH variation has been seen in barnacles (*Balanus improvises)*, increased variance in shell growth and decreased overall shell growth (Eriander, Wrange and Havenhand 2016), whilst the survival and swimming speeds of isopods (*Paradella dianae*) is also impacted (Alenius and Munguia 2012).

1.2.3 *Benthic sediments*

The pH of sediments has received very little attention compared to seawater pH with respect to climate change. However, potential changes due to OA and other climate stressors are important to understand to predict the outcome of organisms inhabiting sediments. The macrobenthos has an important ecological role in reworking sediments, providing nutrients and food to higher trophic groups and allowing the provision of habitats through engineering species (Herman et al. 1999, Van Hoey et al. 2008, Lee 2008, Ragnarsson and Raffaelli 1999). Many factors impact sediment pH, including overlying seawater pH, presence of key bioturbators and sediment type (Widdicombe et al. 2013, Dashfield et al. 2008, Atkinson et al., 2007). The seafloor is an area of intense biogeochemical and mineral dissolution-precipitation reactions which create strong pH gradients near the sediment-overlying water interface, with dramatic variations of up to 2 pH units being seen both horizontally and vertically (Zhu et al., 2006). The effect of acidified water on sediment pH is thought to differ depending upon sediment type. Sandy sediments from the North Sea had very similar pH profiles to both control (pH 8.00) and acidified (pH 7.70) conditions suggesting these sediments were buffered against acidification (Braeckman et al. 2014). However, in permeable sediment, the pH was lower in the upper 1cm of the sediment in OA conditions (Braeckman et al. 2014).

Bioturbation activity can affect the pH of sediments with mixed results in terms of increasing or decreasing pH being seen. The presence of the burrowing urchin in muddy sand, *Echinocardium cordatum*, resulted in more consistent sediment profiles with sediment pH being 7.40 in controlled seawater conditions (pH 8.00) and 7.30 in OA conditions (pH 7.50) compared to the absence of urchins which resulted in a higher

sediment pH, sometimes greater than the overlying water (Dashfield et al. 2008). Similar results, with the same species, were seen in sandy sediments with sediment pH being lower when the urchins were present (Widdicombe et al. 2009). However, the same species in muddy sediments, resulted in pH levels in the sediment to be higher than in sediments without urchins (Widdicombe et al. 2013). This suggests that both the sediment type and presence of a bioturbator are important when looking at sediment pH. The presence of burrows (from *Nereis succinea*) has previously been shown to cause localised changes to the sediment pH, with pH minimum zones, as low as 6.20, forming within a few millimetres of inner burrow walls and becoming more pronounced with time if the burrow remains irrigated and stable (Zhu et al. 2006). In general the pH of sediments are lower than that over the overlying water (Dashfield et al. 2008, Atkinson et al. 2007, Widdicombe et al. 2013) with surface sediments under overlying water of pH 7.80-8.20 having pH levels of between 6.80 and 7.80 (Atkinson et al. 2007). However, a few studies looking at much lower seawater pH (6.80-7.20) have found that sediment pH levels tend to be similar or slightly higher (Widdicombe et al. 2009, Widdicombe et al. 2013).

1.2.4 *Saturation states and calcifying organisms*

Carbonate ions combine with calcium ions (Ca^{2+}) in seawater to form calcium carbonate ($CaCO_3$) and it is this mineral that forms the shells or skeletons of many marine invertebrates (Doney et al. 2009). $CaCO_3$ saturation state, the ion products of calcium and carbonate ions (Doney et al. 2009) depend on seawater temperature, salinity and pressure (Feely et al. 2004), determines the rate of formation or dissolution of these shells and skeletons (Feely et al. 2008). Saturation is a weak function of temperature and salinity but a strong function of pressure (water depth), with deeper waters being more corrosive (Barker and Ridgwell 2012). Formation of calcium carbonate takes place when the saturation state is greater than 1 due to an excess of CO_3^{2-} ions in the seawater forming $CaCO_3$ crystals (Barker and Ridgwell 2012), with dissolution occurring when the saturation state is lower than 1. Below 1, seawater becomes potentially corrosive to unprotected shells or skeletons (Bates et al. 2009).

Calcium ions can be estimated from the seawater salinity as these are closely proportional, meaning that saturation states are mainly affected by variations in carbonate ions. The concentration of carbonate ions can be calculated from DIC (dissolved inorganic carbon (carbon dioxide, bicarbonate and carbonate) (Cole 2013) and TA (total alkalinity) measurements. (Doney et al. 2009, Feely et al. 2008). The $CaCO_3$ crystal structure is also important when looking at saturation states with CO_3^{2-} saturation state being lower for aragonite than calcite, meaning that aragonite is more soluble (Barker and Ridgwell 2012). The result is that organisms whose shells or skeletons are made from this form of $CaCO_3$ will be at a higher risk as the pH continues to fall (Feely et al. 2004).

With the continuing decrease in CO_3^{2-} the depth at which waters become undersaturated is shoaling and will ultimately reach the surface in certain regions (Barker and Ridgwell 2012). Due to the fact that $CaCO_3$ solubility decreases as temperature decreases and pressure increases, saturation states are lowest in cold, deep, high-latitude waters and highest in shallow, warm tropical waters (Doney et al. 2009), with polar regions becoming undersaturated first (Barker and Ridgwell 2012). Surface waters of the Arctic Ocean already experience seasonal under-saturation due to increased sea ice melt (Robbins et al. 2013), river run off and Pacific water intrusion (Azetsu-Scott et al. 2014, Tynan et al. 2016). With the latest predictions from the IPCC special report on the ocean and cryosphere in a changing climate, it is very likely that 16-20% of surface oceans, specifically the Arctic and Southern Ocean, will experience year-round corrosive conditions for aragonite by 2081-2100 (Bindoff 2019). Undersaturation with respect to aragonite has also been seen in estuarine environments. The Puget Sounds, USA, experiences undersaturation reaching aragonite saturation state lows of 0.34-0.97 in the Hood Canal (Feely et al. 2010, Reum et al. 2014).

A range of meta-analyses have been conducted on the effects of OA on calcifying organisms including Hendriks et al. (2010), who found that all calcifying groups tested showed reduced calcification rates with increasing pCO_2 including bivalves, coccolithophores and corals, whilst Wittman and Pörtner, who analysed 167 studies, find negative effects of OA on physiological performance in 153 species (Wittmann and Pörtner (2013) i.e. over 90% of the species studied. This analysis by Wittmann

and Pörtner revealed that the calcifying organism's corals, molluscs and echinoderms, are the most sensitive phyla to OA. Ocean pH value projections for 2100 (RCP 8.5 scenario) show that 63% of echinoderms and 51.6 % of molluscs will be vulnerable, with 27.8 % of crustaceans being negatively affected (Wittmann and Portner 2013). Polychaetes were not included in this analysis however, most likely due to the limited number of OA studies performed for these phyla to date.

Changes to shell growth and structure has been seen across a range of species. One of the 6 known forms of calcium carbonate is amorphous calcium carbonate (Addadi, Raz and Weiner 2003) and functions as a precursor phase to aragonite and calcite production (Weiss et al. 2002, Radha et al. 2010) for the construction of crystalline shells (Addadi et al. 2003), for example, prior to formation of calcite spicules in sea urchin (*Paracentrotus lividus*) (Beniash et al. 1997) or transforming into aragonite in bivalve larvae (*Mercenaria mercenaria* and *Crassostrea gigas*) (Weiss et al. 2002). Dehydrated ACC, the most unstable form of ACC, becomes hydrated (each $CaCO_3$ binds to a water molecule) under super saturation. This more stable form can be transported and deposited, by precipitation, to form crystalline aragonite or calcite for shell formation (Addadi et al. 2003, Weiss et al. 2002). The effects of OA on ACC formation have been seen in the mussel, *Mytilus edulis*. Acidified conditions led to a greater concentration of hydrated ACC, implying less crystalline order in shells (Fitzer et al. 2016). Precipitation of hydrated ACC under OA may be an energetically cost-effective means of producing ACC (Ihli et al. 2014) or shell repair (Addadi et al. 2003). The same species (*Mytilus edulis*) exhibited a decrease in shell growth, to near future pH levels, as well as changes in calcite crystal formation which impacted on shell structure. This change seen in shell structure may impact shell strength and reduce protection against predators (Fitzer et al. 2014).

Changes in shell mineralogy over recent time has also been documented in Californian mussels collected from natural populations, with dramatic changes seen in *Mytilus californianus* over the last 15 years (2000 to 2015), despite consistent mineral structure being seen in the 2500 years prior to the year 2000 (McCoy et al. 2018). An increase in disorder of calcium carbonate shells has been found, with a mosaic pattern (not uniformed) seen and smaller crystal shells, coupled with greater variability between individuals (McCoy et al. 2018). Changes have also been seen in other

taxonomic groups with pteropods (*Limacina helicina*) having displayed reductions in calcification rates and shell dissolution. In the Arctic a 28% reduction in calcification rates was found in organisms exposed to pH levels expected by 2100 (Comeau et al. 2009). Severe shell dissolution has been found to affect 53% of onshore individuals on the Californian coast, with the extent of aragonite under saturation showing a strong positive correlation to the proportion of individuals with damage (Bednarsek et al. 2014). This correlation with under saturation is clear in pteropods, *Limacina helicina antartica*, from the Southern Ocean as well with dissolution of aragonite shells being most severe in under saturated conditions (($\Omega = 0.8$) (Bednaršek et al. 2012). A loosely organised crystal structure was seen with crystals of the crossed-lamellar layer becoming partly eroded meaning shells became prone to fragmentation and increased frailness (Bednaršek et al. 2012). Given that polar areas already experience under saturation, these findings could see such species becoming increasingly affected by ocean acidification.

There is broad agreement that OA will lead to a decrease in coral calcification, however, there is considerable uncertainty about the magnitude of this effect and the factors driving variations in the calcification response (Chan and Connolly 2013). The response of coral calcification to decreasing aragonite saturation state has been reviewed in a meta-analysis by Chan et al., (2013) who found that coral calcification decreases by 15% per unit decrease in aragonite saturation state (Chan and Connolly 2013). On the Great Barrier Reef calcification rates of the massive *Porites* decreased by 21% from 1988 to 2003 (Cooper et al. 2008) with similar rates (just under a third) seen in *Pocillopora damicornis* in the eastern tropic Pacific from 1974 to 2006 (Manzello 2010). When compared to current day pH levels, *Montipora capitata* (from Hawaii) showed a 15-20% reduction in calcification under OA conditions (Jokiel et al. 2008). However, *Stylophora pistillata*, showed that reduction in calcification rate was only observed at the lowest pH of 7.20, where crystal growth was significantly lower than the control (pH 8.0) (Venn et al. 2013). These corals might be able to mitigate OA effects by regulation of pH in the fluid at the tissue-skeleton interface (Venn et al. 2013).

The use of CO_2 vents, which takes the species ability to adapt into account, has enabled studies to look at shifts in communities and species abundance. Many of

these vents have been in the same location for thousands of years, allowing local adaptations to low pH environments (Kelly and Hofmann 2013) .Cold vents off the coast of Italy have shown a 30% reduction in species numbers at a pH of 7.80-7.90, with organisms having an aragonite skeleton (commonly present in this area) being absent at this pH (Hall-Spencer et al. 2008). However, a whole range of algal species proved to be resilient at this low pH (Hall-Spencer et al. 2008), meaning that shifts in calcifying communities towards algae dominated ones could be seen in the near future. Further changes in community structure has been seen in the northeast Atlantic where calcifying red algae (*Corallina officinalis*) cover decreased when exposed to elevated CO_2 levels and non-calcifying red algae (*Chondrus crispus*) cover increased (Hofmann et al., 2012), again adding to the changes in community structure and species abundance that is expected as pH continues to decrease.

The importance of studying all life cycle stages is apparent with gametes and early development appearing to be far more impacted by OA than adult stages with the potential for bottle necks to occur during these stages (Dupont et al., 2010a), especially for those marine organisms which start to calcify during their larval and/or juvenile stages (Ross et al. 2011). The first studies by Kurihara found that CO_2 tolerance differed between life stages and vulnerable stages can differ between species (Kurihara 2008). For example, the larval stages of sea urchins and bivalves and the settlement stages in corals and shrimps seemed to be most vulnerable, which can partly be explained by the fact that shell and skeleton synthesis starts at these stages (Kurihara 2008). Different stages of the larval period were also differently effected by OA in bivalves, with mussel (*Mytilus galloprovincialis*) embryogenesis being unaffected by pH 7.4 up until the trochophore stage where development was then delayed when the shell began to form, with all veliger larvae showing morphological abnormalities and decreased larval height and length (Kurihara et al. 2009). Oysters showed similar, although more severe than mussels at the same pH with decreased normality in veliger larvae, resulting in just 5% normality at this pH (Kurihara, et al., 2007).

Since these studies experimental design has improved and the level of understanding of these physiologies have increased, for example, exposure scenarios became more relevant and were longer. In particular work by Sam Dupont's group highlighted the

importance of looking at all phases of the life cycles as well as working in realistic abiotic and biotic conditions amongst a wide range of taxa (Dupont and Thorndyke 2008). The sea star *Crossaster papposus* produces lecithotrophic larvae, an alternative life history strategy to planktotrophic larvae, which grew faster under OA conditions, showing no effects on survival or skeletogenesis, showing the importance of looking at various life history strategies (Dupont et al., 2010b). Dupont and Thorndyke (2009) also found mixed results amongst and between species, for example, opposite impacts of OA were seen in closely related sea urchins in experiments carried out under the same experimental conditions with *Echinus esculentus*, showing a negative effect on mortality whereas *Strongylocentrotus droebachiensis* showed enhanced developmental success (Dupont and Thorndyke 2009). Stumpp et al., (2011) suggested that sea urchin larvae (*Strongylocentrotus purpuratus*) suffer development delay, due to altered energy budgets, under OA conditions (seawater pCO_2 = 1271 µatm) rather than the previously postulated reductions in size at comparable developmental stages, highlighting the importance of defining a standard frame of reference when comparing parameters between treatments (Stumpp et al. 2011).

1.2.5 *Acid-base disturbances and homeostasis*

Whilst calcifying species are amongst the most sensitive to OA and as such have been the focus of most research, there is now increasing evidence that a range of other physiological and biological processes and hence biota may be impacted. OA has also been found to affect a species' ability to acid-base regulate and increase the energetic demands of homeostasis (Lannig et al., 2010, Miles et al., 2007). Understanding the ability for acid-base regulation is important because intracellular formation and maintenance of the calcium carbonate skeleton is dependent on pH homeostasis (Stumpp et al. 2012).

The maintenance of a contestant intracellular and extracellular pH is crucial for the normal physiology of organisms and cellular metabolism. This is primarily achieved, in the short term, through actively secreting H^+ and through the bicarbonate buffering system where bicarbonate is accumulated in order to buffer the excess protons

generated through respiratory or metabolic respiration (Ishimatsu et al. 2005, Hu et al., 2015). A Na^+/K^+/-ATPase, a universal enzyme found in all animal cells that generates an electrochemical gradient (Hu et al. 2015), pump is used to transfer bicarbonate (Lucu and Towle 2003) and regulate pH (Morth et al. 2011, Henry and Wheatly 1992). In crustaceans pH compensation is via ion exchange, using the Na^+/K^+/-ATPase pump, across the gills, with 93% of bicarbonate being taken up from the surrounding seawater and longer term compensation probably being employed by protein buffering (Rastrick et al. 2014).

The ability to compensate for OA-induced changes in extracellular pH is believed to be a key determinant of an organisms' ability to tolerate near future OA, playing an important role in survival and distribution of a given species (Melzner et al. 2009, Portner 2008, Wittmann and Portner 2013, Calosi et al. 2013a). Any uncompensated changes in extracellular or intracellular pH may lead to metabolic depression or hamper the function of blood oxygen transport (Wittmann and Portner 2013). In order to compensate for these changes (i.e. an increase in acidity due to OA) an organism must either increase its expulsion of excessive protons or employ additional buffering systems, such as uptake of bicarbonate ions, which bind protons (Bollmann et al. 2010). This pH and ion regulation is driven by energy-consuming ion pumps (Wittmann and Portner 2013) which may lead to changes in energy budgets under OA conditions.

Fish are known to be good acid base regulators and are able to compensate for changes to internal pH, primarily through acid-base transfer over the gill epithelium (Claiborne et al., 2002). Activity levels are thought to impact on an organisms ability to compensate as higher metabolic rates and oxygen consumption, leads to higher rates of CO_2 excretion (Melzner et al. 2009), with fish and active marine invertebrates maintaining pH homeostasis (Zlatkin and Heuer 2019). Full compensation has been seen in the California sea hare (*Aplysia californica*) where internal pH was maintained at CO_2 levels of 1200 µatm and the velvet swimming crab (*Necora puber*) via bicarbonate buffering to end of century ocean pH levels (Zlatkin and Heuer 2019, Spicer, Raffo and Widdicombe 2007). It is worth noting that bicarbonate buffering, in the case of the velvet swimming crab was partly supplied by dissolution of the exoskeleton (Spicer et al. 2007). The common limpet (*Patella vulgate*) also experienced visible shell dissolution as it was able to almost completely compensate

using the shell as a bicarbonate source (Marchant et al., 2010). Other species have been less able to cope with a reduction in seawater pH and not achieve full compensation. Both mussels (*Mytilus galloprovincialis*) and sea urchins (*Psammechinus miliaris*) use bicarbonate buffering in response to decreasing seawater pH, however, despite bicarbonate increases incomplete compensation was found in both species, with mussels showing a reduction in pH_e from 7.55 to 7.36, in response to seawater pH values of 7.30 and 7.44 respectively (Michaelidis et al. 2005, Miles et al. 2007).

The responses of organism's acid base regulation to can vary dramatically, even within the same class. These stark differences can be highlighted using sea urchins. *Strongylocentrotus dröebachiensis*, whose extracellular fluids showed a reduction in pH, show no ability to compensate via bicarbonate ions (Spicer et al. 2011). The purple tipped urchin (*Psammechius miliaris*) demonstrated an increase in bicarbonate, however, this increase was not enough for the organism to fully compensate to the decrease in seawater pH (Miles et al. 2007). Finally, the urchin *Paracentrotus lividus* was able to maintain a relatively stable pH due to an increase in bicarbonate (Lewis et al. 2016).

1.2.6 *OA impacts on benthic organisms*

Benthic communities will be affected by climate change through a wide range of abiotic (storms and habitat loss) and biotics effects (shift in species and larval supply) as well as wider effects such as rising temperatures, OA and anthropogenic interactions (Birchenough et al. 2015). Changes in species distribution can be due to factors such as sea level rise, temperature increases as well as the ability for species to survive in acidified waters. Rising seawater temperatures can cause poleward shifts in some species towards cooler regions (Birchenough et al. 2015), for example, the northern range of the top shells *Osilinus lineatus* and *Gibbula umbilicalis* has extended (Mieszkowska et al. 2006) and the warm water barnacle *Solibodalanus fallax* is now found in European waters (Southward et al. 2004).

Significant changes in structure, diversity and abundance of organisms has been seen in response to OA (Linares et al. 2015, Widdicombe et al. 2015, Hale et al. 2011). A negative impact from CO_2 seabed leakage was seen in the diversity of species, abundance and biomass of the microbenthic community, which was not restricted to calcifying organisms but was experienced by the majority of animals (Widdicombe et al. 2015). In the Mediterranean Sea, a pH drop from 8.10 to 7.90 (due to CO_2 vents) also caused dramatic shifts in the distribution and dominance of key benthic ecosystems. Forests of kelp, usually found at 65m or below, replaced habitats dominated by calcifying organisms at 40m, with only aragonite-calcifying algae able to survive (Linares et al. 2015). Further shifts away from calcifying dominated systems are seen off the cost of Japan. Here decreases in pCO_2 result in algal dominated systems, accompanied by biodiversity loss and major simplification of the ecosystem (Agostini et al. 2018). The addition of seawater warming to OA levels caused lower species abundance and diversity compared to lower temperatures, with species specific responses being recorded. Molluscs showed the largest reduction in abundance, with arthropods being moderately reduced and annelids being mostly unaffected by these conditions (Hale et al. 2011).

1.2.7 *OA impacts on polychaetes*

Studies focusing on the effects of OA on polychaetes initially focused on behavioural traits, looking at burrowing ability under reduced pH conditions. Here, there was no impact of a 5 week exposure to OA conditions on the size or structure of burrows in the polychaete *Alitta virens* (Widdicombe and Needham 2007a). A few studies have used CO_2 vents to represent naturally occurring low pH areas as well as a pH gradient. The abundance and recruitment of polychaetes was found to decrease along with seawater pH in CO_2 vents off Italy with the disappearance of both calcifying and non-calcifying polychaetes (Gambi et al. 2016), despite a wide range of polychaetes being able to survive under acidified conditions (Cigliano et al. 2010). A species-specific response amongst closely related species was seen with decreasing pH resulting in an increase in the abundance of filter feeders and herbivores, while sessile polychaetes disappeared in the extreme low pH zones (Gambi et al. 2016). Three species of polychaetes have been found to exhibit high tolerance to low pH-high pCO_2

conditions with *Amphiglen mediterranea*, *Platynereis dumerilli* and *Syllis prolifera* being abundant along the pH gradient, with significantly increased abundance in the most acidified areas (Ricevuto et al. 2014). *Platynereis dumerilli*, was able to physically adapt to vent conditions, being genetically different from nearby populations, however, this adaptation is not ubiquitous among all tolerant species with *Amphiglen mediterranea* exhibiting physiological plasticity instead as a viable strategy for successful colonisation (Calosi et al. 2013b).

A combination of stressors has been looked at with *Alitta virens* being exposed to OA (750/1000 µatm) and warming (+4^{o}C) on a long-term scale (18 months) with the effects of both stressors and their interaction not being detectable in the short term (7 days). However, over time they manifest through changes in growth, bioturbation and bioirrigation with changes intimately linked to species responses to seasonal variations which, depending upon timing, can either exacerbate or buffer the effects (Godbold and Solan 2013). This study highlights the need to look at long term studies as well as short term exposures.

Studies looking at physiological impacts of OA conditions are limited, but the few that have studied these impacts have shown that polychaetes can be sensitive to low pH levels, including decreased energy reserves, reduced capacity to regenerate tissue and increased oxidative stress (Freitas et al. 2017, Freitas et al. 2016b, Pires et al. 2015). Despite an increase in their antioxidant defence, the polychaete *Hediste diversicolor* was unable to prevent lipid peroxidation (Freitas et al. 2017). Given the importance of acid-base regulation in determining species responses to varying seawater pCO_2/pH conditions there is surprisingly little known for the taxonomic group. In one of the few studies to look at this, Wage et al., found that acid-base balance was maintained in *Platynereis dumerilii* via the up regulation of the NHE transport protein (Na^+/H^+ exchangers, used to carry excess ions out), suggesting that existing protein activity was insufficient to regulate pH under acidified conditions (Wage et al. 2015).

It is important to include all life stages when looking at potential impacts, especially early stages as these are particularly sensitive with the polychaete worm *Ophryotrocha sp.* having reduced fecundity as well as slower development of embryos and larvae (Verkaik et al., 2017). However, in multi-generational exposures of

Ophryotrocha labronica this species was found to be tolerant to lowered pH (7.68) and elevated pCO_2 (1137 µatm) conditions from generation F3 onwards, despite an initial significantly lower fecundity in F1 and F2 females (Rodriguez-Romero et al. 2016). *Arenicola marina* larvae had no reduction in survival under reduced seawater pH, however, this organism did exhibit reduced fertilisation success and lower sperm motility (Campbell et al. 2014). Inter-individual variation on sperm swimming has been seen in *Galeolaria caespitosa* with the majority of males having reduced sperm swimming, however, robust sperm swimming behavior was seen in some males under near-future ocean acidification (pH 7.80) (Schlegel et al. 2014), again highlighting that responses to OA is not only species specific but that differences can also been seen between organisms of the same species.

1.3. OA is occurring in a contaminated ocean

Increasing OA is not the only stressor marine organisms are currently being exposed to, with multiple anthropogenic threats affecting marine environments globally. These include the warming of sea surface temperatures (Hoegh-Guldberg and Bruno 2010, Pachauri and Reisinger 2007) invasion of exotic species and increased disease (Hoegh-Guldberg and Bruno 2010, Stachowicz et al. 2002, Harvell et al. 2009), extreme weather, sea level rises (Oppenheimer et al. 2019), increasing harmful algal blooms and pathogenic organisms (Bindoff et al. 2019) and pollution (Halpern et al. 2008). OA may interact with coexisting stressors and could increase the susceptibility of marine organisms and communities to disease and invasion of exotic species (Hoegh-Guldberg and Bruno 2010). Our oceans are also being exposed to plastic pollution, with an estimated 10% of the 200 million tons of plastic produced annually ending up in the ocean (Vannela 2012) impacting negatively on marine life (Galloway et al., 2017) as well as decreasing the efficiency and productivity of fisheries and causing large economic costs (Beaumont et al. 2019). On top of this anthropogenic noise is now recognized as a world-wide problem with a broad range of negative effects been shown on a variety of taxa (Williams et al. 2015).

Almost half of the global population lives within the coastal zone (45%) (Mee 2012), with the population density here 2.5 times above the global average (Crossland and

Crossland 2005). This has increased the pressure on coastal waters due to the wide range of pollutants finding their way into these systems. Due to the high level of pollutants in coastal waters the potential for OA to alter the way pollutants induce toxicity (Roberts et al. 2013) is of concern as here pollutants occur in high enough concentrations to cause harm to biota. The toxicity of all weak acids and bases varies with their dissociation status, with the undissociated form usually being more toxic (Saarikoski and Viluksela 1982). Many of the contaminants in coastal waters are ionisable and are sensitive to changes in pH with changes in pH changing the ionized fraction of chemicals and altering the uptake in organisms (Karlsson et al. 2017). The decrease in concentration of hydroxide and carbonate due to the reduction in pH from OA will change the speciation of metal ions in seawater (Byrne 2002). In surface waters, hydroxides and carbonates are predicted to decrease by 82% and 77% respectively with a rapid reduction expected after the year 2200 (Millero et al. 2009). One such metal expected to significantly alter its speciation under OA is copper. Copper (II) forms strong complexes with carbonate and a change in pH will lead to an increase in copper free ions (Millero et al. 2009). These free ions are known to be more toxic than the complex forms of metals (Allen et al., 1980). It has been predicted that over the next 100 years there will be a 115% increase of free copper ions in estuarine waters due to OA, coupled with the increase in temperature (Richards et al. 2011). Hence OA has the potential to alter the bioavailability of pH sensitive metals, such as copper, to biota and therefore potentially alter their toxicity responses.

Ocean acidification is unlikely to occur in isolation with climate change resulting in multiple stressors such as warming, nutrient change, hypoxia and stratification of the water columns, all which have the ability to interact with each other in different ways (Wake 2019, Boyd et al. 2018). In order to understand what future oceans may look like consideration of possible permutations of stressors is needed (Wake 2019) with selection of these stressors depending upon the organism of interest (Boyd et al. 2018). It has been suggested that when choosing stressors certain considerations must be made, including examination of known interactions, ensuring stressors mimic change and are kept within environmentally relevant levels (Boyd et al. 2018). Given that it is not possible to test all stressors, a combination of OA and copper pollution was chosen due to the fact that metal speciation, in particular copper, is expected to

change due to pH changes and because polychaetes are a key sediment dwelling bioturbator which is often found in sediments with high levels of copper pollution.

1.4. Copper – mechanisms of toxicity and responses

Metals remain very common aquatic pollutants, especially in coastal waters and sediments where they can be found in high concentrations, often above their EC_{50} concentrations (Bryan and Langston 1992). For example, copper can be found in concentrations ranging from low levels of 0.004 µM (Jones and Bolam 2007) to much higher levels of 1.61 µM (Bryan and Gibbs 1983a). This is mainly due to human activities (Pascal et al. 2010) such as effluent discharge, agriculture, nutrient run off, oil and chemical spills and mining activities (Lewis 1995). In addition to being a metal that exhibits major changes in speciation under OA relevant pH changes, copper is also highly prevalent in coastal waters and sediments. Copper is a naturally occurring trace metal due to geological weathering (Phillips 1980), essential for biological functions such as normal growth and metabolism, by allowing many critical enzymes to function properly (Harris 2001). At elevated concentrations however, copper is toxic to organisms directly via the production of reactive oxygen species (ROS) such as O^{-} and $HO^{-\cdot}$ or indirectly via covalent bonding of free ionic copper to macromolecules. It is known to cause lipid peroxidation, genotoxicity and larval teratogenicity (Stohs and Bagchi 1995, Regoli et al., 1998). Due to anthropogenic activities, copper is now one of the most ubiquitous and widespread contaminants found in coastal waters and sediments (Eisler 1998) and the amount of copper present is significantly greater than the natural geological rate (Phillips 1980). As a result of its prevalence in aquatic environments and its threat to aquatic organisms, copper has been ranked as the metal posing the highest threat for aquatic invertebrates (Donnachie et al. 2014).

One of the main toxic mechanisms of copper is due to oxidative stress generation through Fenton and Haber-Weiss reaction. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced by these reactions causing oxidative stress. Metals such as iron and copper promote the formation of free radicals such as superoxide and hydroxyl radicals and nonradical molecules such as hydrogen

peroxide causing damage to DNA, proteins, lipids and carbohydrates (Sharma et al. 2012, Das and Roychoudhury 2014).

$Cu^{2+} + O_2^{-\cdot} \longrightarrow Cu^{+} + O_2$

$Cu^{+} + H_2O_2 \longrightarrow Cu^{2+} + OH^{\cdot} + OH^{-}$ Fenton reaction

$O_2^{\cdot} + H_2O_2 \longrightarrow O_2 + OH^{\cdot} + OH^{-}$ Haber Weiss reaction

Oxygen radicals may attack DNA at either the sugar, leading to base loss and single strand breaks (Imlay and Linn 1988) or the base which generally involves hydroxyl radical addition to double bases (Dizdaroglu 1993) with this radical being known to react with all purine and pyrimidine bases as well as the deoxyribose backbone (Halliwell and Gutteridge 1999). When there are high levels of ROS present, enhanced lipid peroxidation takes place (Sharma et al. 2012) with the double bonds found in polyunsaturated fatty acids being particularly sensitive to attack by ROS (Smirnoff 1995) with a single hydroxyl radical resulting in peroxidation of many polyunsaturated fatty acids due to a chain reaction starting with the removal of a hydrogen atom from the double bond (Smirnoff 1995). Malondialdehyde (MDA) is one of the final products of peroxidation in phospholipids and is responsible for cell membrane damage (Halliwell and Gutteridge 1999) increasing membrane fluidity and permeability (Sharma et al. 2012).

Metallothioneins (MT) are soluble low molecular weight proteins which have a high affinity for particular trace metals including copper, cadmium, zinc and mercury (Rainbow 1997). Synthesis of this protein is induced by exposure to these trace metals and the subsequent binding of the metals, through cysteine residues (Ruttkay-Nedecky et al. 2013), reduces their cytotoxic effects (Rainbow 1997). In aquatic invertebrates MTs appear to play a role in the routine metabolic handling of copper and zinc but also in the detoxification of excess amounts intracellularly of these metals (Amiard et al. 2006). For example, in a copper resistant population of *Hediste diversicolor* MT-like proteins were significantly higher compared to nearby populations, not resistant to copper (McQuillan et al. 2014).

A few studies have now investigated the potential for ocean acidification to alter the toxicity effects of copper in marine invertebrates, and these have generally found that OA does indeed increase the toxicity of copper. However, these altered toxicity responses vary in magnitude according to both species and life history stage, suggesting that the observed altered toxicity is not simply being driven by the change in metal speciation. For example, a study by Lewis et al. (2016), showed that OA (using a pH of 7.71, pCO_2 1480 µatm) significantly increases the copper toxicity responses of the mussel *Mytilus edulis* and the purple sea urchin *Paracentrotus lividus*, but that the level of response differed between these two species. Copper-induced DNA damage was increased in both species, but the relative increase in DNA damage was 4 times higher in mussels compared to the sea urchins, which the authors proposed may be due to their differing abilities to acid-base regulate. (Lewis et al. 2016). The species ability to acid-base regulate may determine the OA – copper interaction. In the polychaete *Arenicola marina*, Campbell et al., found that copper toxicity to early life history stages was increased by OA (Campbell et al. 2014) with similar results found in *Hydroides elegans* (Gopalakrishnan et al., 2007) but with difference in the interaction between OA and copper observed across the different life history stages examined.

Since most contaminants, including copper, accumulate in sediments and concentrations of heavy metals in sediments usually exceed those of the overlying water by between 3 to 5 orders of magnitude (Bryan and Langston 1992), sediment dwelling organisms are often exposed to the highest levels of any contaminant. Therefore, it is important to look at the potential for OA-metal interactions in sediment dwelling organisms such as polychaetes.

1.5. Polychaetes

Copper has been found to cause increased DNA damage in *Alitta virens* (Watson et al., 2018) as well as decreasing burrowing activity in *Hediste* (formally *Nereis*) *diversicolor* which the authors suggested may be down to metabolic or physiological disturbances (Bonnard et al., 2009). *H.diversicolor* from a contaminated mining site in

America had significantly reduced weight compared to a nearby population from a reference site, however, no changes in gene expression was seen despite the tolerance of this species at this location, which may reflect the importance of other strategies such as metal cation sequestration (Breton and Prentiss 2019). The same species from Restronguet Creek, UK, where the population is known to be genetically adapted to survive in these contaminated sediments (Pook, Lewis and Galloway 2009), have copper-containing lysosomes in their epidermal cells as well as other lysosomes which appear to be a major detoxifactory store for accumulated copper (Mouneyrac et al. 2003)

However, only a few studies to date have looked at the impact of copper under OA conditions. *Pomatoceros Lamarckii* larvae have been shown to be sensitive under OA conditions with their susceptibility being significantly increased by copper (10^{-4} µM), leading to decreased larval survival, however, fertilisation success was unaffected (Lewis et al., 2013a). The ability for OA to alter copper toxicity has also been seen in *Arenicola marina* where sperm DNA damage and early larval survival showed greater toxicity responses at reduced seawater pH (7.77 and 7.47) compared to control conditions (Campbell et al. 2014).

Given the ecological importance of polychaetes coupled with the fact that OA has been shown to alter copper toxicity in some species it is important that we further the knowledge in this field.

1.6. Aim of thesis

This literature review has highlighted the potential for ocean acidification to interact with the speciation of the common contaminant copper and therefore potentially also its uptake and toxicity effects on coastal species. The magnitude of OA-induced increases in copper toxicity effect appears to vary in a species-specific way. A key group of coastal marine invertebrates likely to experience copper pollution under varying seawater pH conditions are polychaetes, yet to date this interaction has not been studied. The aim of this thesis is therefore to look at the effects of OA and copper as combined stressors, on the physiological and toxicity responses of two coastal

polychaete worms, *Alitta virens* and *Hediste diversicolor*, living in both contaminated and clean environments. This thesis examines the relationship between the acid-base physiology and copper toxicity responses of these two ragworms under combined OA and copper exposures under two different exposure scenarios; first under a standardised laboratory ecotoxicological testing scenario, using water-bourne exposures, and then a more environmentally realistic exposure set up using sediment-based exposures and simulating tidal immersion and emersion.

First, to gain insight into the current state of knowledge and look for emerging paradigms of OA contaminant interactions, I conduct a systematic review of the literature for studies that have examined changes in contaminant-induced toxicity under OA conditions in marine invertebrates (chapter 2). I then conduct a meta-analysis on all existing data to examine the magnitude and direction of the key biological responses measured in these studies to determine if there are any clear patterns in both the direction and size of the OA-effect size. A clear pattern of the effect of OA on contaminant toxicity may be useful in predicting the changes and the susceptibility of taxa in the future, however, marine invertebrates are known to differ in their physiological responses to OA which might influence these interactions in varying ways across taxas.

In chapter 3 I use a water born exposure and standardised ecotoxicological methodologies to test the hypothesis that OA will increase the toxicity of copper using *Alitta virens*, focusing on a range of physiological and ecotoxicological responses. This work will enable direct comparisons with previous OA-copper exposure studies in two other marine invertebrate species (mussels and urchins) with differing acid-base physiologies, and therefore add to our understanding of the role of an organism's ability to both acid-base regulate and mount an oxidative stress response in determining the OA-copper toxicity outcome.

Following on from this, in chapter 4, I then use this same exposure scenario to compare two different populations of *Hediste diversicolor*, one being genetically adapted to survive in contaminated sediments and resistant to high levels of copper and zinc and the other being a nearby non-resistant population. I address the 'energy

limited tolerance to stressor' theory which proposes that bioenergetics plays a role in tolerance to stressors. I expose these population to both OA and copper treatments (singularly and together) and compare responses, through effect sizes, between populations across a range of physiological and toxicity end points. This chapter aims to understand if there are any energetic costs associated with copper resistance and how different populations may respond in the face of future climate scenarios.

Finally, in chapter 5, I look at the impacts of both copper, OA and the combination of both under a more environmentally realistic scenarios using *Alitta* virens in a sediment-based exposure. This exposure will use a tidal cycle to mirror the conditions that this species experience in their natural habitat, creating immersion and emersion periods similar to the site these polychaetes were collected from. Biological end points will be measured along with sediment pH to compared to the overlying seawater and gain greater insight into the sediment conditions that benthic organisms inhabit. This will provide insight into whether environmental context influences the OA-copper interaction for polychaetes, to better understand how this ecologically important group may respond to an acidifying ocean.

Chapter Two

A meta-analysis of the current evidence into the role of seawater pH and carbonate chemistry in the toxicity of chronic and emerging global contaminants to marine biota.

2.1. Introduction

The scale of anthropogenic impacts on our planet have meant that no area of the world's oceans are unaffected by human influence (Halpern et al. 2008). A wide range of contaminants are still entering global oceans, due to human activities, from land based sources via rivers and the atmosphere to the ocean where they may accumulate and recycle in sediments and organisms (Schiedek et al. 2007). These contaminants include trace metals, chemicals, persistent organic compounds, agricultural run-off, pharmaceuticals via sewage effluent and waste disposal and macro/micro plastic debris (Ridgway and Shimmield 2002, Beman et al., 2005, Gaw et al., 2014, Cozar et al. 2014, Roose et al. 2011). Worldwide, the production of chemicals is increasing with a total production volume expected to double in comparison to the levels produced in the year 2000 levels by 2024. About 30,000 of the chemicals currently on the EU market have a production volume of higher than one tonne per year and many have been on the market for more than 20 years. These substances can, and often do, end up in rivers, estuaries and seas with potentially damaging effects on marine organisms, ecosystems and processes. (Roose et al. 2011) The OSPAR intermediate assessment report, 2017, concluded that despite reduced releases of contaminants from land-based sources and offshore oil industries in the North Sea since 2010, concerns still remain in some areas where there are high levels of mercury, lead and CB118 (PCB congener) as well as increasing concentrations of PAHs and cadmium in open waters (OSPAR 2017). Pharmaceuticals, designed to be active in organisms even at low concentrations (Serra-Compte et al. 2018), have received less attention than metals to date. However, a recent review found that pharmaceuticals have been detected in the environment in 71 countries, with 631 different substances being found (der Beek et al. 2016).

The chemistry of global oceans is now changing at an unprecedented rate due to the rise in atmospheric CO_2, leading to a net decrease of CO_3^{2-} and a net increase in HCO_{3-} and H^+ concentrations. This results in a decrease of seawater pH with open ocean pH levels predicted to decrease by up to 0.29 units by 2018-2100 (Bindoff et al. 2019). This change in ocean pH could lead to potential changes in how contaminants interact and cause toxicity due to altering their speciation and bioavailability (Zeng, Chen and Zhuang 2015). Coastal and estuarine ecosystems have a much more variable chemistry with pH fluctuating by as much as 0.50-0.99 units over tidal, daily and seasonal timescales (Duarte et al. 2013, Hofmann et al. 2011) thus any further decrease in pH could exacerbate the toxicity of contaminants further. These ecosystems are also where the a large proportion of the world's populations lives, with 2.4 billion people (41% of global population) living within coastal limits and half of all coastal countries having 80-100% of their total population within 100 km of the coastline (Martinez et al. 2007), hence these ecosystems are both economically important as well as vulnerable to further anthropogenic impacts.

Many of the chemical contaminants present in estuarine and coastal waters are ionisable compounds and are therefore sensitive to changes in pH. For example, it has been estimated that between 85 and 95% of active pharmaceutical ingredients are ionisable (Manallack 2009). This presents the possibility for any change in seawater pH to affect the behaviour, fate and uptake of such contaminants and for interactions between the process of global OA and any contaminants present in aquatic systems. For example, changes in seawater pH change the organic and inorganic speciation of metals in surface ocean waters, with the decrease in concentrations of hydroxide and carbonate ion affecting the solubility, adsorption, toxicity and redox processes of metals (Millero et al. 2009, Stockdale et al. 2016). It is predicted that metals forming strong complexes with carbonate, including Cu^{2+}, will be most strongly affected by pH decrease, resulting in an increase in free ion forms (Millero et al. 2009, Zeng et al. 2015). Changes in pH can also affect the rate of oxidation and reduction of metals, with decreasing pH leading to an increased amount of reduced metal, which are more toxic to biota than oxidised metals (Zeng et al. 2015). The change in metal speciation associated with OA can also affect organic degradation. Biologically available iron (Fe(III)) is likely to be reduced by 10-20% by

2100, compared to current day levels (Shi et al. 2010), having harmful effects of the degradation of organic pollutants and oil (Zeng et al. 2015). This is due to the fact that iron is an important contributing factor to the detoxification of hydrocarbons (Zeng et al. 2015) as the enzymes which are essential for more microbial pathways of PAH and oil degradation require a metal cofactor, often being iron (Bugg 2003).

OA is not just an open ocean problem, with acidification of coastal sediments enhancing the release of metals, including Cd, Fe, Zn, Co, Pb, Ni and Cu, to the overlying waters (de Orte et al. 2014, Roberts et al. 2013, Atkinson et al. 2007) thus having the potential to become more toxic to organisms. For example, in sediments on the southwest coast of Spain, concentrations of lead, zinc, copper, cobalt and iron all increased in pore water under low pH treatments, with iron increasing by up to 82% as pH decreased from 7.50 to 5.50 (de Orte et al. 2014). However, it is worth noting that protons can compete with these potentially toxic metals for key binding sites associated with these biotas (Tipping et al. 2003).

The uptake of chemicals in organisms depends mainly on their lipophilicity (Erickson et al. 2006), the ability for a chemical to dissolve in lipids, and is a key physiochemical parameter linking solubility, membrane permeability and therefore chemical absorption, potency and distribution (Stocks 2013). Lipophilicity is much higher for weakly acidic compounds than weakly basic ones (Erickson et al. 2006). Under low pH levels the toxicity and bioconcentration factors were higher for acids than bases in freshwater; the factor at which this changed with pH was correlated with the lipophilicity of the compound for ionizing organic chemicals (Rendal, Kusk and Trapp 2011). The ionisation of a compound decreases its lipophilicity, compared to neutral compounds and can therefore reduce its toxicity (Rendal et al. 2011) and changes in pH can change the ionized fraction of chemicals, which can greatly change the uptake in organisms (Karlsson et al. 2017). For example, the uptake rate of the ionized form of both Diclofenac and Fluoxetine were more than three orders of magnitude lower than the corresponding neutral forms of these molecule. A reduction in pH changed the proportion of ionized forms, with Diclofenac being in complete ionization at pH 8.40, dropping to 93.9% ionization at pH 5.20, varying the uptake to *Lumbriculus variegatus* by a factor of 168 in freshwater (Karlsson et al. 2017). Unlike neutral compounds, in ionisable compounds an ion trap can occur, where non-dissociated

molecules rapidly cross the cell membrane and become trapped inside the cell when they dissociate, which may lead to accumulation of compounds in cells (Rendal et al. 2011).

As a result of increasing awareness of the potential interactions between ocean acidification and the fate, behaviour and ultimately toxicity of environmental contaminants, there have been a number of studies over the last decade looking at this experimentally, for a number of marine invertebrates. A key question remains whether these interactions of compounds and OA can be predicted by the speciation changes of the contaminants within the OA pH range, and whether these are consistent across taxa or groups as this will enable some generic predictions to be made. To assess current knowledge and investigate any such patterns in response, a systematic review of existing knowledge of the effects of pH changes on toxicity of contaminants to marine invertebrates was carried out. A meta-analysis of the studies resulting from the review was conducted to determine any overall trends.

2.2. Methods

A systematic search (figure 2.1) was carried out during November 2019 on Web of Science with the following topics; ocean acidification (OA) and pollution, OA and metals, OA and contaminants, OA and pharmaceuticals, OA and copper, OA and pollutant, OA and chemical, OA and pesticide and OA and insecticide. The number of hits was then recorded and papers were included, after reading, if they met all criteria. The studies must have included research looking at the combined effects of OA and a contaminant on an invertebrate species. We limited the range of pH to a minimum of 7.1 and the pH of exposures must have been lowered with CO_2 gas, not hydrochloric acid to capture OA relevant changes driven by pCO_2 and pH, rather than pH alone. Papers were systematically reviewed and excluded either after reading the title, the abstract or the full paper. In total 44 studies were included.

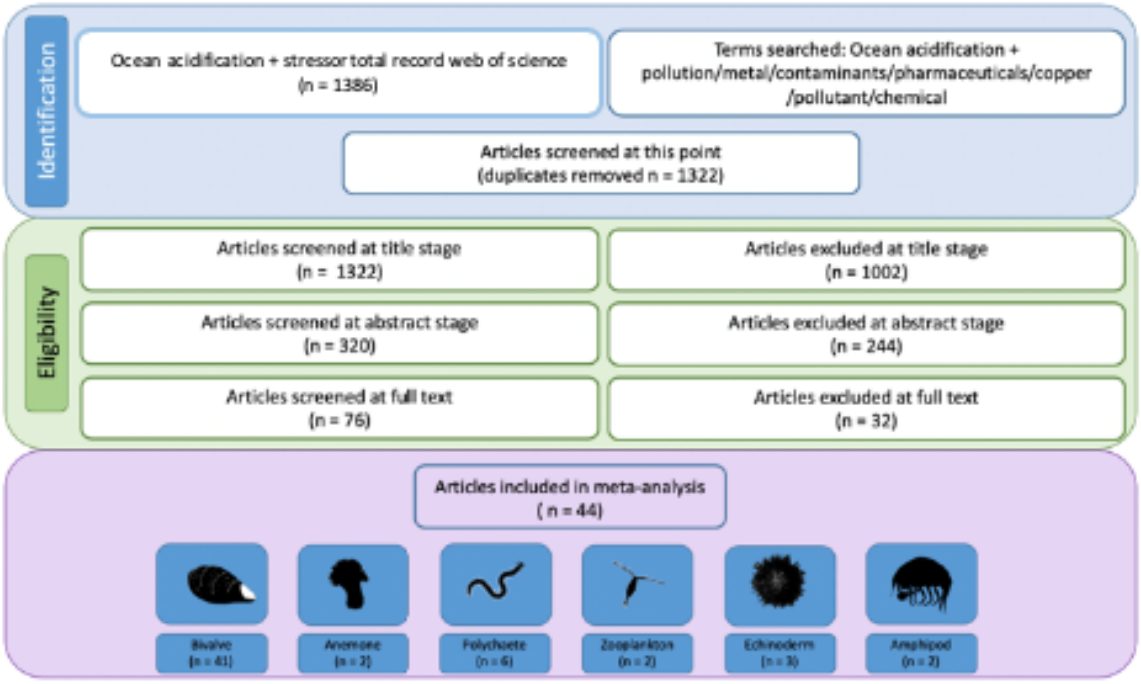


Figure 2.1. Overview of the systematic review methodology applied, including which articles were excluded and included at each stage of the search. The final 44 papers included were broken down into species groups (some papers used two different species/taxonomic groups which were counted separately).

From the included papers, contaminants were broken down into five groups of contaminants; metals, oil, pharmaceuticals, metal mixtures and PAH's. The effects on a total of six taxonomic groups of marine invertebrates of these contaminants, in conjunction with OA, was measured in 7 endpoints; mortality, metal accumulation, oxidative stress (broken down into superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) and lipid peroxidation (LPO)), growth rate, metabolic rate, DNA damage and immune response (measured as phagocytosis rate).

2.2.1 *Data analysis*

To enable the results of the different studies to be compared, the OA 'effect sizes' of the contaminant response were calculated using percentage increase or decrease in toxicity response as a result of the pH change from ambient conditions to future conditions. The control group (ambient pH 8.10 and contaminant) for each study was

compared to the OA and contaminant group for each end point measured and these numbers were used for the percentage increase/decrease calculation. Where raw data were not given or could not be attained via emailing authors, an online tool to extract numerical data from figures, Web Plot Digitizer, was used (Rohatgi 2012). Resulting effect size for each end point was plotted against the corresponding change in pH to normalise all data as different pH ranges were used in each study. Regression analysis was performed on the final effect size data to look at the relationship between pH effect size on each end point and change in pH units. This was carried out using SPSS.

2.3. Results

2.3.1. *Breakdown of studies*

The systematic approach yielded 44 papers that matched our search criteria. Of the studies conducted by these papers the large majority (73%, n = 41) conducted on bivalves (Figure 2.2). These studies include the species *Mytilus edulis*, *Mytilus galloprovincialis* and *Crossostrea gigas* as the most popular choice of test bivalves. Polychaetes represent the next most studied group with 11% of papers studying polychaetes, mainly *Hediste diversicolor*. The remaining studies were split relatively evenly between anemone, zooplankton, echinoderms and amphipods (each making up between 3 and 5% of studies). Some papers used two different groups of organisms or two different species in the same study, which were counted separately, hence, the number of groups included is greater than the number of papers included.

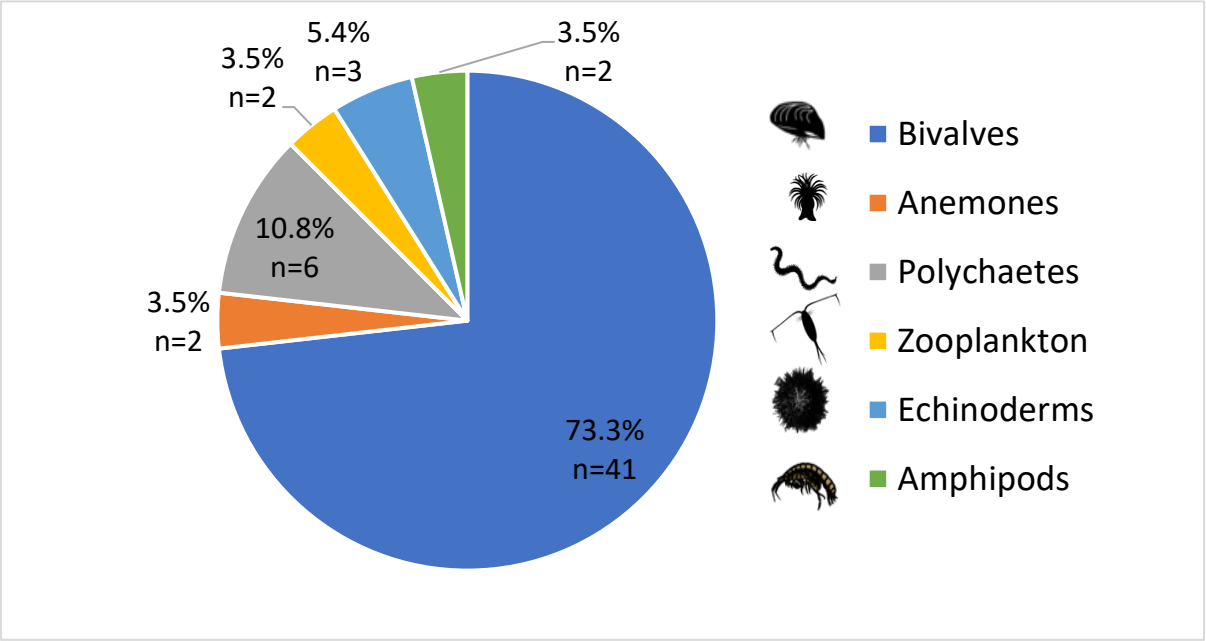


Figure 2.2. A breakdown of the six different taxonomic groups used in the included studies, with bivalves making up 73%.

In total the toxicity of 13 different contaminants has been studied in combination with OA, these comprise of; copper, cadmium, mercury, zinc, nickel, lead, nano-titanium dioxide, arsenic, carbamazepine, diclofenac, oil, benzo[a]pyrene and a metal mixture (in sediments). Of the contaminants examined in combination with ocean acidification to investigate combined impacts, the majority of studies have been conducted on metals (72%, Figure 2.3), with copper (44%) and cadmium (31%) being most regularly used in these studies. Pharmaceuticals, carbamazepine being the most common, and metal mixtures were the next most studied contaminant. A metal mixture represents contaminated sediments which contained a mixture of multiple metals for the exposure. Studies using oil and PAH's (benzo[a]pyrene) represented just 4% each.

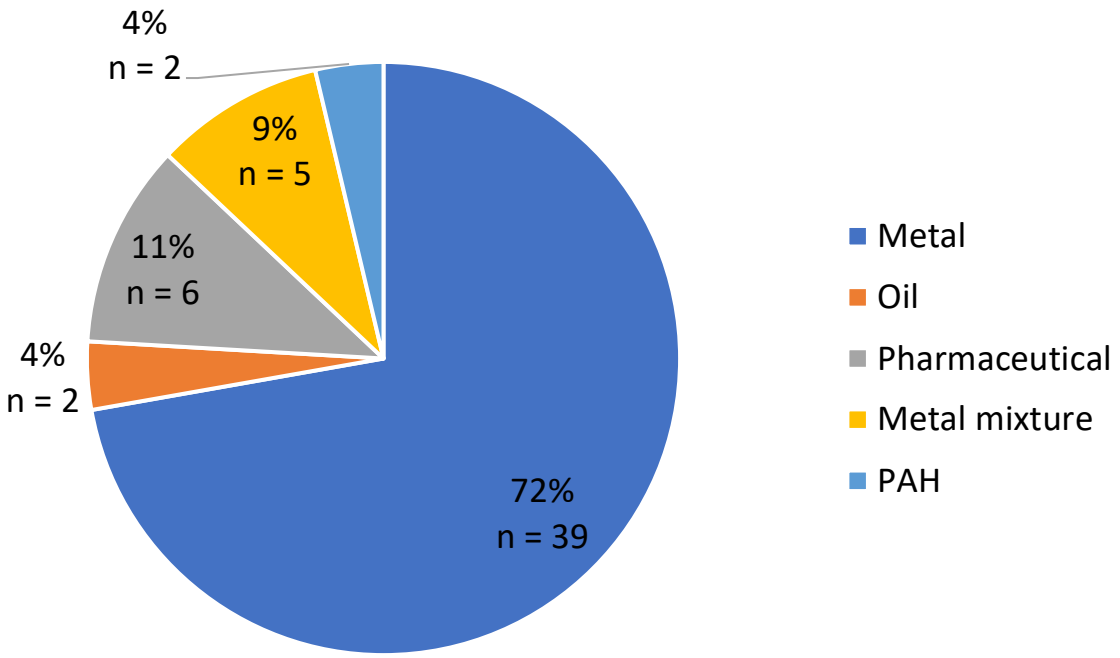


Figure 2.3. A breakdown of the contaminants used across the included studies with metals making up the majority.

2.3.2. *Biological end points*

The majority of studies have measured metal accumulation as an end point, with studies on bivalves making up most of these (Figure 2.4). Oxidative stress was the next most measured end point, however, it is worth noting that oxidative stress was broken down into four sub categories which were not all measured in each study. Bivalves were again the most studied organisms, making up 68% of studies. Mortality is regularly measured in studies and was measured across a wide range of taxonomic groups; bivalves (43%), polychaetes, zooplankton, echinoderms and amphipods. The remaining four end points went in the following order for amount of studies; DNA damage > metabolic rate > immune response > growth rate. All studies using immune response as an end point were using bivalves as a study species.

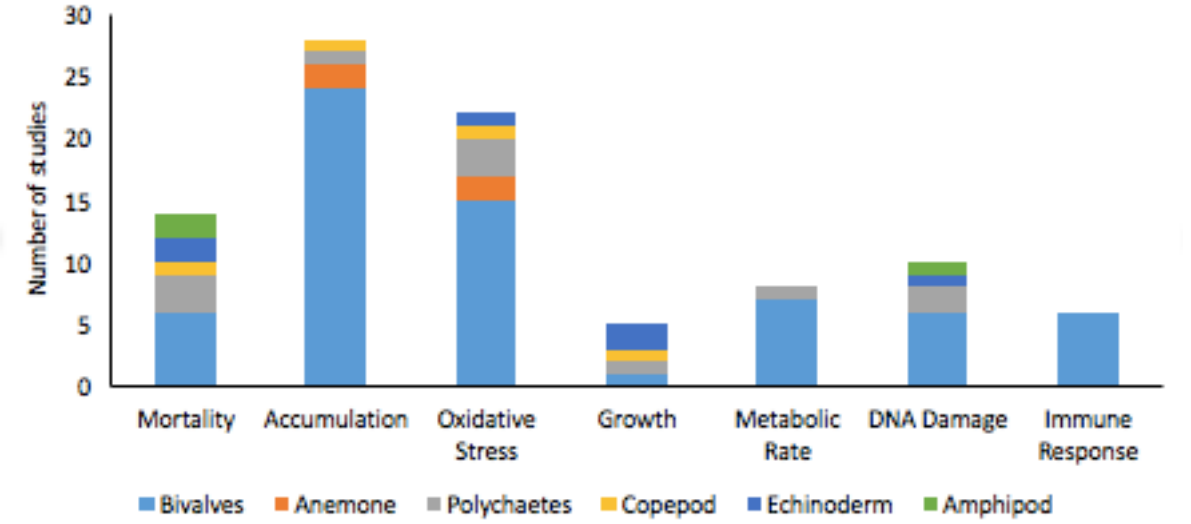
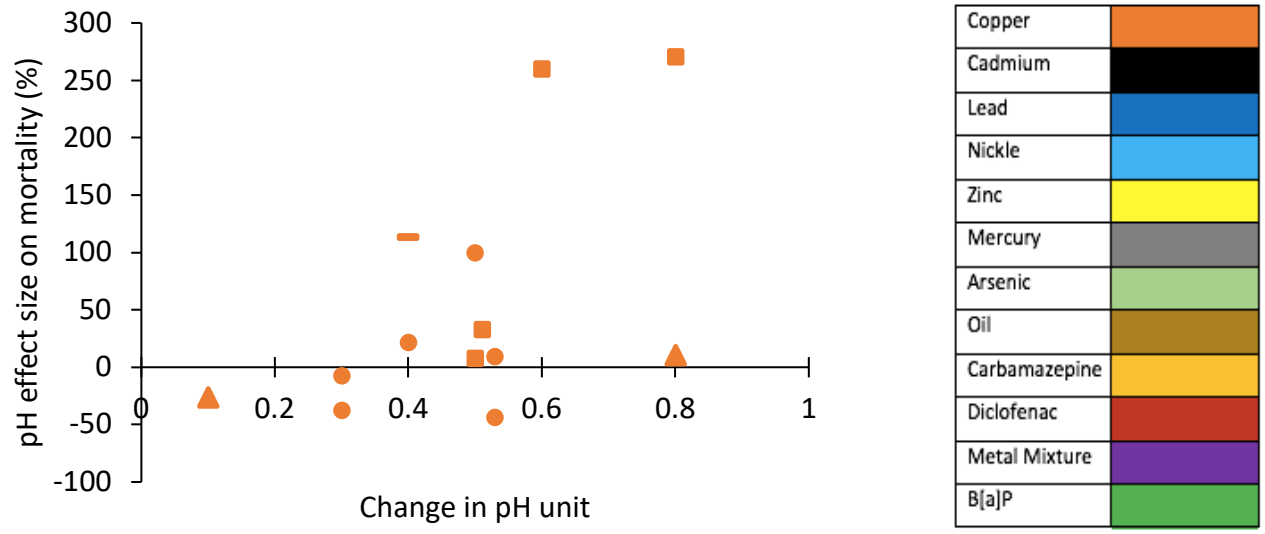


Figure 2.4. A breakdown of the seven end points; mortality, contaminant accumulation, oxidative stress, growth, metabolic rate, DNA damage and immune response, measured in each study and in which taxonomic groups these studies used.

2.3.3. *Mortality*

The pH effect size on mortality for copper toxicity responses range from -43.74% to 270.99% with 69% of the mortality responses increasing as the change in pH became greater (i.e. a larger pH decrease). Regression analysis revealed a slight increase but revealed no relationship between mortality effect size and pH change across studies (linear regression, $R^2 = 0.828$, $p = 0.062$). Mortality effect size and pH change with all other contaminants showed no trend and again regression analysis showed no significant trends (linear regression, $R^2 = 0.046$, $p = 0.362$).

A



B

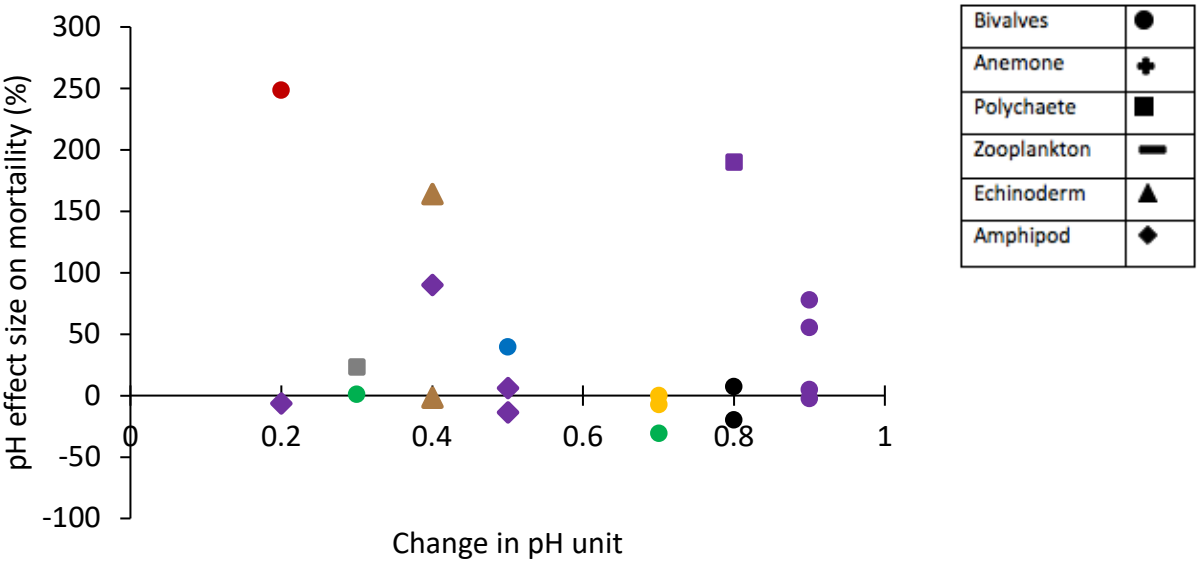
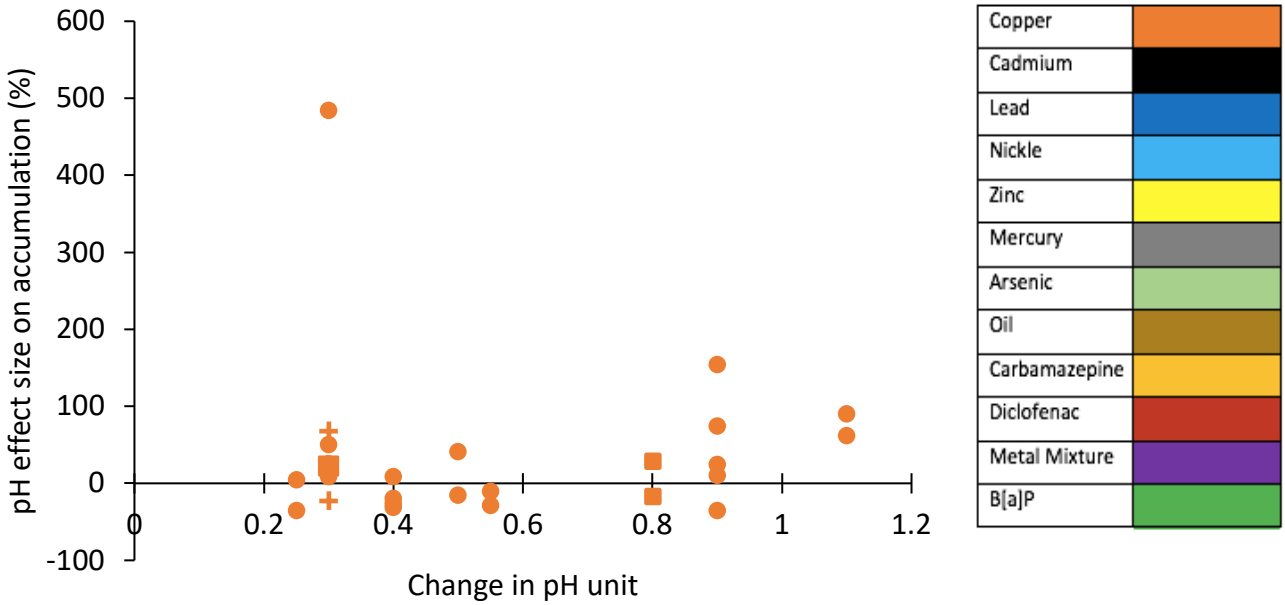


Figure 2.5. pH effect size on mortality in different taxonomic groups under different contaminant exposures. Mortality to copper exposure (A) is shown in the top graph, with mortality to other contaminants shown below (B).

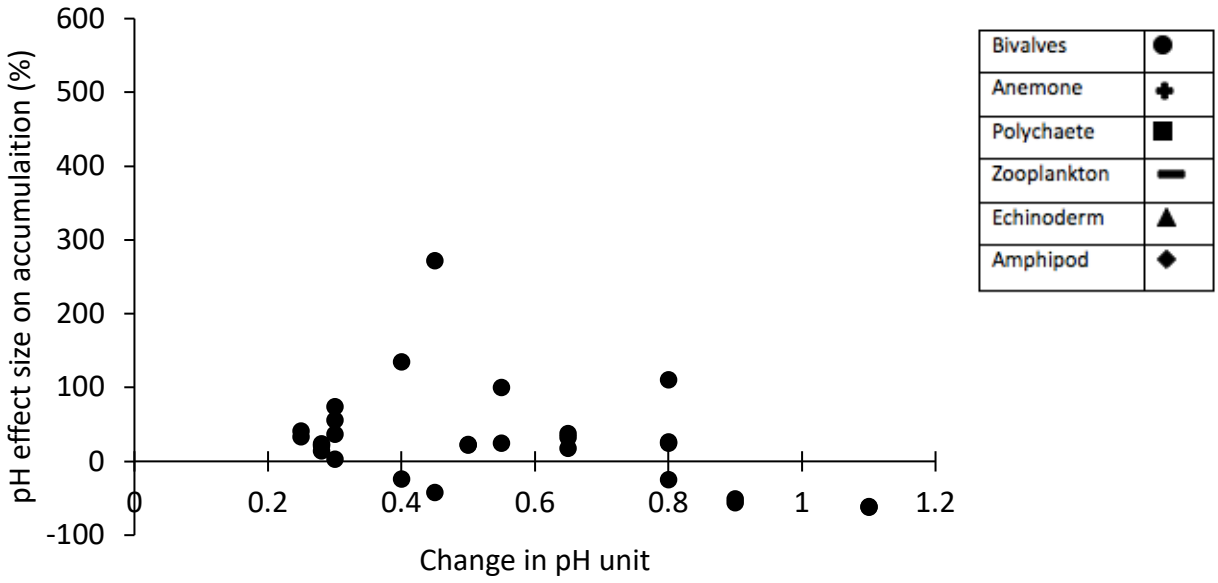
2.3.4. *Accumulation*

Contaminant accumulation was split into three sections; copper, cadmium and all other contaminants. For copper accumulation there was no trend as the change in pH became greater (linear regression, $R^2 = 0.000$, $p = 0.999$). This was the case for both cadmium (linear regression, $R^2 = 0.080$, $p = 0.190$) and all other contaminants (linear regression, $R^2 = 0.048$, $p = 0.295$) which also showed no significant correlation.

A



B



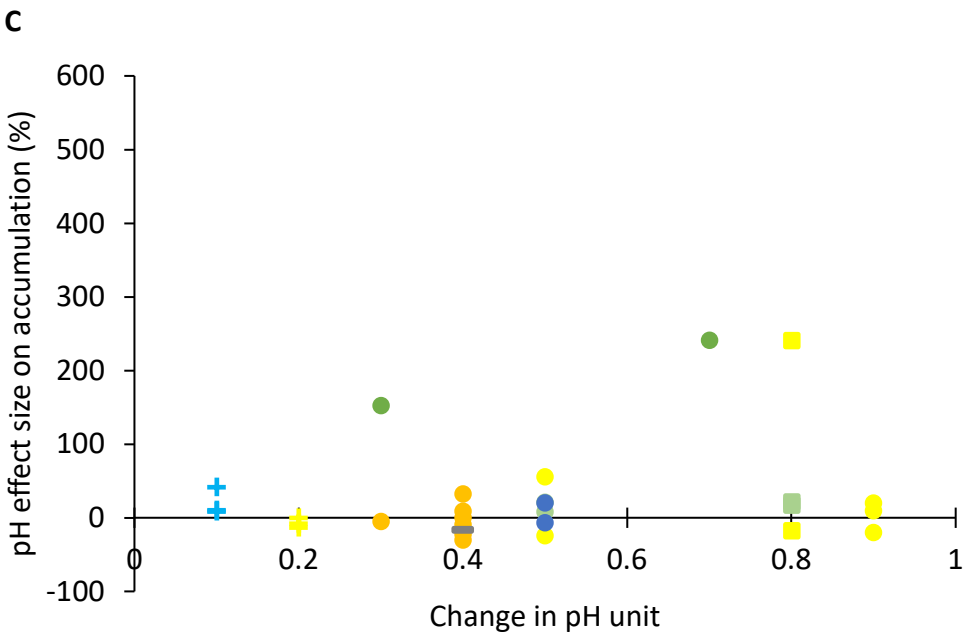
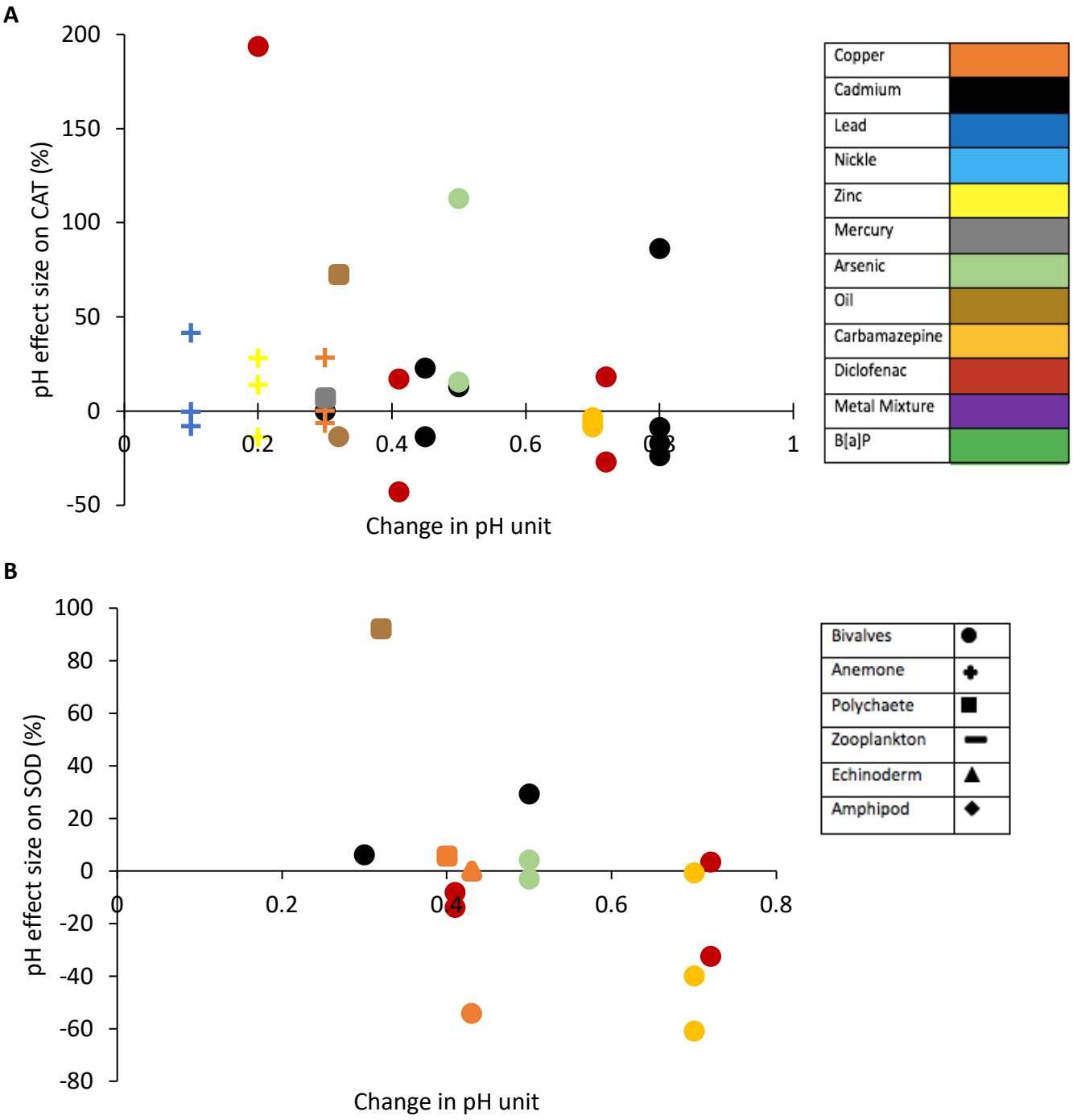


Figure 2.6. pH effect size on accumulation in different taxonomic groups under different contaminant exposures. Results were split into 3 graphs based on contaminants; copper accumulation (A), cadmium accumulation (B) and all other contaminants (C).

2.3.5. *Oxidative stress*



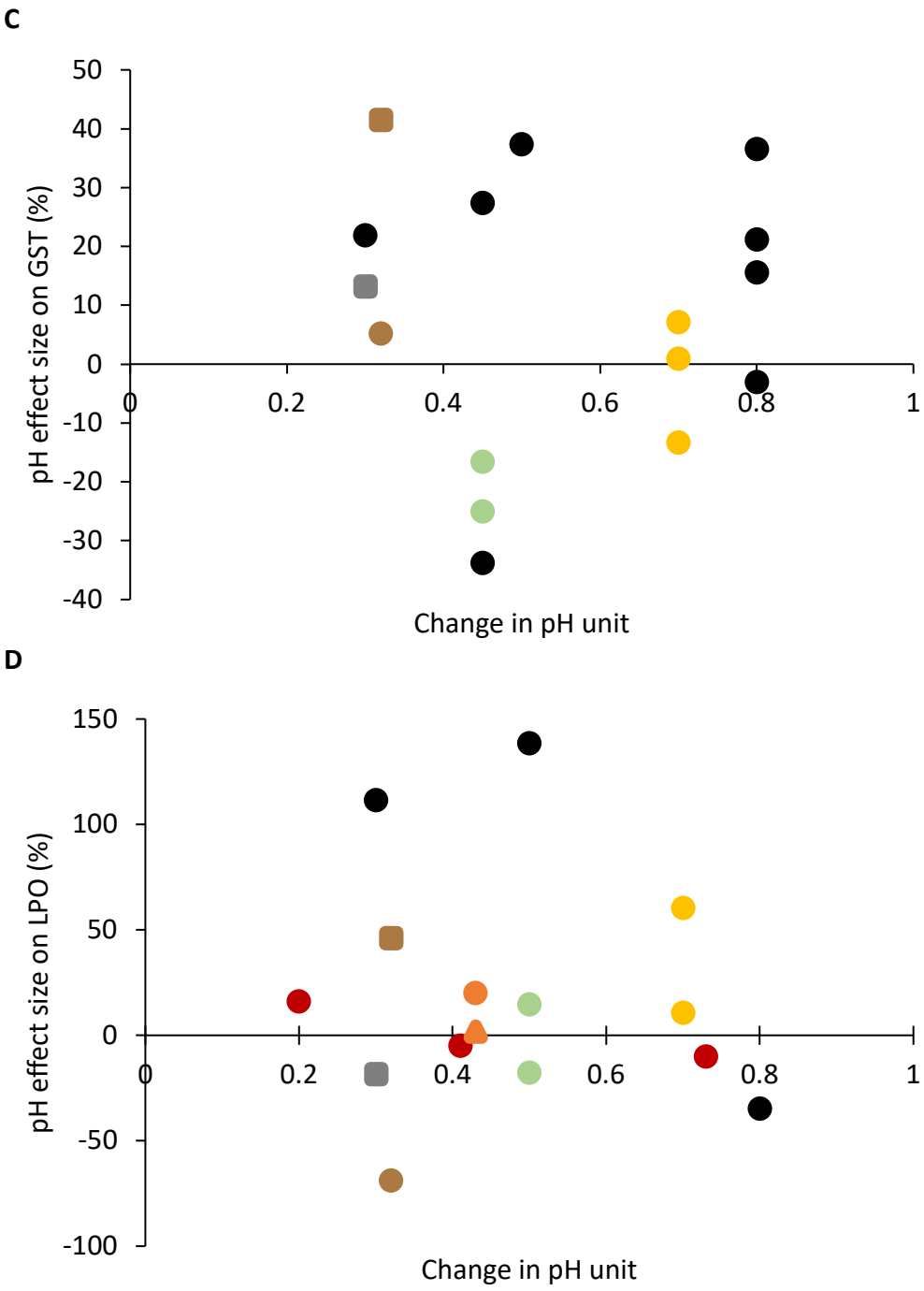


Figure 2.7. pH effect size on oxidative stress endpoints in different taxonomic groups under different contaminant exposures. Oxidative stress end points were spilt into four categories; catalase (A), superoxide dismutase (B), glutathione s-transferases (C) and lipid peroxidation (D).

All four oxidative stress measurements, SOD, CAT, GST and LPO, showed no trend with changes being both positive and negative over a wide range. The largest range

of a contaminant was seen in diclofenac where pH effects size on CAT increased by 193.6% at a 0.2-unit pH change (decrease) but a decrease of -42.9% in effect size was seen at a 0.4-unit change. Regression analysis revealed that there was no significant correlation in SOD (linear regression, $R^2 = 0.243$, $p = 0.062$), CAT (linear regression, $R^2 = 0.035$, $p = 0.322$), GST (linear regression, $R^2 = 0.000$, $p = 0.995$) or LPO (linear regression, $R^2 = 0.008$, $p = 0.745$), with GST having an extremely low R^2 value, indicating no relationship at all.

2.3.6. *DNA damage*

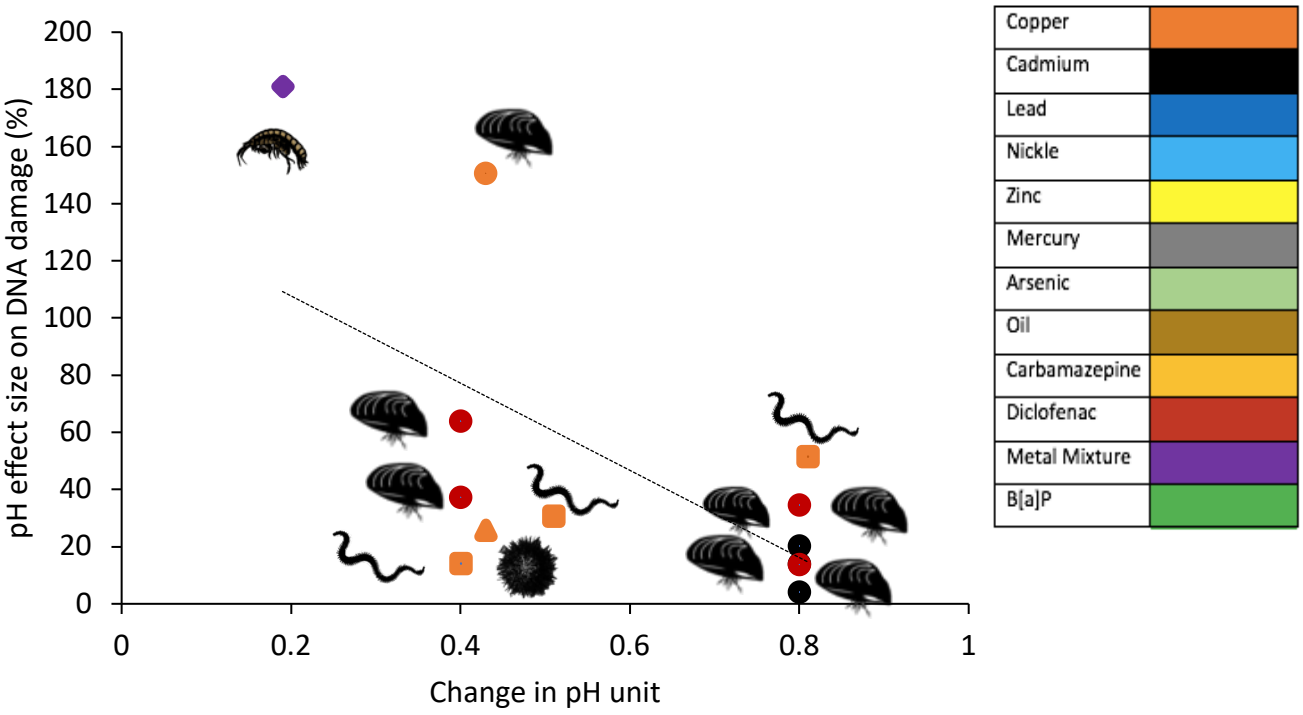


Figure 2.8. pH effect size on DNA damage in different taxonomic groups under different contaminant exposures. Trend line represents a significant correlation between change in DNA effect size and change in pH units.

The DNA damage effect size with contaminants showed a negative trend, indicating a decrease in DNA damage as the pH decrease became larger. This negative correlation was significant with regression analysis revealing a significant relationship between DNA damage effect size and pH change (linear regression, $R^2 = 0.371$, $p =$

0.036). across all studies (all taxonomic groups and all compounds). When broken down into taxonomic groups, polychaetes and bivalves showed opposing trends. Bivalves show a negative trend, similar to the overall trend of all taxonomic groups and regression analysis reveals a close to significant correlation between DNA effect size change and change in pH units (decrease in pH) (linear regression, $R^2 = 0.482$, $p = 0.056$). However, polychaetes have a positive trend, with DNA damage effect size increasing as the change in pH gets greater. Again, this is not significant (linear regression, $R^2 = 0.961$, $p = 0.127$).

2.3.7. *Growth*

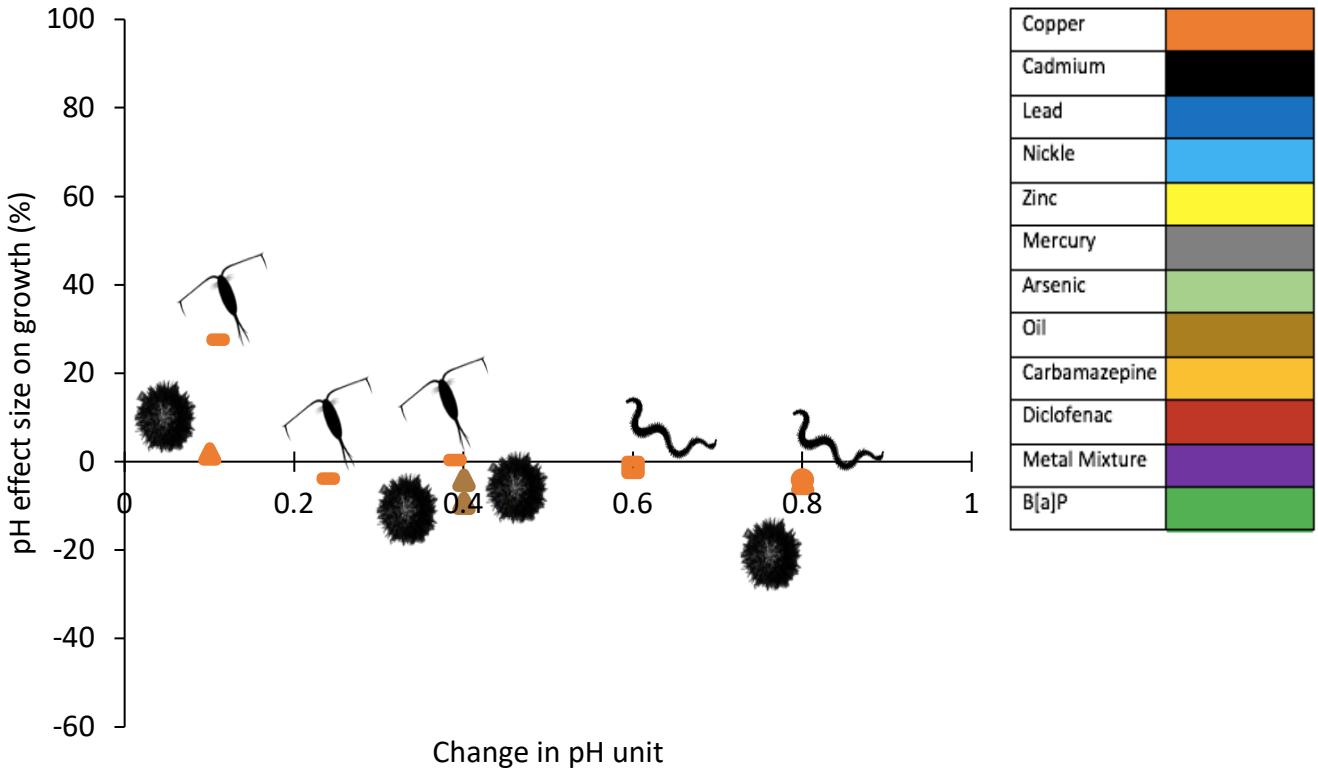


Figure 2.9. pH effect size on growth (body length) in different taxonomic groups under different contaminant exposures.

Changes in growth rate showed no trend, with all but one of the effect size changes being between -10% and 2%. Regression analysis showed no significant correlation

between changes in growth rate effect size and changes in pH (linear regression, R^2 = 0.260, p = 0.132).

2.3.8. *Immune response*

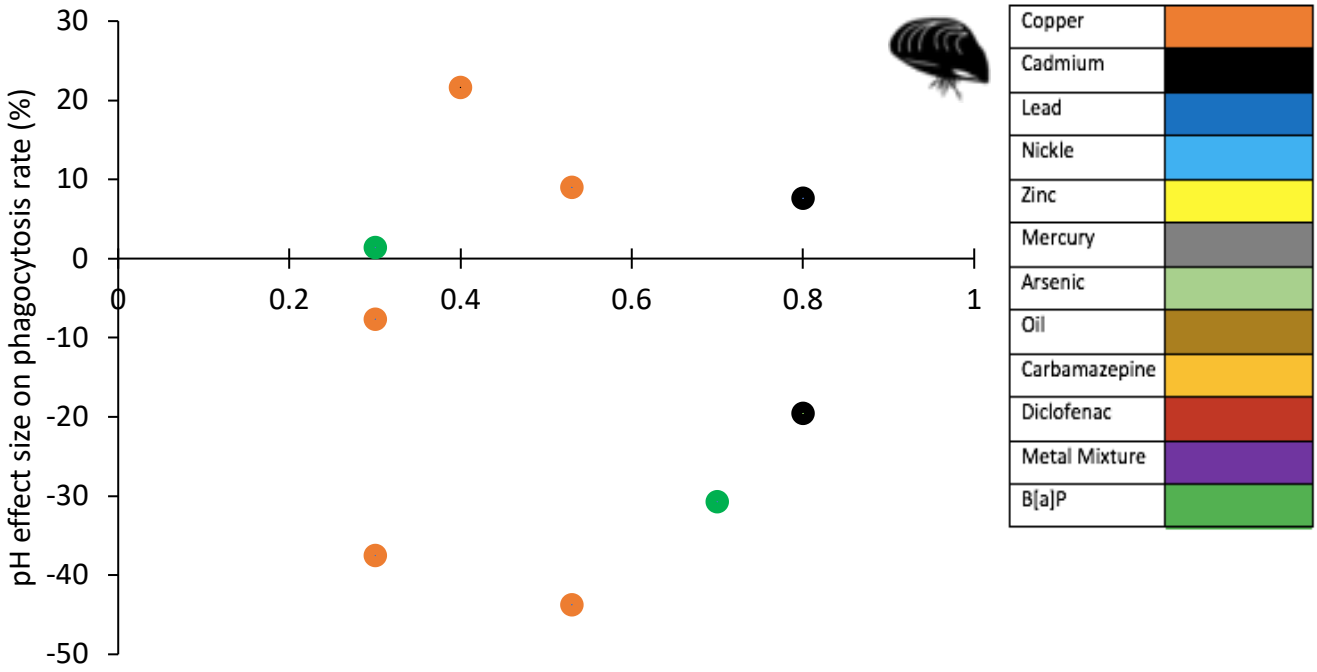


Figure 2.10. pH effect size on immune response (measured as phagocytosis rate) in different taxonomic groups under different contaminant exposures. All studies with this end point used bivalves as their species.

Changes in immune response effect size in bivalves showed no trend in response to changes in pH in the three different contaminants used (copper, cadmium and B[a]P). Regression analysis showed a very weak R^2 value, indicating no correlation which was also not significant (linear regression, R^2 = 0.004, p = 0.873).

2.3.9. *Metabolic rate*

Author	Class	Measured	Contaminant	ΔpH	Result
Freitas et al.	Polychaete	ETS (nmol-min/g FW)	Mercury	0.3	↑
Ivanina et al.	Bivalve	Total MO_2 (umol O_2 min^{-1} 10^{-6} cells	Copper	0.3	↑
Ivanina et al.	Bivalve	Total MO_2 (umol O_2 min^{-1} 10^{-6} cells	Copper	0.3	↓
Ivanina et al.	Bivalve	State 3 O_2 consumption rate (nmol O_2 $min^{-1}mg^{-1}$protein)	Copper	0.4	↓
Ivanina et al.	Bivalve	State 3 O_2 consumption rate (nmol O_2 $min^{-1}mg^{-1}$protein)	Cadmium	0.4	↑
Ivanina et al.	Bivalve	State 3 O_2 consumption rate (nmol O_2 $min^{-1}mg^{-1}$protein)	Copper	0.4	↑
Ivanina et al.	Bivalve	State 3 O_2 consumption rate (nmol O_2 $min^{-1}mg^{-1}$protein)	Cadmium	0.4	↑
Ivanina et al.	Bivalve	State 4 O_2 consumption rate (nmol O_2 $min^{-1}mg^{-1}$protein)	Copper	0.4	↓
Ivanina et al.	Bivalve	State 4 O_2 consumption rate	Cadmium	0.4	↓

		(nmol O_2 $min^{-1}mg^{-1}$protein)			
Ivanina et al.	Bivalve	State 4 O_2 consumption rate (nmol O_2 $min^{-1}mg^{-1}$protein)	Copper	0.4	
Ivanina et al.	Bivalve	State 4 O_2 consumption rate (nmol O_2 $min^{-1}mg^{-1}$protein)	Cadmium	0.4	
Ivanina et al.	Bivalve	Total MO_2 (umol O_2 min^{-1} 10^{-6} cells	Copper	0.5	
Ivanina et al.	Bivalve	Total MO_2 (umol O_2 min^{-1} 10^{-6} cells	Copper	0.5	
Hu et al.	Bivalve	Respiration (mg O_2 h^{-1} g^{-1})	Nano-titanium dioxide	0.8	

Table 2.1. pH effect size on metabolic rate in different taxonomic groups under different contaminant exposures. Red indicates a decreasing trend in metabolic rate, represented by a downwards arrow, blue indicates no change in metabolic rate and green indicates an increased trend in metabolic rate, represented by an upwards arrow.

The pH effect size on metabolic rate showed a mixed response. Only one studied used either mercury or nano-titanium dioxide with effect size increasing in polychaetes exposed to mercury but decreasing in bivalves using nano-titanium dioxide. The majority of studies exposed bivalves to either copper or cadmium as a contaminant, with copper causing an overall decreasing trend in effect size and cadmium showing mixed responses.

2.4. Discussion

This meta-analysis of studies looking at the effect of OA (a CO_2-induced lowering of seawater pH) on the contaminant response for a range of taxonomic groups has revealed to date there have been 44 studies looking at this potential interaction. The majority of studies have focused on metals and have used bivalves, in particular mussels, as a test organism. This is likely to be due to that fact that metals show clear speciation change within the pH range of OA (Millero et al. 2009) and that metals remain a prevalent contaminant in coastal and estuarine sediments (Bryan and Gibbs 1983a). Mussels are a key species used in monitoring and assessment of contaminants and are biological indicators of water quality, for example, the NOAA Mussel Watch Programme in the USA, monitors the nation's coastal waters for chemical contaminants, including more than 200 contaminants of emerging concern (Kimbrough et al. 2008).

The analysis of pH-effect sizes revealed no clear trends for all but one of the toxicity end points used across these studies. When looking at all contaminants together there was a significant negative trend for OA effect size on DNA damage, with DNA damage decreasing as the pH decreased. This is the opposite relationship of what might be expected and this trend may be an artefact of the limited data available. However, these data are heavily dominated by diclofenac and copper, which appear to be inducing differing responses within this data set. There was also clear taxonomic group specific response for this end point with polychaetes and bivalves showing opposite trends. Again, the limited number of studies limit the ability to interrogate these trends across contaminants of different speciation behaviours.

A theory from Rendall et al. (2011) found that ionisable organic compounds in freshwater are less toxic in their ionized state due to reduced lipophilicity. They also found that pH can affect the toxicity and bioaccumulation of acids and bases with acids becoming more toxic as pH decreases whereas the opposite was seen for bases, as they become more toxic with increasing pH (Rendal et al. 2011). Changes were most apparent for weak acids when the pH minus the pKa (acid dissociation constant) was between -1 and 3 (i.e. pKa's between 8.70 and 4.70 for OA scenarios) but it did also occur for acids with pKa's outside this range to a lesser extent on average. For weak

bases the effect of pH was greatest when the pH minus the pKa was between -3 and 1 and again changes in toxicity outside this range occur (Rendal et al. 2011). Diclofenac is a weak acid with a pKa of 4.15 therefore slight toxicity increases may be seen under OA conditions. Diclofenac was not readily used in studies looked at in this meta-analysis, however, when it was no significant trends were seen in either oxidative stress or DNA damage under pH decrease. However, this theory is harder to fit for other contaminants used. For example, carbamazepine has a wide range of pKa values making it hard to make any predictions. It is important to remember that Rendal's (2011) findings are only related to organic compounds and are focused on freshwater, making it less applicable to marine studies and exclude any inorganic compounds, such as metals which make up the majority of the contaminants used. The marine environment represents a complex environment with many factors affecting the toxicity changes under different pH's including organism's physiology and mechanistic effects. It is also possible that the pH range for OA studies may not be large enough to produce any effect in compounds with large pKa ranges.

Metals that are chloride speciation dominated, such as cadmium, are expected to see little, if any change in speciation as decreases in pH will not change the chloride concentration (Millero et al. 2009, Stockdale et al. 2016). However, the pH effect size on accumulation shows that 74% of data points show an increase under changes (decreases) in pH. Other end points measured show a range of changes, both positive and negative including immune response. Here, there was only two data points, both at a 0.80-unit pH change, showing an increase in effect size of 7.65% but also a decrease in effect size of -19.53%. Both these data points (from different studies) used the same species, *Mytilus galloprovincialis*, however, these two studies took place at different times of the year and as a result the mussels were exposed to different natural temperatures. The study resulting in a negative effect size took place in January, with an exposure temperature of 10^{o}C whereas the positive effect size had an exposure temperature of 20 ^{o}C, taking place in June.

A key factor that limits any ability to interrogate the relationship between changes in seawater pH and toxicity responses is the lack of any 'dose response curve' style approaches. The majority of studies used the same pH unit changes which affects the ability to undertake robust regression analysis, limiting the ability to look at

relationships with any power. This meta-analysis found that 68% of all the pH data points used covered just 4 values, with the majority of these being a 0.30, 0.40 or 0.50 pH unit change, representing near future conditions. The need for studies to use multiple pH levels is clear for obtaining a mechanistic understanding of what changes might be expected, especially as pH decrease will take place gradually.

The ability of some organisms to maintain internal pH via accumulation of bicarbonate levels may serve to protect against copper toxicity as free copper (II) ions form a wide range of complexes with bicarbonate ions (Stiff 1971), thus potentially reducing the proportion of toxic free copper ions (Lewis et al. 2016). In addition to this, organisms maintaining a stable pH may mitigate the OA effect and therefore no change in metal speciation may be seen. The sea urchin, *Paracentrotus lividus*, showed increased levels of bicarbonate in response to an OA-copper (0.1 μM) treatment, compared to mussels (*Mytilus edulis*) which may help explain the difference seen in DNA damage between these two species with the sea urchin showing lower levels of damage (Lewis et al. 2016). Other physiological interactions may also occur in a species-specific manner that all contribute to the overall toxicity response. Since many studies don't include acid-base physiological parameters in their measurements, it's difficult to assess this for many of the species included here. Much less is known about responses and toxicity to pharmaceuticals, however, this is an emerging area of concern.

This impact of pH on marine organisms' interaction with chemicals is not likely to be restricted to man-made pollutants, such as pharmaceuticals, pesticides and plasticizers, but may also influence any molecule dissolved in the sea that can be protonated, from marine drugs to biomolecules used for signalling (to locate food and partners or to deter predators) (Roggatz et al. 2019). There is increasing evidence that there may be a direct impact of pH on information-carrying signalling cues and receptor proteins, since many of these molecules also possess functional chemical groups that are sensitive to pH, including hydroxyl, carbonyl, carboxyl, amine, phosphate and sulfide groups (Roggatz et al. 2019). Therefore, disruption in marine chemical communication may be seen which could significantly impact the ecological network (Roggatz et al. 2019). For example, pheromones, signalling molecules and

even sperm:egg recognition proteins may all be influenced by changes in seawater pH, however, there is very little current understanding of this.

There is a clear need for more studies to be undertaken, particularly on non-metals as data is limited. Although patterns may have been seen in organic compounds in freshwater, Rendal's (2011) theory is unlikely to fit with contaminants in seawater meaning making predictions is hard. This meta-analysis has found no clear trends in changes to contamination toxicity under OA conditions with variable responses to different contaminants and in different species. This poses a major challenge for the field of ecotoxicology in understanding both the current and future pCO_2/pH interactions driving a species toxicity repose to contaminants in the coastal environment. Understanding the physiological and ecological mechanisms underpinning these species-specific physiological responses will help to fill in some of these knowledge gaps and improve predictions about changes likely to be seen under future ocean conditions.

Chapter Three

Ocean acidification buffers the physiological responses of the king ragworm *Alitta virens* to the common pollutant copper.

This chapter has been published in Aquatic Toxicology in May 2019 with co-authors Dr Ceri Lewis and Cameron Hird.

I set up and ran the experiment, sampled organisms, analysed the data and wrote the paper. Dr Ceri Lewis helped with editing and submitting this paper. Cameron Hird helped out on the sampling days.

3.1. Introduction

Ocean acidification (OA) is now widely regarded as one of the major threats to marine organisms globally (Doney et al. 2009, Dupont and Portner 2013, Gattuso et al. 2015). Increased atmospheric carbon dioxide concentrations have led to an average global ocean pH decrease of 0.10 units since the industrial revolution with a further decrease of 0.30 - 0.43 units predicted by the end of this century (Fabry et al. 2008, Bao et al. 2012). Coastal ecosystems are more complex and variable than open oceans, being governed by interactions between processes on land, in the open ocean, the atmosphere and the biological processes of the system itself (Aufdenkampe et al. 2011). Multi-decadal trends in coastal pH reveal fluctuations of about 0.50 pH units over tidal, daily and seasonal timescales, with OA already influencing this trend by an additional 0.10 unit decline (Duarte et al. 2013) and this variability is predicted to intensify as atmospheric CO_2 rises (Kwiatkowski and Orr 2018). There is now a huge body of evidence that support the paradigm that the levels of OA predicted for the end of the century will have negative impacts on a wide range of species and physiological processes (Kroeker et al. 2010, Dupont et al. 2010a, Wittmann and Portner 2013, Wang et al. 2018, Cao et al. 2018).

Whilst initial studies focused on the negative impact of OA on calcification (Orr et al. 2005, Riebesell et al. 2000), OA has also been found to affect a wide range of other physiological and behavioural processes, including an organisms' ability to acid-base regulate and an increased energetic demand of homeostasis (Lannig et al. 2010, Miles et al. 2007), hence OA can also negatively impact many non-calcifying species. Marine animals acutely subjected to seawater with elevated pCO_2 experience a corresponding extracellular acidosis (Portner 2008) and whilst many fish and crustaceans are able to regulate these acid-base perturbations by the elevation of extracellular bicarbonate ions (HCO_3^-) other invertebrates are less able to acid-base regulate and experience acidosis under OA conditions. Despite their ecological importance, only a few studies to date have looked at the physiological impacts of OA on non-calcifying, sediment-dwelling polychaetes, despite their ecological importance. Early studies on the king ragworm *Alitta (Nereis) virens* found no impact of OA on mortality or burrowing behaviour (Widdicombe and Needham 2007b), however subsequent studies over longer exposures revealed OA effects do manifest

over time, reducing growth, bioturbation and bioirrigation behaviour in *A. virens* that, in turn, affect nutrient generation (Godbold and Solan 2013). Physiological end points may be more sensitive to OA. In the harbour ragworm *Hediste diversicolor* increased oxidative stress, measured as lipid peroxidation and elevated antioxidant enzyme activity (SOD) was observed when exposed to reduced seawater pH_{NBS} of 7.50 compared to ambient pH conditions (8.10) (Freitas et al. 2017).

Increasing OA is not the only stressor that marine organisms are currently being exposed to, with multiple anthropogenic threats affecting marine environments globally. Of particular relevance for coastal species is the potential for OA to alter the bioavailability and toxicity of certain pollutants (Roberts et al. 2013). Metals, in particular copper, continue to be some of the most wide-spread environmental contaminants, found at elevated concentrations in the majority of estuarine and coastal environments (compared to open ocean) as a result of local mining (past or present), road run-off, effluent discharges or use in antifouling paints and nanoparticles, with evidence that concentrations in sediments are currently increasing (Watson et al. 2018). Copper can be found in coastal waters at concentrations ranging from low levels of 0.004 µM (Jones and Bolam 2007) to much higher levels of 1.61 µM (Bryan and Gibbs 1983b). A comprehensive review by Bryan and Langston (1992) reported copper levels for 19 estuaries around the U.K reporting sediment levels for copper of 7 mg kg^{-1} to 648 mg kg^{-1} of dry weight sediment with extreme values of 2389 mg kg^{-1} for Restronguet Creek, Cornwall. Pore water is the key exposure route for organisms living and feeding within sediment (Chapman et al. 2002). More recent work by Pini et al., (2015) measured pore water concentrations across 7 sites along the English Channel, reporting concentrations for copper of 0.68 µg L^{-1} to 1.85 µg L^{-1}. Sediment characteristics, sediment organic content and the complexation of copper with organic matter present in seawater are all known to also play a role in determining the bioavailability of copper and zinc to sediment dwelling polychaetes (Pini, Richir and Watson 2015, C.M.G. 2000).

The speciation of many metals in seawater is pH sensitive, hence the decrease in seawater pH and subsequent changes in hydroxide, carbonate and bicarbonate ion

concentrations due to OA will change the speciation of many of the metal ions commonly present in coastal seawater (Byrne 2002, Stockdale et al. 2016). Copper (II) ions form strong complexes with carbonate and a change in pH will lead to an increase in the more toxic copper free ions (Millero et al. 2009, Stockdale et al. 2016) such that an increase of 48 - 115% in free copper ions due to OA, coupled with the increase in sea surface temperature is predicted for the end of the century (Richards et al. 2011, Stockdale et al. 2016). Hence OA might be expected to alter the bioavailability of pH sensitive metals, such as copper, to marine biota and therefore potentially alter their toxicity responses. A number of studies have now investigated the potential for OA to alter the toxicity effects of copper in marine invertebrates, with many of these studies supporting the hypothesis that the toxicity of copper to biota is higher when exposed under OA conditions (e.g. (Lewis et al. 2016, Lewis et al. 2013a, Siddiqui and Bielmyer-Fraser 2015). However, these altered toxicity responses under OA conditions reported across studies vary in magnitude according to both species and life history stage, and are not consistent across the different endpoints measured, suggesting that the observed altered toxicity is not simply being driven by the change in metal speciation (Campbell et al. 2014, Gopalakrishnan et al. 2007, Lewis et al. 2016, Scanes et al. 2018). Since most contaminants, including copper, accumulate in sediments and concentrations of heavy metals in sediments usually exceed those of the overlying water by between 3 to 5 orders of magnitude (Bryan and Langston 1992) sediment dwelling organisms are often exposed to the highest levels of any contaminant. Therefore, it is important to look at the potential for OA-metal interactions in sediment dwelling organisms such as polychaetes, which often dwell in polluted sediments (Lewis and Galloway 2008).

The king ragworm, *Alitta* (formally *Nereis*) *virens,* is an ecologically and commercially important sediment dwelling polychaete (Watson et al. 2017) found in coastal waters and estuaries (Kristensen, Jensen and Andersen 1985). Here, using a suite of representative physiological (acid-base balance) and toxicological (DNA damage and oxidative stress) endpoints, we test the hypothesis that copper toxicity will increase under OA conditions relative to those experienced under ambient (i.e. current) seawater pCO_2 conditions in polychaetes, as has been observed in other taxa (e.g.

molluscs and echinoderms) by examining the responses of the polychaete *A. virens* to combined OA and copper exposures.

3.2. Methods

3.2.1. Animal collection and maintenance

Immature (assessed by colour and transparency) adult *Alitta virens* specimens (ranging from 0.3 – 1.2g) were collected from Starcross, Devon, England (50°37'36.5"N 3°26'47.2"W) during February 2016 by carefully digging them from the intertidal mud (mid-shore) with a fork. Starcross has been reported as having estuarine water copper levels in the range ~ 0.5 – 2.5 µg L^{-1} (i.e. ~0.009 - 0.04 µM (Langston et al. 2003). Worms were maintained in a holding tank in natural sediment from the collection site in a temperature controlled room at 15 °C for 48 hours before the experiment started. Sediment grain size for this site was mostly within the 125-250 µm range. The worms were then transferred into individual 2 L tanks, each with a small tube in for burrowing. Each tank contained well aerated artificial seawater and was kept at a salinity of 34 ppt. The salinity was monitored daily using a salinity probe (SevenGo Duo, pH/conductivity meter SG23). The tanks were kept covered for the duration of the exposure to simulate the darkness the animal would experience when burrowing.

3.2.2. Seawater manipulation

Artificial seawater (Tropic Marine©) was used to fill the individual tanks. Seawater pH_{NBS} values of 8.10 (with resulting pCO_2 of 453 µatm) and 7.70 (pCO_2 1305 µatm) were targeted, representing current and near future OA treatments respectively according to the IPCC WGI AR5 RCP 8.5 scenario (Stocker 2013, Meinshausen et al. 2011). Full seawater chemistry is provided in Table 1. Oxygen saturation states were measured every other day for each treatment and were maintained as fully saturated across all treatments by bubbling air into each individual tank. Seawater

pH_{NBS} in the OA conditions was maintained at 7.70 using an Aalborg Mass Flow Controller GFC set at the correct ratio of air to CO_2 and bubbled separately into each individual tank. Seawater pH (SevenGo Duo, pH/conductivity meter SG23), temperature and salinity (SevenGo Duo, pH/conductivity meter SG23) were measured daily in the holding tanks and the individual exposure tanks.

Water samples (36 for DIC, 14 for copper) were taken throughout the two-week experiment. Samples were taken every third day, to cover both just before and just after water changes (where re-dosing occurred) to best represent the exposure condition experienced by the worms over this period. Water samples for DIC analysis were preserved (0.04 % of final volume) with 4 % mercuric chloride for storage prior to analysis (Dickson 2007a) whilst samples for metals analysis were added to acid-washed 50 ml tubes and acidified using 50 µl of concentrated hydrochloric acid. Seawater DIC analysis was carried out using a bespoke system based on that described by Friederich et al. (2002) and using Dickson seawater `standards, as described in detail in Lewis et al. (2013a). Total alkalinity (TA) and pCO_2 were calculated from the measured values of pH_{NBS} and DIC using CO2sys, applying the constants from Mehrbach et al., (1973) and the KSO_4 dissociation constants from Dickson (1990b). The concentrations of copper in the seawater samples were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) using an Agilent 7900 Spectrometer utilising a collision cell with helium as the collision gas to minimise polyatomic interferences. Copper standards, matrix matched to the samples, at concentrations of 1, 10 and 100 mg/l (15.74, 157.37 and 1573.66 umol/l) respectively, were used for instrument calibrations. Analysis employed in-line addition of scandium as an internal standard and calibration of the ICP-MS was validated by the use of a quality control standard of 10 mg/l.

3.2.3. Experimental design

Ten worms per treatment (maintained in individual tanks) were transferred to each of the following four treatments; (1) ambient conditions (pH_{NBS} 8.10) with no added copper, (2) ambient conditions (pH_{NBS} 8.10) with 0.25 µM copper sulphate added, (3)

OA conditions (pH_{NBS} 7.70, the expected pH under RCP 8.5, IPCC AR5) with no added copper, (4) OA conditions (pH_{NBS} 7.70) with 0.25 µM copper sulphate added. Individuals were kept at 15 °C for 14 days in their own tank. A concentration on 0.25 µM of copper sulphate was used as initial experiments using lower concentrations of copper found no toxicity response in this species. Therefore, a concentration equivalent to a polluted site, such as Restronguet Creek (Bryan and Gibbs 1983b) was used to induce a sub-lethal response to enable relative responses across pCO_2 treatments to be compared. Partial water changes and re-dosing of exposure water with the treatment nominal copper concentration (pre equilibrated to the treatment pCO_2) were performed every three days to maintain the copper concentration.

Following the 14-day exposure each individual worm was analysed for all endpoints measured. Worms were removed from their tanks and samples of coelomic fluid were collected from each individual worm using an 18-gauge needle and 1 ml syringe carefully inserted into the coelomic cavity of the organism, working from the anterior region to the posterior region. Coelomic fluid was immediately analysed for acid-base physiology (below) and an aliquot taken for use in the Comet assay. Worms were then snap-frozen in liquid nitrogen for later use in the oxidative stress assays. Prior to use, frozen worms were defrosted then homogenised in PBS buffer using a hand-held homogeniser, centrifuged at 10,000 g for 20 minutes then the supernatant frozen until use at -20 °C.

3.2.4. Acid-base physiology

The pH_{NBS} of *A. virens* (10 worms per treatment) coelomic fluid samples (taken as above) was measured immediately after sampling at 15 °C in microcentrifuge tubes using a Metrohm 826 pH mobile pH electrode and meter calibrated using NBS buffers. From this fluid, 50 µl was stored in a micro capillary tube sealed with paraffin oil and Critoseal® sealant and were analysed for TCO_2 the same day as collection using a Mettler Toledo 965D Carbon Dioxide Analyser. Fifty microliters of a 10 mM $NaHCO_3$ standard was used between each sample for calibration purposes. Acid-base parameters were then calculated using a modified version of the Henderson-

Hasselbalch equation, as described in Lewis et al., 2016 paper (Lewis et al. 2016). This used previously calculated constants from Truchot, 1976, based on the crab, *Carcinus maenas* (Truchot 1976).

3.2.5. DNA damage

DNA damage was measured as single strand breaks using the comet assay. From the coelomic fluid collected as described above, 100 µl from each individual (10 worms per treatment) was used immediately for the comet assay. It was combined with 100 µl of phosphate buffer and centrifuged. The comet assay was then run according to the methods described by Lewis & Galloway (Lewis and Galloway 2008), under alkaline conditions at 5°C. Briefly, the supernatant was removed and 180 µl of low melting point agarose was added, this was then pipetted out onto a frosted slide, previously coated in high melting point agarose, and left to cool. The slides were placed in Lysis solution for 1 hour at 4^{o}C and then into the electrophoresis tank. Here the slides were covered with electrophoresis solution for 40 minutes before the current (25 V) was switched on for 30 minutes. Finally, the slides were rinsed in neutralising buffer before being stained with SYBRSafe (1 µL in 10 mL TBE buffer) and viewed using a fluorescence microscope (excitation: 502 nm, emission 530 nm). One hundred cells per replicate worm were quantified for DNA damage using COMET IV Software (Perceptive Instruments Ltd.), which measures the percentage of DNA present in the comet tail for each cell as the measure of DNA damage.

3.2.6. Oxidative stress endpoints

Superoxide dismutase (SOD) is an enzyme which is essential in the defence against oxidative damage (McCord et al., 1971). The SOD assay generates O_2- and uses nitroblue tetrazolium (NBT) which changes colour, from clear to purple, when it comes in contact with a free radical. SOD inhibits this colour change hence levels can be quantified by determining the level of inhibition of this colour change in a sample compared to a standard (Beaucham and Fridovic 1971) and normalised to protein content using standardised Bradford protocol (Bradford 1976) Initially 5 µl of homogenised samples or standards were added to 96-well plates along with 30 µl of

buffer A (2.28 g/500 ml Na_2CO_3 and 1.18 g/ 500 ml $NaHCO_3$) and 195 µl substrate solution B (0.1 mM xanthine, 0.1 mM EDTA, 0.05 mg BSA and 0.025 mM NBT). Free radicals were created using xanthine oxidase (4.95 units/ml in a 1:80 dilution in buffer A), where 10 µl was added to the microplate immediately prior to reading at a wavelength of 573 nm.

Lipid peroxidation was determined using the thiobarbituric acid reactive substances (TBARS) assay (Camejo 1998) which quantifies malondialdehyde, a secondary product of lipid peroxidation, via its reaction with thiobarbituric acid (Lewis et al. 2016). In microcentrifuge tubes, 100 µl of homogenised samples or standards (see below) were added along with 300 µl of PBS + EDTA (372.24 mg EDTA in 1L PBS), 150 µl thiobarbituric acid (1.95g in 150ml NaOH), 100 µl trichloroacetic acid (50 g/100 ml DI water) and 20 µl butylated hydroxytoluene (22 mg/100 ml ethanol). All microcentrifuge tubes were vortexed and incubated at 60^{o}C for 60 minutes, after 60 minutes microcentrifuge tubes were centrifuged for 7 minutes at 13000 rmp. In a 96-well plate either 200 µl of standards or 100 µl of sample + 100 µl PBS + EDTA were added in triplicate to the corresponding wells and the plate was read using a microplate reader at a wavelength of 530 nm. Results were compared to a standard curve prepared using 1,1,3,3-tetraethoxypropane (a stabilized form of MDA) and normalised to protein content using standardised Bradford protocol (Bradford 1976).

3.2.7. Statistical analysis

All data was analysed for normality using the Shapiro-Wilk test. DNA damage (%) was first normalised using the arcsine transformation. Normal data (DNA damage, coelomic pCO_2, bicarbonate and pH) was analysed using a 2-way analysis of variance (ANOVA) general linear model with ‘pH’, ‘copper’ and ‘pH x copper’ as fixed factors. Non-normal data (TBARS and SOD) was analysed using Scheirer-Ray-Hare, an extension to a Kruskall-Wallace test as a non-parametric method. Tukey’s post-hoc test was carried out on all data. All statistical analysis was performed using SPSS software.

3.3. Results

3.3.1. Seawater chemistry

The carbonate chemistry of the seawater from the four treatments together with total copper levels for each of the exposures are summarised in Table 3.1. Whilst there was loss of copper to the experimental system during the exposures, possibly due to being bound to the plastic tanks, a one-way t-test confirmed that there was no significant difference in measured seawater copper concentrations for the ambient (pH_{NBS} 8.10) copper and OA (pH_{NBS} 7.70) copper treatments (independent-sample t test; t=-0.601, p=0.951). The two treatments with added copper had significantly higher copper concentrations than those with no added copper (independent-sample t test; for pH 8.1 t=1.389, p=0.004; for pH 7.7 t=1.814, p=0.039). The seawater for all treatments was fully saturated with oxygen throughout the 14 days (monitored daily).

3.3.2. Acid-base physiology

Coelomic fluid pCO_2 in *Alitta virens* under ambient seawater pH_{NBS} of 8.10 was 2.03 ± 0.34 mmHg. Exposure to OA conditions, pH_{NBS} 7.70, for 14 days did not cause any increase in the worms' coelomic fluid pCO_2, with values of 2.03 ± 0.21 mmHg measured in worms exposed to this treatment (two-way ANOVA for pH, F = 1.757, P = 0.193). The presence of copper induced a significant decrease in pCO_2 in both the ambient and OA treatments (two-way ANOVA for copper F = 6.694, P = 0.014, Figure 3.1.A). Under ambient conditions, pH_{NBS} 8.10, pCO_2 levels fell to 1.15 ± 0.078 mmHg, a 43% decrease. A smaller decrease of 14% was seen in OA conditions (pH_{NBS} 7.7) where coelomic fluid pCO_2 decreased to 1.75 ± 0.181 mmHg. There was no significant interaction factor (two-way ANOVA for pH*copper F = 1.847, P = 0.183; Figure 3.1.A).

Coelomic fluid bicarbonate levels showed a similar pattern to the pCO_2 levels (Figure 3.1.B). Under ambient seawater pH/pCO_2 conditions bicarbonate levels were 5.74 ± 1.07 mmol l^{-1}. OA, pH_{NBS} 7.70, caused a significant increase in coelomic fluid bicarbonate levels to 6.77 ± 0.79 mmol l^{-1} (two-way ANOVA for pH F = 5.681, P =

0.023). The addition of copper resulted in a decrease of bicarbonate levels in both seawater treatments. At an ambient pH_{NBS} of 8.10, bicarbonate levels decreased to 3.18 ± 0.23 mmol l^{-1}. A decrease was also seen at pH_{NBS} of 7.70, although it was not as large. Here, bicarbonate levels fell to 5.49 ± 0.36 mmol l^{-1}. The results show that there was a significant effect from the addition of copper (two-way ANOVA for copper F = 7.509, P = 0.009). There was no significant interaction term between OA and copper (two-way ANOVA for pH*copper F = 0.882, P = 0.371, Figure 3.1.B).

There was no significant effect of exposure to OA (two-way ANOVA for OA F = 3.159, P = 0.084) on *A. virens* coelomic fluid pH_{NBS}, which was measured as 7.78 ± 0.05 and 7.84 ± 0.23 under ambient seawater pCO_2/pH and OA conditions respectively (Figure 3.1.C). Exposure to copper also had no effect on coelomic fluid pH (two-way ANOVA for copper F= 0.003, P = 0.957), and there was no interaction between OA and copper (two-way ANOVA for pH*copper F = 0.001, P = 0.979). These acid-base physiology data can be visualised together as a Davenport Diagram, as in Figure 3.2 (Davenport 1974). Plotting the data in this way highlights the difference in acid-base status of *A. virens* in the ambient pCO_2 plus copper treatment compared to the other three treatments.

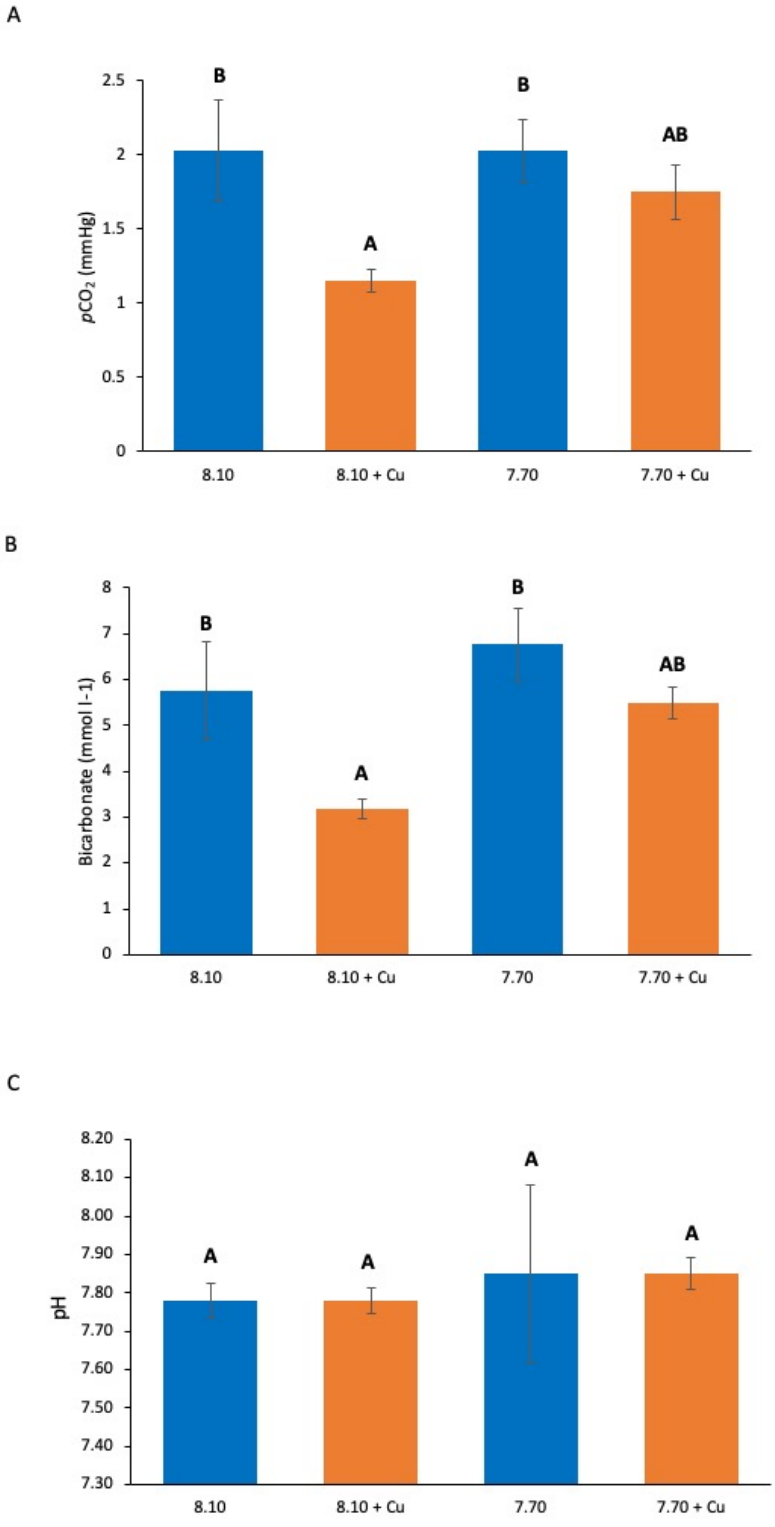


Figure 3.1. Acid-base parameters in the coelomic fluid of *Alitta virens* following a 14 day exposure with or without 0.25µM copper. Mean (±SE). (A) Coelomic fluid pCO_2 (n=40), (B) Coelomic fluid bicarbonate concentrations (n=40), (C) Coelomic fluid pH_{NBS} (n=40). Differing letters denote significant differences between treatments (Tukey's post-hoc: $p<0.05$).

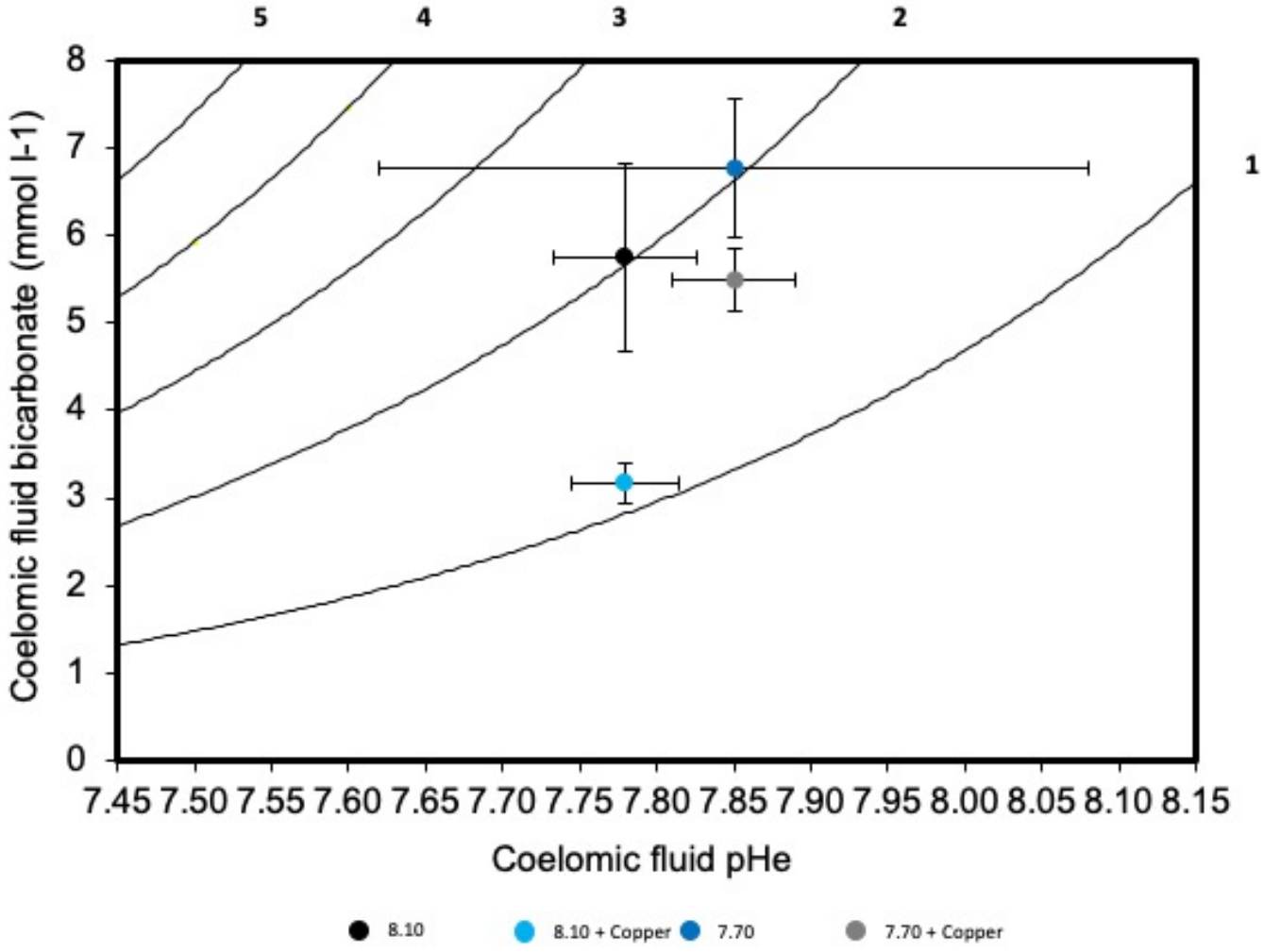


Figure 3.2. Davenport diagram presenting the relationship between pH, pCO_2 and bicarbonate ion concentrations in the coelomic fluid of *Alitta virens* at 15°C & 35ppt, following a 14 day exposure, under the four different treatment conditions, demonstrating the alkalisation effect of copper exposure under ambient conditions. Lines represent isopleths of equal pCO_2 (mmHg).

3.3.3. DNA damage

Exposure to 0.25 µM copper under both seawater pH/pCO_2 treatments led to a significant increase in DNA damage in *A. virens* coelomocytes (Figure 3.3 A; two-way ANOVA for copper, F = 31.106, P < 0.001), with % DNA damage increasing from 18.7 ± 1.49 % (ambient pH/pCO_2 no copper) to 29.8 ± 1.9% (ambient pH/pCO_2 with copper) and 34.0 ± 3.2% (OA with copper) respectively. Worms in the OA treatment had

similar levels of DNA damage to the controls of 17.7 ± 2.79 %., with no significant effect of pH on DNA damage measured (two-way ANOVA for pH, F = 0.519, P = 0.476). However, there was no significant difference in DNA damage between the two copper treatments and no significant interaction term between OA and copper (two-way ANOVA for pH*copper, F = 1.187, P = 0.283, Figure 3.3 A).

3.3.4. Oxidative stress

Activity of the anti-oxidant enzyme SOD significantly increased under exposure to OA conditions, from 0.137 ± 0.03 units per mg of protein under ambient pH/pCO_2 to 0.361 ± 0.06 units per mg of protein under OA (Figure 3.2 B, Scheirer-Ray-Hare for pH, H = 1.617, P = 0.008). There was no significant effect of the addition of 0.25 µM of copper under either seawater pH/pCO_2 treatments (Scheirer-Ray-Hare for copper, H= 0.382, P = 0.171), with SOD activity of 0.176 ± 0.06 units per mg of protein (ambient pH/pCO_2 with copper) and 0.186 ± 0.03 units per mg of protein (OA with copper) respectively, and no significant interaction term between pH and copper on SOD activity (Scheirer-Ray-Hare for pH*copper, H = 0.629, P = 0.082, Figure 3.2 C).

There were no significant effects of either copper or OA on the levels of lipid peroxidation, measured as the amount of malondialdehyde, in *A. virens* (Figure 3.2 C; Scheirer-Ray-Hare for pH, H = 0.191, P = 0.660; for copper, H = 0.003, P = 0.960; for pH*copper, H = 0.704, P = 0.400). A slight increase in lipid peroxidation from 5.03 ± 1.57 to 6.12 ± 2.37 nM per mg of protein was observed with the addition of copper for the ambient pH/pCO_2 scenarios. This slight trend was reversed under OA conditions, however, where the levels of lipid peroxidation decreased slightly when copper was added (but not significantly) from 6.12 ± 2.37 nM per mg of protein (OA no copper) to 4.01 ± 0.78 nM per mg of protein (OA with copper).

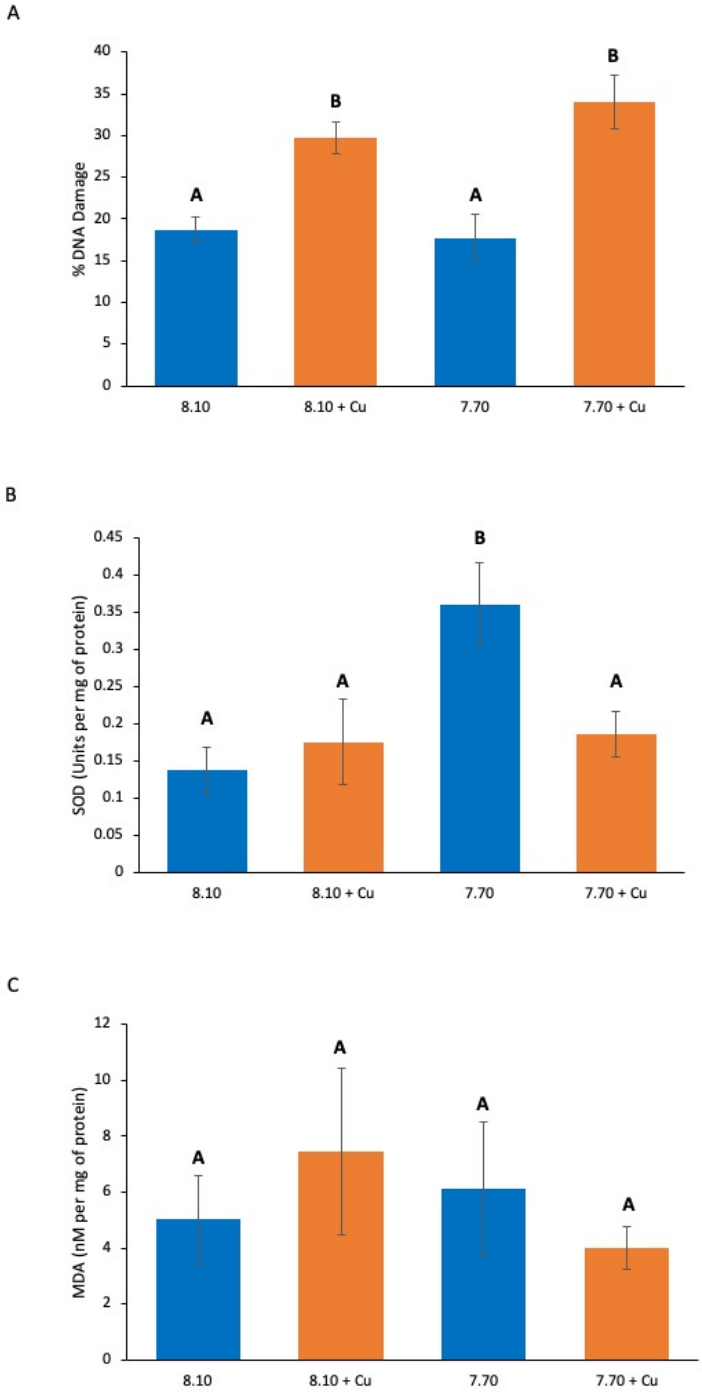


Figure 3.3. Oxidative stress indicators in *Alitta virens* following a 14-day exposure with or without 0.25µM copper. Mean (±SE). (A) The percentage DNA damage measured as single strand breaks (n=40), (B) The activity of superoxide dismutase (SOD) in units per mg of protein (n=40), (C) The measure of lipid peroxidation using TBARS activity (n=40). Differing letters denote significant differences between treatments (Tukey's post-hoc: $p<0.05$).

3.4. Discussion

In the ecologically and commercially important polychaete, the king ragworm *Alitta virens,* ocean acidification (OA) conditions do not cause an increase in the toxicity effects induced by exposure to copper relative to those experienced under ambient (present day) pCO_2/pH seawater conditions. This is the opposite effect to that observed for a number of other coastal invertebrate species, where a relative increase in copper toxicity under OA has been reported (e.g. (Campbell et al. 2014, Freitas et al. 2016a, Roberts et al. 2013, Siddiqui and Bielmyer-Fraser 2015). Here, *A. virens* was surprisingly robust to both short-term OA exposure and relatively high copper contamination. No significant increase in the internal pCO_2 of the worms was observed in response to the elevated pCO_2 levels in their surrounding seawater under the OA exposure. This aligns with previous studies using CO_2 vents as proxies for OA that reveal non-calcifying polychaetes (e.g. *Syllis prolifera* often do well under the lower pH conditions nearest the vents (Cigliano et al. 2010). Whilst exposure to nominal 0.25 µM copper (representative of a highly contaminated site) induced elevated levels of DNA damage in the worm's coelomocytes, this was not significantly altered when experienced under OA conditions. In fact, the opposite effect to that hypothesised was observed, with OA appearing to buffer the impacts of copper toxicity on *A. virens*, with a significant reduction in the levels of copper-induced lipid peroxidation and acid-base disturbance measured in the adult worms when experienced under OA conditions.

The addition of 0.25 µM copper under ambient seawater pCO_2/pH conditions induced a 1.6-fold increase in DNA damage in the coelomocytes of *A. virens*. Genotoxic responses of *A. virens* to copper have been previously observed in long-term sediment exposures, where 9 month exposures (to nominal copper concentrations of 70 - 575 mg kg^{-1}) resulted in significant increases in DNA damage accumulating (Watson et al. 2018). Despite high levels of DNA-damage accumulating worms maintained positive growth over this period, although some mortality was observed (Watson et al. 2018). Thriving populations of *A. virens* have been found living in polluted sediments with coelomocyte DNA damage levels averaging ~35% (Lewis and Galloway 2008) and the response of *A. virens* to known genotoxins has been demonstrated to be about 50% lower than for two other polychaete species tested. Whilst copper exposure often

leads to increased lipid peroxidation (e.g. (Brown et al. 2004, Maria and Bebianno 2011) and changes in activity of the anti-oxidant enzyme SOD (Xu et al. 2018), here we found no significant changes in lipid peroxidation or SOD activity in *A. virens* in response to copper under ambient seawater pH/pCO_2 conditions. This again is in contrast to the responses previously reported for *M. edulis* and *P. lividus* (Lewis et al. 2016), further supporting the idea that this *A. virens* is relatively robust to copper contamination. Our data aligns with the suggestion that *A. virens* can regulate copper uptake, leading to low tissue concentrations even when exposed to high bioavailable concentrations (Pini et al. 2015, Watson et al. 2018).

We found no significant acid-base disturbance in *A. virens* during exposure to OA conditions. The influence of OA on the acid-base physiology of marine fauna is well documented (Portner et al., 2004, Widdicombe and Spicer 2008) whereby animals subjected to seawater with elevated pCO_2 experience a corresponding increase in internal pCO_2 in the blood or haemolymph driving an extracellular acidosis. Whilst most fish and crustaceans are able to regulate these acid-base perturbations by elevating their extracellular bicarbonate ions (HCO_3^-) (Spicer et al. 2007) many other invertebrates, such as mussels and some urchin species, are less able to acid-base regulate (Collard et al. 2013, Gazeau et al. 2013) and experience acidosis under elevated seawater pCO_2. This lack of increase in coelomic fluid pCO_2 under OA conditions is counter-intuitive, given that *A. virens* are a simple, soft bodied organism with gas exchange occurring, via diffusion, across the general integument (Gomme 1984). We used artificial burrow (gas permeable plastic tubes) in our experiments as habitat for the worms which may influence the pCO_2 conditions immediately surrounding the worms. Polychaetes are known to irrigate their burrows via active or passive irrigation which may alter under OA conditions and also play a role in the pCO_2 levels that build-up within the tubes. Hence a combination of microhabitat conditions and behavioural responses may act to buffer the worms from the changes in the seawater pCO_2 but this requires further work to elucidate.

The addition of 0.25 µM copper, however, did significantly alter the acid-base balance of the worms, causing a significant decrease in their coelomic fluid pCO_2 and bicarbonate levels. No corresponding change to the coelomic fluid pH was observed

suggesting that the reduction in coelomic fluid bicarbonate levels balanced out the reduced pCO_2 effect, preventing alkalosis. A blood alkalosis in response to copper exposure has been previously reported in the sea urchin *P. lividus* (Lewis et al. 2016) and in Rainbow Trout *(Oncorhynchus mykiss)* (Wang et al. 1998) whilst studies in crabs have reported an acidosis of the haemolymph in response to copper exposure (Weeks, Jensen and Depledge 1993, Boitel and Truchot 1989). The mechanisms underpinning these differing responses have not been elucidated. Rather than OA increasing the relative toxicity of copper to *A. virens* as was hypothesised, OA conditions have altered the physiological and toxicological response of the worms to copper, reducing the copper toxicity effects on lipid peroxidation and acid-base disturbance. This is evident looking at the Davenport diagram (Figure 3.2), where the copper only treatment is quite distinct from the other three treatments, whilst the 'copper and OA' worms have over-lapping acid-base status to the worms were no copper was added exposed to elevated copper. A protective effect of hypercapnia on copper toxicity has also been suggested by the work of Larsen et al., (1997) in the cod *Gadus morhua*. This is contrary to what would be predicted if toxicity were driven by the availability of the copper (II) ion (Cu^{2+}) ion alone, since metal speciation models predict an increase in the more toxic free copper (II) ion with a decrease in seawater pH (Millero et al. 2009).

Other studies looking at interactions between OA and the toxicity of pH-sensitive metals report a range of contrasting responses, from strong increased toxicity under OA (Lewis et al. 2016, Roberts et al. 2013, Campbell et al. 2014, Freitas et al. 2016a, Scanes et al. 2018) to no change in toxicity under OA (Dorey et al., 2018, Lewis et al. 2013a, Scanes et al. 2018) and now reduced toxicity under OA. This raises the question as to what biological mechanisms underpin these different responses. Our data further supports the developing paradigm that the acid-base physiology of a species/ life history stage plays an important role in determining any OA-copper interaction. In *A. virens* here there was no OA effect on the internal pCO_2 or pH of the worms, therefore speciation of copper within the worms would theoretically be the same under the OA and the ambient pH/pCO_2 treatments. In the sea urchin *P. lividus,* a relatively good acid-base regulator, a small increase in copper toxicity under OA was observed (Lewis et al. 2016), whereas the poor acid-base regulator *M. edulis* (common

mussel) OA conditions induced a much larger relative increase in copper toxicity alongside an acidosis of the haemolymph (Lewis et al. 2016).

Hence an organisms' ability to acid-base regulate and the pH of the extracellular fluid may be key to determining both the direction and magnitude of any interaction between OA and copper toxicity. Differences in the uptake and accumulation of copper may also play a role in species differences, with OA known to also influence trace metal accumulation (Breitbarth et al. 2010, Lacoue-Labarthe et al. 2009, Lacoue-Labarthe et al. 2011). The idea that physiology can determine the interaction between copper and another environmental stressor, in this case salinity, has previously been suggested. Grosell et al.,(2007) reviewed data for copper toxicity responses of marine fish and invertebrates under varying salinities and found four orders of magnitude differences between species in their sensitivity to copper in seawater. The authors argued that the majority of these differences could be attributed to differences in physiology (in this case turnover rates of the Na^+ ion) rather than water chemistry (Grosell et al. 2007). Given the variety of species with differing physiologies that will be experiencing coastal pollution as OA progresses it is vital to gain a better understanding of how these factors interact to determine species tolerance to combined anthropogenic stressors as climate change progresses.

Table 3.1. Seawater carbonate chemistry and copper levels for all experimental treatments over the exposure period. Data presented as mean ± standard error. Temperature, salinity, and pH were measured daily in each replicate tank. DIC (n = 9) and copper concentration (n = 7) were measured every three days. The other carbonate parameters were calculated using CO2sys (Lewis and Wallace 1998).

Treatment	Temperature (°C)	pH_{NBS}	Salinity	Copper (µM)	Copper (µM) Min/max	TA (µmol/kg)	pCO_2 (µatm)	HCO_3^- (µmol/kg)	CO_3^{2-} (µmol/kg)	ΩCa	ΩAr
8.10 + Cu	14.03 ± 0.05	8.19 ± 0.01	34.07 ± 0.03	0.046 ± 0.0177	0.037/0.065	2790.4 ± 47.3	453.3 ± 7.9	2325.3 ± 40.5	194.9 ± 3.4	4.7 ± 0.1	3.0 ± 0.1
8.10	13.91 ± 0.05	8.19 ± 0.01	34.05 ± 0.03	0.016 ± 0.016	0.007/0.022	2801.9 ± 60.9	453.3 ± 10.1	2336.9 ± 52.2	194.9 ± 4.4	4.7 ± 0.1	3.0 ± 0.1
7.70 + Cu	14.08 ± 0.04	7.77 ± 0.01	34.13 ± 0.04	0.061 ± 0.0196	0.045/0.079	2756.5 ± 44.9	1310.9 ± 21.6	2556.3 ± 42.2	81.7 ± 1.3	2.0 ± 0.0	1.3 ± 0.0
7.70	14.12 ± 0.05	7.77 ± 0.01	34.20 ± 0.05	0.019 ± 0.0018	0.008/0.024	2743.8 ± 50.9	1305.9 ± 24.5	2546.5 ± 47.8	81.7 ± 1.5	2.0 ± 0.0	1.3 ± 0.0

Chapter Four

A population comparison of the polychaete *Hediste diversicolor* demonstrates that copper resistance alters the metabolic responses to ocean acidification and copper as combined stressors.

4.1. Introduction

Many ecologically and economically important marine species live in coastal ecosystems that are already under stress from numerous sources of anthropogenic pollution (Islam and Tanaka 2004, Crowe et al. 2000). Estuaries are strongly susceptible to pollution from anthropogenic input via rivers, marine traffic and coastal construction (Nicolaus et al. 2015). Metals, and in particular copper, continue to be some of the most wide-spread environmental contaminants and can still be found at elevated concentrations in the majority of estuarine and coastal environments (compared to open ocean) as a result of local mining (past or present), road run-off, antifouling paints and effluent discharges (Matthiessen, Reed and Johnson 1999, Boxall et al. 2000, Sainz, Grande and de la Torre 2004). For example, individual docks in Essex and Suffolk, England, can account for 820-7400 kg yr^{-1} of copper input from ships and yachts (Matthiessen et al. 1999). Estuaries are an important sink for many of these contaminants, with heavy metals in particular showing high affinities for fine-grained estuarine sediments (Lee and Cundy 2001). Metal concentrations in the particulate form tend to be 3-5 orders of magnitude higher than in the dissolved form (Comber and Gunn 1995), meaning that the bulk of metals tend to accumulate within the sediments (Salomons and Förstner 1984). Of these metals, copper has been ranked as the highest threat metal (and indeed aquatic contaminant) for aquatic invertebrates due to its prevalence in aquatic environments (Bryan and Langston 1992, Watson et al. 2018) and its known toxicity effects, causing lipid peroxidation, genotoxicity and larval teratogenicity.

These coastal waters are now additionally experiencing global environmental change as a result of increasing atmospheric carbon dioxide levels, including increased sea

surface temperatures, increasing dissolved carbon dioxide and hence decreased pH levels, against this background of pollution (Harley et al. 2006, Doney et al. 2012). Organisms living in coastal and estuarine waters are therefore being exposed to multiple stressors. According to the IPCC'S Special Report on the 'Ocean and Cryosphere in a Changing Climate' (IPCC 2019) the open oceans pH levels have declined by 0.017-0.027 units per decade since the late 1980s and are predicted to decrease by up to 0.29 units (RCP 8.5) by 2081-2100 (Bindoff et al. 2019). Many anthropogenic contaminants are pH sensitive, hence the change in seawater pH associated with the process of ocean acidification has the potential to alter the bioavailability and toxicity, via changes in speciation, of these pollutants (Roberts et al. 2013, Byrne 2002, Stockdale et al. 2016). Copper (II) ions form strong complexes with carbonate and a change in pH will lead to an increase in the more toxic copper free ions (Millero et al. 2009, Stockdale et al. 2016) such that an increase of 48 - 115% in free copper ions due to OA, coupled with the increase in sea surface temperature is predicted for the end of the century (Richards et al. 2011, Stockdale et al. 2016).

A number of studies have recently reported increased copper toxicity responses under OA conditions in a range of marine invertebrate species, including higher larval mortality (in polychaete worms) (Lewis et al. 2013a), elevated anti-oxidant enzymes (sea anemone) (Siddiqui and Bielmyer-Fraser 2015) and increased damage to DNA and lipids (in mussels and sea urchins)(Lewis et al. 2016) when compared to ambient pH/pCO_2 conditions. However, it is important to note that responses to these stressors can be species dependant, possibly at least in part related to the acid base physiology of the species which determines extra cellular pH (Lewis et al. 2016), or differ according to life history stages, with larval stages often showing increased sensitivity compared to adults. In Chapter 3 I demonstrated that the King Ragworm, *Alitta virens*, under short term two week exposures, showed no increase in copper toxicity under OA conditions with OA appearing to buffer the impacts of the copper, reducing the level of copper-induced lipid peroxidation and acid-base disturbance in the worms (Nielson et al., 2019). The responses of individuals to multistressors may also be influenced by an organisms previous exposures history, for example, exposure to low pH or copper, as demonstrated in the work of Lewis et al that revealed Arctic copepods that migrate vertically through variable pH/pCO_2 conditions under sea ice on a daily

bases were much more resistant to near-future OA than a smaller copepods without daily vertical migration (Lewis et al. 2013b).

Ultimately, species survival under changing global stressors will depend on their ability to adapt to these multiple selective pressures. Current populations might harbour phenotypic variation in response to stressors and the extent to which this has a heritable, genetic basis can indicate the potential for evolutionary responses (Sunday et al. 2014, Foo et al. 2012). The responses of individuals to environmental stressors may also be influenced by an organisms previous exposures history to them, as demonstrated in the work of Lewis et al that revealed Arctic copepods that migrate vertically through variable pH/pCO_2 conditions under sea ice on a daily bases were much more resistant to near-future OA than a smaller copepods without daily vertical migration (Lewis et al. 2013b). The effect of different habitats on responses to increased pCO_2 has also been seen in barnacles (*Amphibalanus improvisus*), with populations living in areas of naturally fluctuating pCO_2 being more tolerant than populations living in areas with a stable pCO_2 (Pansch et al. 2014). This latter example is likely a result of local adaptation.

Local adaptation, i.e. the differential reproduction or survival of genotypes in their environment such that better adapted genotypes become more frequent (Sanford and Kelly 2011; Collins, Boyd and Doblin 2020), resulting in these populations having a higher fitness in their native habitat than more distant ones (Kawecki and Ebert 2004), has been demonstrated for copper or elevated seawater *p*CO2/reduced pH as single stressors. Such examples can be studied to provide useful insights into the potential and underpinning mechanisms for adaptations to global change. For example, larvae from the purple sea urchin, *Strongylocentrotus purpuratus,* from the coast of California routinely exposed to low seawater pH as a result of the upwellings along that coast displayed pronounced genetic changes in genes for biomineralization, lipid metabolism and ion homeostasis, enabling them to survive and continue to calcify in the face of changing pCO_2 levels (Pespeni et al. 2013). Embryos and larvae from the same species, *Strongylocentrotus purpuratus,* also along the Californian coast, showed local adaptations to changing pH levels (Evans et al. 2017). Population comparisons found that those populations exposed to low pH levels more often responded by expressing genes within major ATP-producing pathways at a greater

levels than those populations which encountered low pH seawater less often (Evans et al. 2017). The polychaete *Ophryotrocha labronica* was reported to rapidly restore fitness within two generations under lower pH seawater conditions after an initial reduction of survival (Rodriguez-Romero et al. 2016). Having a better understanding of local adaptations will improve the accuracy of predictions made about climate change (Sanford and Kelly 2011) and comparing different populations of the same species from a range of differing environmental conditions enables the influence of this local adaptation to be tested.

One well documented example of local adaptation to elevated metal pollution is the population of copper tolerant *Hediste* (*(*formally *Nereis*) *diversicolor* found at Restronguet Creek, which survive levels of copper contamination of the sediments as a result of the mining heritage of the region shown to be lethal to other populations (Bryan and Gibbs 1983a). This population of worms demonstrate significantly different gene expression, including significantly higher gene transcripts encoding membrane copper ion transporters and metallothioneins-like proteins, compared to populations living in less polluted environments (McQuillan et al. 2014). The effect of chronic copper pollution on different populations of the polychaete *Laeonereis acuta* resulted in organisms from a polluted site being more susceptible to oxidative stress conditions than those from an unpolluted population (Geracitano et al. 2004). However, the ability for an organism to cope with multiple stressors may pose a far great challenge, especially if they act synergistically. Ultimately, populations adapted to one stress (e.g. chronic pollution) could have reduced genetic diversity/reduced standing genetic variation in the genes required to adapt to the second stressor (e.g. OA), inflating the risk of extinction (Dutilleul et al. 2017, Rogell et al. 2009).

Being adapted to one stressor already, could pose a different set of challenges when being faced by multi stressors and therefore single-stressor experiments may not be appropriate in assessing the effects of climate change in marine habitats (Wernberg, Smale and Thomsen 2012). Multiple stressors can have larger impacts than the sum of individual stressors (Lange and Marshall 2017, Brown et al. 2014) especially if the stressors act synergistically (Crain et al., 2008). The negative impacts of multi stressors, compared to single stressors, can be seen across a wide range of areas, in a wide range of species. For example, the meta-analysis of Przeslawski et al. (2015),

of multi-stressors on early marine life stages showed that synergistic interactions are the most common (65% of individual tests) with larvae generally being more vulnerable than embryos to a combination of thermal and pH stress (Przeslawski et al. 2015). Gunderson et al., (2016) review also found that the majority of interactions are synergistic but that the impact of multiple stressors depends critically on the intensity and timing of each stressor. Their concept, adapted from (Todgham and Stillman 2013), states that when two stressors don't coincide, and therefore physiological responses don't overlap, an additive effect is more likely to occur. However, if the stressors occur simultaneously or in quick succession (stressors overlap) then synergistic effects are most likely (Gunderson et al. 2016).

Sub-lethal responses to environmental stressors often come at an energetic cost to organisms. For example, climate change stressors (combined elevated temperature and reduced seawater pH) have been shown to lead to a decreased energetic investment of females into their eggs resulting in smaller egg sizes in a number of marine invertebrate species, which then leads to a prolonged larval stage potentially resulting in increased chance of predation and mortality (Foo and Byrne 2017). In the resistance of *Hediste diversicolor* from Restronguet Creek, Pook et al., (2009) observed that resistant worms living in high levels of the metals copper and zinc were smaller in size with a reduced fecundity, smaller size at reproduction (so likely earlier reproduction in this semelparous worm) and lower sugar and lipid reserves than worms from nearby non-resistant populations. This was hypothesised by the authors to be due to the energetics costs of defence and repair from the high copper levels such as the production of metallothionein proteins and DNA repair (Pook et al. 2009). Tolerance to stress can also be energy limited (Sokolova 2013, Pansch et al. 2014) as a result of elevated metabolic costs, activation of defences and repair mechanisms, reduced food assimilation and/or stress induced impacts on ATP production. Sokolova's 'energy limited tolerance to stressors' hypothesis uses energy metabolism as an integrator of the effects of multiple stressors as bioenergetics plays a central role in the tolerance to environmental stress. The balance between the input and expenditure of energy is a key requirement for existence and environmental stress can affect the optimal allocation of energy by modulating energy demands for survival (Sokolova 2013), hence multiple stressors may exert greater energetic demands and potentially result in trade-offs in energetic partitioning. The importance of energy

availability to organisms under CO_2 stress has been shown in the barnacle *Amphibalanus improvisus*, where enhanced food availability can increase the resilience of individuals (Pansch et al. 2014).

To investigate whether adaptation to a local stressor (a contaminant) effects an organism's response to a second, global stressor, we compare the responses to the combined stressors of ocean acidification and copper in the metal resistant Restronguet Creek population of the common ragworm, *Hediste diversicolor* to those of a near-by population without this metal resistance. *H. diversicolor* is a soft mud dwelling organism inhabiting the shallow waters of European and North American coasts (Pocklington and Wells 1992) playing an important role as a keystone species in sediment habitats. *Hediste diversicolor* can often be found in contaminated environments, but the population found in the estuarine waters of Restronguet Creek has been shown to be resistant to the toxic effects of copper (Bryan and Gibbs 1983a, Mouneyrac et al. 2003), with LC50 levels of 2193 µg l^{-1}, which is more than double that of two other populations living nearby in Froe Creek, Cornwall and in the Teign Estuary, Devon, which had an LC50 of 1022 µg l^{-1} and 1053 µg l^{-1} respectively (Pook et al. 2009). This resistance has been demonstrated as being heritable (Weis 2014) and has been attributed to changes in gene expression, with copper resistant *H. diversicolor* from Restronguet having significantly more gene transcripts encoding copper homeostasis proteins and putative metallothionein-like proteins (McQuillan et al. 2014). The effects of OA, on top of the costs of high metal concentrations in these populations is unknown.

Here, the hypothesis that a multiple stressor exposure will exert a greater stress, due to restricted energy reserves, on the metal-resistant population of *H. diversicolor* from Restronguet Creek than for a non-resistant population is tested by comparing a suite of toxicity, physiological and metabolic responses of worms to copper under the additional stress of ocean acidification in worms from the copper resistant Restronguet Creek population with those from a nearby but non-resistant population. We were unable to use a common garden experiment due to the high levels of copper the Restronguet Creek worms are exposed to, therefore, we will look at the relative responses of the two different populations to combined stressors. This population

comparison will allow us to test the influence of local adaptation (copper resistance) and the resulting responses to both OA and copper.

4.2. Methods

4.2.1. *Animal collection and maintenance*

Non–resistant adult *Hediste diversicolor* were collected by hand from Exton, Devon, England (50.6681° N, 3.4381° W) during May 2016 and resistant adults from Restronguet Creek, Cornwall (50.2091° N, 5.0874° W), England during June 2016 by carefully digging them from the mud with a fork. The worms were maintained in three main tanks in a temperature controlled room of 15°C for 48 hours before the experiment started. The main tanks were filled with seaweed from the collection site and well aerated artificial seawater at a salinity of 22 ppt. The worms were transferred into individual 400 ml glass beakers, with two worms per beaker and two small glass tubes to act as artificial burrows for the 10-day exposure to prevent over-exertion of the worms during the exposure. Each beaker contained well aerated artificial seawater and was kept at a salinity of 22 ppt. The salinity was monitored daily using a salinity probe (SevenGo Duo, pH/conductivity meter SG23). The beakers were kept covered for the duration of the exposure to simulate the darkness the animal would experience when burrowing in their natural sediment.

4.2.2. *Seawater chemistry*

Artificial seawater (Tropic Marine©) was kept in two 90 L holding tanks with the individual beakers being filled from these. Seawater pH_{NBS} values of 8.10, 7.70 were targeted, representing current and near future ocean acidification treatments respectively according to the IPCC WGI AR5 RCP 8.5 scenario (Stocker 2013, Meinshausen et al. 2011) Seawater pH_{NBS} in the control was maintained by bubbling air into each individual tank. Seawater pH_{NBS} in the OA conditions was maintained at 7.70 using an Aalborg Mass Flow Controller GFC set at the correct ratio of air to CO_2 and bubbled into each individual tank. Seawater pH (SevenGo Duo, pH/conductivity meter SG23), temperature and salinity (SevenGo Duo, pH/conductivity meter SG23) were measure daily in the holding tanks and individual tanks.

In order to be able to compare the metal resistant Restronguet populations' response to combined OA and copper exposures with those of the non-resistant Exton population, copper concentrations that elicited a comparable effect size when experienced as a single stressor were used. The LC50 calculations generated by Pook et al., (2009) demonstrated worms from the metal resistant Restronguet Creek population have an LC50 of 2193 µg l^{-1} which was approximately double compared to nearby non-resistant populations (LC50s of 1022 µg l^{-1} and 1052 µg l^{-1}). Based on the responses to copper demonstrated in Chapter 3 (Nielson et al., 2019) and a preliminary trail, nominal copper concentrations of 0.25 µM for the non-resistant population and 0.5 µM for the resistant Restronguet population were therefore used.

Water samples for copper and DIC analysis were taken throughout the 10-day exposure from randomly selected tanks from each treatment, to cover both just before and just after water changes where re-dosing occurred (i.e. providing the highest and lowest points of the exposure dose). Water samples for DIC analysis were preserved (0.04 % of final volume) with 4 % mercuric chloride for storage prior to analysis (Dickson 2007b) whilst samples for metals analysis were added to acid-washed 50 ml tubes and acidified using 50 µl of concentrated hydrochloric acid. Seawater DIC analysis was carried out using a bespoke system based on that described by Friederich et al., (2002) and using Dickson seawater standards, as described in detail in Lewis et al., (2013a). Total alkalinity (TA) and pCO_2 were calculated from the measured values of pH_{NBS} and DIC using CO2sys, applying the constants from Mehrbach. et al. (1973) and the KSO_4 dissociation constants from Dickson (1990a). The concentrations of copper in the seawater samples were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) using an Agilent 7900 Spectrometer utilising a collision cell with helium as the collision gas to minimise polyatomic interferences. Copper standards, matrix matched to the samples, at concentrations of 1, 10 and 100 mg/l respectively, were used for instrument calibrations. Analysis employed in-line addition of scandium as an internal standard and calibration of the ICP-MS was validated by the use of a quality control standard of 10 mg/l.

4.2.3. *Experimental design*

Twenty worms per treatment (two per individual tank, each tank with an individual air/gas supply (for OA treatments)) were transferred to the following treatments at 15°C for 10 days; (1) ambient conditions (pH_{NBS} 8.1) with no added copper, (2) ambient conditions (pH_{NBS}8.1) with 0.25 µM (non-resistant population) or 0.5 µM (resistant population) copper sulphate added, (3) OA conditions (pH_{NBS} 7.7) with no added copper, (4) OA conditions (pH_{NBS} 7.7) with 0.25 µM/0.5 µM copper sulphate added. Salinity, temperature and pH were monitored daily. Worms were fed, with crushed trout pellets, on day 2 and at the beginning of day 6 and water changes to re-dose with copper were performed on day 3 and the end of day 6.

On day 7 of the 10-day exposure one individual worm per beaker (ten per treatment) was removed and a feeding assay performed (see below) to determine individual feeding rate and then returned to its exposure beaker. On day 9, one individual worm per beaker (ten per treatment) was removed from its exposure beaker and samples of coelomic fluid were collected from each individual worm. Samples were collected using an 18-gauge needle and 1 ml syringe carefully inserted into the coelomic cavity of the organism, working from the anterior region to the posterior region. The coelomic fluid was used for the comet assay and the worm was snap frozen in liquid nitrogen for the SOD/TBARS assays (see below). Following the end of the 10-day exposure the remaining worms (10 per treatment) were removed from their tank and placed into individual 100 ml beakers for analysis of oxygen consumption rates (see below). Oxygen consumption rate was recorded for each individual worm (details below) and the worms were transferred to individual pre-weighed weighing boats, before being placed in an oven at 60°C for 24 hours. The dry weight of each worm was then calculated and recorded.

4.2.4. *Feeding assay*

Post exposure, individual worm feeding rates were assessed using an assay adapted from (Moreira et al. 2005) and previously demonstrate as sensitive in *H. diversicolor* to contaminant responses (Hird et al. 2016). Prior to the assay, 100 24h old brine shrimp (*Artemia franciscana*) nauplii were counted out, stored in a microcentrifuge

tube and frozen. Worms were then transferred at the start of the assay into individual 100 ml beakers containing 80 ml of fresh artificial seawater taken from the same holding tank with a corresponding pH (i.e. treatment pH). One-hundred defrosted nauplii were then added to each beaker and kept in darkness for 1 hour. At the end of one-hour worms were removed, rinsed and placed back to the exposure beaker. The remaining nauplii were rinsed off and counted by eye to determine the feeding rate as nauplii consumed h^{-1}.

4.2.5. *Comet assay*

DNA damage was measured as single strand breaks using the comet assay, according to methods described by Lewis and Galloway (2008), under alkaline conditions at 5^{o}C. From the coelomic fluid collected as described above, 100 µl from each individual was combined with 100 µl of phosphate buffer and centrifuged. The supernatant was removed and 180 µl of low melting point agarose was added, this was then pipetted out onto a frosted slide, previously coated in high melting point agarose, and left to cool. The slides were placed in Lysis solution for 1 hour at 4^{o}C and then into the electrophoresis tank. Here the slides were covered with electrophoresis solution for 40 mins before the current (25 V) was switched on for 30 minutes. Finally, the slides were rinsed in neutralising buffer before being stained with SYBRSafe (1 µL in 10 mL TBE buffer) and being viewed under ultraviolet fluorescence (excitation: 502 nm, emission 530 nm). One hundred cells per individual worm were quantified for DNA damage using COMET IV Software (Perceptive Instruments Ltd.), which measures the percentage of DNA present in the comet tail for each cell as the measure of DNA damage.

4.2.6. *Oxidative stress endpoints*

Superoxide dismutase (SOD) is an enzyme which is essential in the defence against oxidative damage (McCord et al. 1971). The SOD assay generates O_2- and uses nitroblue tetrazolium (NBT) which changes colour, from clear to purple, when it comes in contact with a free radical. SOD inhibits this colour change hence levels can be quantified by determining the level of inhibition of this colour change in a sample compared to a standard (Beaucham and Fridovic 1971). Initially 5 µl of homogenised

samples or standards were added to 96-well plates along with 30 µl of buffer A (2.28 g/500 ml Na_2CO_3 and 1.18 g/ 500 ml $NaHCO_3$) and 195 µl substrate solution B (0.1 mM xanthine, 0.1 mM EDTA, 0.05 mg BSA and 0.025 mM NBT). Free radicals were created using xanthine oxidase (4.95 units/ml in a 1:80 dilution in buffer A), where 10 µl was added to the microplate immediately prior to reading at a wavelength of 573 nm.

Lipid peroxidation was determined using the thiobarbituric acid reactive substances (TBARS) assay (Camejo 1998) which quantifies malondialdehyde, a secondary product of lipid peroxidation, via its reaction with thiobarbituric acid (Lewis et al. 2016). In microcentrifuge tubes, 100 µl of homogenised samples or standards (see below) were added along with 300 µl of PBS + EDTA (372.24 mg EDTA in 1L PBS), 150 µl thiobarbituric acid (1.95g in 150ml NaOH), 100 µl trichloroacetic acid (50 g/100 ml DI water) and 20 µl butylated hydroxytoluene (22 mg/100 ml ethanol). All microcentrifuge tubes were vortexed and incubated at 60^{o}C for 60 minutes, after 60 minutes microcentrifuge tubes were centrifuged for 7 minutes at 13000 revolutions per minute. In a 96-well plate either 200 µl of standards or 100 µl of sample + 100 µl PBS + EDTA were added in triplicate to the corresponding wells and the plate was read using a microplate reader at a wavelength of 530 nm. Results were compared to a standard curve prepared using 1,1,3,3-tetraethoxypropane (a stabilized form of MDA) and normalised to protein content using standardised Bradford protocol (Bradford 1976).

4.2.7. *Metabolic rate*

Metabolic rate was measured as a proxy to assess the energy expenditure of *Hediste diversicolor*. Metabolism of substrates requires oxygen and the amount of oxygen used for each energy substrate is proportional to the amount of energy produced, therefore, oxygen consumption can be used, as a proxy, to measure metabolic rate. Metal resistance has been demonstrated to come at an energetic cost in *H. diversicolor* (Pook et al. 2009) and therefore measuring the energy expenditure of these resistant worms in the face of additional stressors is key to understanding their response. Ammonia excretion rate was also measured to enable a final O:N ratio to be calculated.

Oxygen consumption was measured, according to the methods of Hird et al.,(2016) using an optical meter (FireStingO_2, Pyro-Science) with a robust sensor, which was calibrated using fully aerated seawater (100% air saturation) and a saturated sodium sulphite solution (0% oxygen saturation). Worms were transferred into individual beakers, with plastic lid, containing 75 ml of fully saturated seawater (at 15 ^{o}C) and the initial oxygen level was recorded. The worms were left in darkness for 3 hours with final readings then taken and the change in oxygen was calculated. Control beakers (no worms, 2 per treatment), containing 75 ml of seawater were also run to allow correction for diffusion and bacteria oxygen consumption. These changes were subtracted from the exposure readings. Readings were corrected for temperature (15^{o}C), salinity (22ppt) and barometric pressure as standard, as all factors can impact oxygen concentration and results were standardized by worm dry weight. The dry weight was calculated post exposure, after rinsing in deionized water and drying for 24h at 60 ^{o}C (Hird et al. 2016).

Ammonia concentration was determined using a colorimetric assay (Ivancic and Degobbis 1984), modified for microplates (Urbina et al. 2014), measuring absorbance at 660 nm and compared to a standard curve generated using ammonia chloride standards (0, 20, 40, 60, 80, 100, 200 and 300 µM). Initial, from the start of oxygen measurements, and final, after the final oxygen reading (3 hours), ammonia concentrations were measured and the differences between them determined the ammonia excretion rate. These results were standardized post exposure by the dry weight (g), exposure volume (L) and time (h) (Urbina et al. 2014).

O:N ratios were determined using metabolic and ammonia excretion rates (Taboada et al. 1998, Bayne 1999), enabling us to gain an insight into the type of energy substrate being used by these organisms. The ratio was calculated by firstly dividing the oxygen consumption by 2 and then dividing this result by the ammonia excretion rate, giving the amount of oxygen consumed versus the amount of ammonia excreted. The ability to use certain substrates is genetically controlled (Lovett and Felder 1990), with lower values indicating a switch to primarily protein metabolism. An increase in O:N values indicates that a mixture of proteins and lipids are used (Taboada et al. 1998, Brito et al. 2000).

4.2.8. *Statistical analysis*

All statistical analysis was carried out using SPSS software. All data was first analysed for normality using the Shapiro-Wilk test. All data for DNA damage (% data) was first normalised using the arcsine transformation. Data for SOD, TBARS, O:N ratio and metabolic rate (Restronguet data only) was normalised using the log transformation and Ammonia data (Restronguet data only) using the square root transformation. All data was analysed using a 2-way analysis of variance (ANOVA) general linear model with 'pH', 'copper' and 'pH x copper' as fixed factors with a Tukey's post hoc being carried out on all data.

To compare OA and copper combined responses across the two populations to test our hypothesis, the effect size of the combined stressors compared to the controls (i.e. pH_{NBS} 8.10 with no added copper) was calculated. For this, the percentage change was calculated between the control treatment (8.10, no copper) and the combined stressor treatment (7.70 with copper). For the control treatment the mean value was used, and a percentage change worked out from this value against all individual values from the individual stressor treatment. All percentage changes were plotted, and an independent samples t-test was carried out to compare the means of the effect size in the Exton population to the Restronguet population for each end point.

4.3. Results

4.3.1 *Seawater chemistry*

The seawater carbonate chemistry from all treatments, along with copper levels are summarised in Table 4.1.

4.3.2. *Feeding assay*

In the non-resistant Exton population OA exposure significantly affected feeding rate, measured as the number of brine shrimp eaten per hour, with a decrease in pH causing an increase in the feeding rate. Under ambient conditions, with a pH_{NBS} of

8.10, worm feeding rate was 48 ± 8 Artemia per worm, per hour. Under OA conditions feeding rate was significantly higher at an average of 59 ± 5 artemia per worm per hour. For the copper treatments, under ambient pH conditions average worm feeding rate was 34 ± 7 Artemia per worm per hour, again this rose to 57 ± 8 Artemia per worm per hour at the lower pH (two-way ANOVA for pH, F = 5.508, P = 0.025). The addition of copper didn't cause a significant effect, although a decrease in feeding rate was seen when compared to the treatments without copper (two-way ANOVA for copper, F = 1.353, P = 0.252). There was no significant OA*copper interaction (two-way ANOVA for pH*copper, F = 0.598, P = 0.455) (Figure 4.1 A).

In the resistant Restronguet population, feeding rates were ~1.5 times higher than the non-resistant population and were relatively stable across treatments, with an average feeding rate of 77 ± 4 per worm per hour under ambient conditions. There was no significant difference found between the different pH levels with feeding rate in OA being 77 ± 6 Artemia per worm per hour (two-way ANOVA for pH, F = 1.679, P = 0.204). The addition of copper also did not cause any significant differences with feeding rates in the resistant worms, being slightly lower, 70 ± 4 Artemia per worm per hour, in the 8.10 pH_{NBS} treatment and slightly higher in the OA treatment at 81 ± per worm per hour (two-way ANOVA for copper, F = 0.266, P = 0.609). There was no significant OA*copper interaction observed (two-way ANOVA for pH*copper, F = 1.652, P = 0.207) (Figure 4.1 B).

The average effect size of exposure to combined OA and copper for feeding rate was positive for both populations, such that on average there was a small increase in feeding under combined OA copper exposures. There was no significant difference between the two different populations (T test, t=0.733, p=0.478). The feeding rate of both populations increased when exposed to both OA and copper (compared to control conditions), with the average effect size increase in feeding rate in the non-resistant population being slightly higher at 18.2% compared to 4.9% in the resistant population. The non-resistant population had higher variability with a larger range in values (Figure 4.1 C).

4.3.3. *Metabolic rate*

Metabolic rates in both populations seemed relatively unaffected by either copper or OA as stressors and maintained stabile across all treatments, showing no significant differences. In the non-resistant Exton population, there was no significance effect of either pH (two-way ANOVA for pH, F = 2.223 P = 0.145) or copper ((two-way ANOVA for copper, F = 0.943 P = 0.338). The metabolic rate did decrease slightly with the addition of copper under OA conditions (pH_{NBS} 7.70) from the highest rate of 20.03 ± 2.26 μM O_2 gDW^{-1} h^{-1} in the ambient pH_{NBS} (8.10) treatment without copper to the lowest rate of 15.21 ± 1.58 μM O_2 gDW^{-1} h^{-1} in the OA and copper treatment, but this was not significant. There was no significant OA*copper interaction present (two-way ANOVA for pH*copper, F = 0.076, P = 0.785) (Figure 4.1 D).

In the resistant Restronguet population there was also no significant effect of OA conditions (two-way ANOVA for pH, F = 2.188 P = 0.149) or copper (two-way ANOVA for copper, F = 0.015 P = 0.903) with values similar to the non-resistant population seen. Metabolic rate followed the same pattern in the ambient treatment as the non-resistant population, with the addition of copper lowering the metabolic rate from 19.35 ± 2.13 μM O_2 gDW^{-1} h^{-1} to 17.33 ± 1.83 μM O_2 gDW^{-1} h^{-1}. However, the opposite was seen at a pH_{NBS} of 7.70 where the addition of copper raised the metabolic rate from 20.48 ± 1.31 μM O_2 gDW^{-1} h^{-1} to 23.11 ± 3.65 μM O_2 gDW^{-1} h^{-1}. There was no significant OA*copper interaction (two-way ANOVA for pH*copper, F = 0.330, P = 0.570) (Figure 4.1 E).

The effect size of exposure to combined OA and copper for metabolic rate was significantly different between the metal resistant Restronguet population and the non-resistant Exton population (T test, t=-2,216, p=0.041), with average effect size going in different directions. The effect size in the resistant Restronguet population showed a positive metabolic rate response, with an average percentage increase of 19.70%, whilst in the non-resistant Exton worms on average showed a negative metabolic response, with a significant reduction to -24.02% (Figure 4.1 F).

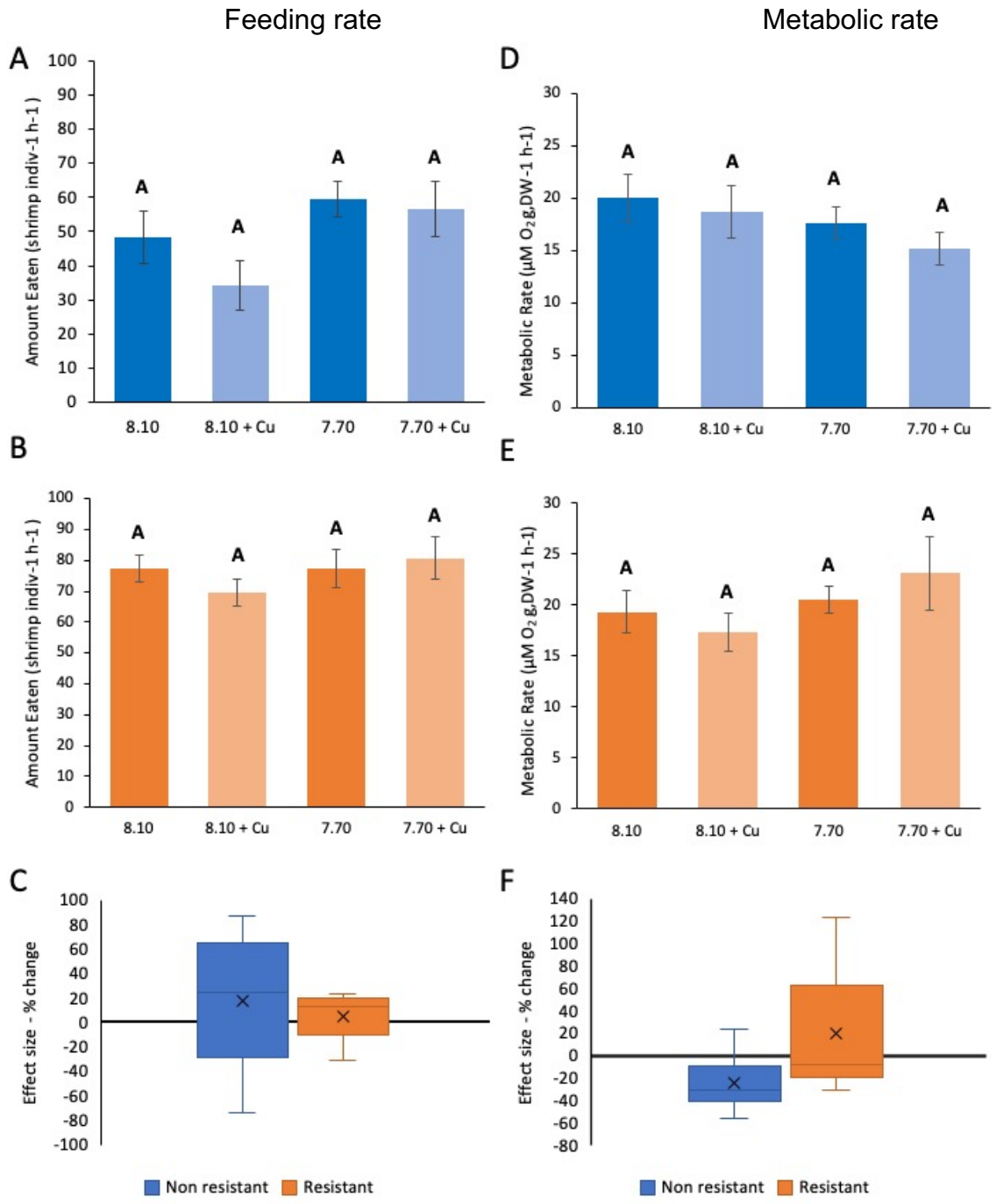


Figure 4.1. The feeding rate (A+B) and metabolic rate (D+E) of non-resistant and resistant populations of *Hediste diversicolor* in ambient (pH_{NBS} 8.10) and OA (pH_{NBS} 7.70) conditions with or without the addition of copper. Mean (±SE). The effect size (C+F) compares the percentage change in feeding rate and metabolic rate between control conditions (pH_{NBS} 8.10 without copper) and the combined stressor condition (pH_{NBS} 7.70 with copper) in both the non-resistant and resistant populations (X represents the mean with the line representing the median value).

4.3.4. *Ammonia*

Ammonia excretion differed significantly between treatments for both populations. In the non-resistant population under ambient conditions the ammonia excretion rate was 1.1 ± 0.13 μM gDW^{-1} h^{-1}. This fell to 0.72 ± 0.09 μM gDW^{-1} h^{-1} when the pH_{NBS} was reduced to 7.70. A similar pattern was seen in the copper treatments, where ammonia excretion fell from 1.46 ± 0.14 μM gDW^{-1} h^{-1} at 8.1 pH to 0.61 ± 0.10 μM gDW^{-1} h^{-1} in the OA treatment. The effect of pH was significant (two-way ANOVA for pH, $F = 28.684$ $P < 0.001$). The addition of copper did not have a significant effect (two-way ANOVA for copper, $F = 1.128$ $P = 0.296$). Under ambient conditions ammonia excretion rose, however, the opposite was seen under OA conditions with a slight decrease. The OA*copper interaction, however, was significant (two-way ANOVA for pH*copper, $F = 4.411$ $P = 0.043$). (Figure 4.2 A).

In the resistant population pH again had a significant effect (two-way ANOVA for pH, $F = 4.176$ $P = 0.050$), with excretion rates decreasing as the pH decreased. The excretion rate under ambient conditions was 0.70 ± 0.10 μM gDW^{-1} h^{-1}, at a pH_{NBS} of 7.70 this fell to 0.38 ± 0.03 μM gDW^{-1} h^{-1}. In the copper treatments the ammonia excretion rate decreased from 1.34 ± 0.33 μM gDW^{-1} h^{-1} at pH 8.1 to 1.06 ± 0.18 μM gDW^{-1} h^{-1} under OA conditions. However, unlike the Exton population the addition of copper also had a significant effect (two-way ANOVA for copper, $F = 19.117$ $P < 0.001$), causing a large increase in excretion rate in both pH treatments. There was no significant OA*copper interaction (two-way ANOVA for pH*copper, $F = 0.319$, $P = 0.576$). (Figure 4.2 B).

The effect size of exposure to combined OA and copper for ammonia excretion rate was significantly different between the metal resistant Restronguet population and the non-resistant Exton population (T-test, $t=-3.562$, $p=0.005$). In the non-resistant population the average percentage change in ammonia excretion was negative -44.48%. In the Restronguet worms, however, there was a positive percentage change in ammonia excretion of 53.63%. Worms from the resistant population also had a higher variability response (Figure 4.2 C).

4.3.5. *O:N ratio*

For the non-resistant Exton population, the decrease in pH_{NBS} from 8.10 to 7.70 caused an increase in the O:N ratio for both non copper and copper treatments. In the treatments without copper the O:N ratio rose from 38.6 ± 3.9 under ambient conditions to 51.3 ± 3.2 under OA conditions. The treatment groups with copper showed the same pattern with ambient conditions having an O:N ratio of 25.1 ± 2.3 and OA conditions have a larger ratio of 65.6. This increase was significant (two-way ANOVA for pH, $F = 22.529$ $P < 0.001$). The addition of copper was not significant, with the O:N ration falling slightly under ambient and rising slightly under OA conditions (two-way ANOVA for copper, $F = 1.527$ $P = 0.225$). There was a significant interaction between OA and copper (two-way ANOVA for pH*copper, $F = 4.674$ $P = 0.038$). (Figure 4.2 D).

In the resistant Restronguet population there was a also a significant effect of pH (two-way ANOVA for pH, $F = 17.354$ $P < 0.001$) with the O:N ratio rising from 60.7 ± 6.99 under ambient conditions to 115.5 ± 12.5 under OA conditions. The addition of copper also caused a significant effect with copper treatments being lower in both pH treatments, especially in the OA treatments where the O:N ratio dropped from 115.5 ± 12.5 to 51.5 ± 10.1 (two-way ANOVA for copper, $F = 33.704$ $P < 0.001$). There was no significant OA*copper interaction (two-way ANOVA for pH*copper, $F = 0.087$, $P = 0.771$). (Figure 4.2 E).

The effect size of exposure to combined OA and copper for O:N ratio was significantly different between the metal resistant Restronguet population and the non-resistant Exton population (T-test, $t=-2.448$, $p=0.026$). The average effect size went in opposite directions with the non- resistant population showing a positive percentage change of 75.67%, whilst the resistant Restronguet population had on average a negative O:N ratio of -15.23% (Figure 4.2 F).

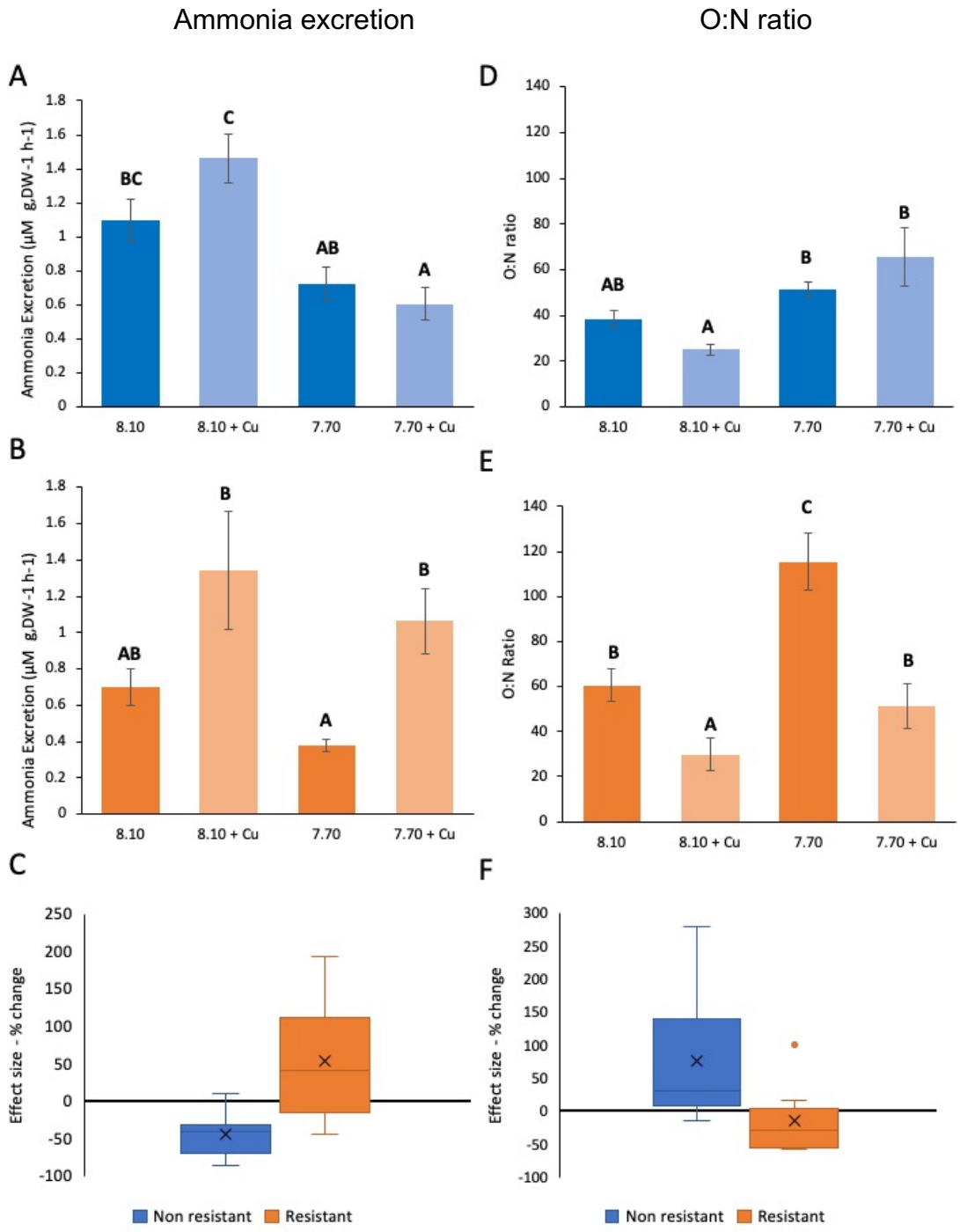


Figure 4.2. The ammonia excretion rate (A+B) and O:N ratio (D+E) of non-resistant and resistant populations of *Hediste diversicolor* in ambient (pH_{NBS} 8.10) and OA (pH_{NBS} 7.70) conditions with or without the addition of copper. Mean (±SE). Differing letters represent significant differences between the four treatments based on Tukey's post-hoc ($p<0.05$). The effect size (C+F) compares the percentage change in ammonia excretion rate and O:N ratio between control conditions (pH_{NBS} 8.10 without copper) and the combined stressor condition (pH_{NBS} 7.70 with copper) in both the non-resistant and resistant populations. (X represents the mean with the line representing the median value and orange dot represents an outlier point).

4.3.6. *SOD*

For both populations SOD activity was unaffected by both OA and copper. In the non-resistant population copper did cause an increase in SOD activity under ambient conditions from 4.63 ± 0.64 units per mg of protein to 5.64 ± 1.00 units per mg of protein with the opposite being seen under OA conditions where SOD activity fell from 4.07 ± 0.63 units per mg of protein to 3.47 ± 0.58 units per mg of protein. However, there was no significant difference (two-way ANOVA for copper, F = 0.333, P = 0.857). There was also no significant effect of either pH (two-way ANOVA for pH, F = 2.753, P = 0.107) or the OA*copper interaction effect between pH and copper (two-way ANOVA for pH*copper, F = 0.437, P = 0.153). (Figure 4. A).

In the resistant population there was also no significant effect of pH (two-way ANOVA for pH, F = 0.824, P = 0.370). Although copper caused a decrease in SOD activity in both treatments, falling from 4.60 ± 0.72 units per mg of protein to 4.07 ± 0.60 units per mg of protein in ambient conditions and from 4.06 ± 1.00 units per mg of protein to 3.15 ± 0.78 units per mg of protein under OA conditions, this decrease was not significant (two-way ANOVA for copper, F = 0.802, P = 0.377). There was no significant OA*copper interaction (two-way ANOVA for pH*copper, F = 0.055, P = 0.816). (Figure 4.3 B).

The effect size of exposure to combined OA and copper for SOD activity was not significantly different between the metal resistant Restronguet population and the non-resistant Exton population with both populations showing a negative percentage change (T-test, t=-0.212, p=0.835). In the non-resistant population the average percentage change in SOD activity was negative -24.95%. In the Restronguet worms, the negative average percentage decrease was slightly larger at -29.50% (Figure 4.3 C).

4.3.7. *TBARS*

Levels of lipid peroxidation on the whole was lower in the resistant population than the non-resistant population. In the non-resistant population there was no significant effect from either pH (two-way ANOVA for pH, F = 2.944, P = 0.096) or copper (two-way

ANOVA for copper, F = 3.492 P = 0.071) despite copper causing a decrease in levels of lipid peroxidation from 26.85 ± 4.23 nM per mg of protein to 23.49 ± 3.36 nM per mg of protein under ambient conditions and from 23.32 ± 3.14 nM per mg of protein to 15.83 ± 1.67 nM per mg of protein. There was no significant OA*copper interaction (two-way ANOVA for pH*copper, F = 0.490, P = 0.489). (Figure 4.3 D).

For the resistant population, unlike the non-resistant population, there was significant effects from both stressors. A significant increase with OA was found (two-way ANOVA for pH, F = 4.396, P = 0.044) with levels of lipid peroxidation increasing from 13.50 ± 1.02 nM per mg of protein under ambient conditions to 16.37 ± 1.27 nM per mg of protein under OA conditions. There was also a significant effect of copper (two-way ANOVA for copper, F = 8.867 P = 0.005) causing a decrease in both pH conditions. There was no significant OA*copper interaction (two-way ANOVA for pH*copper, F = 0.195, P = 0.662). (Figure 4.3 E).

The average effect size of exposure to combined OA and copper for TBARS was negative for both populations, with on average a decrease in lipid peroxidation under combined OA copper exposures, compared to control conditions. There was a significant difference between the two different populations (T test, t=2.805, p=0.014) with the average effect size decrease being significantly lower in the non-resistant population at -41.81% compared to -6.74% in the resistant population (Figure 4.3 F).

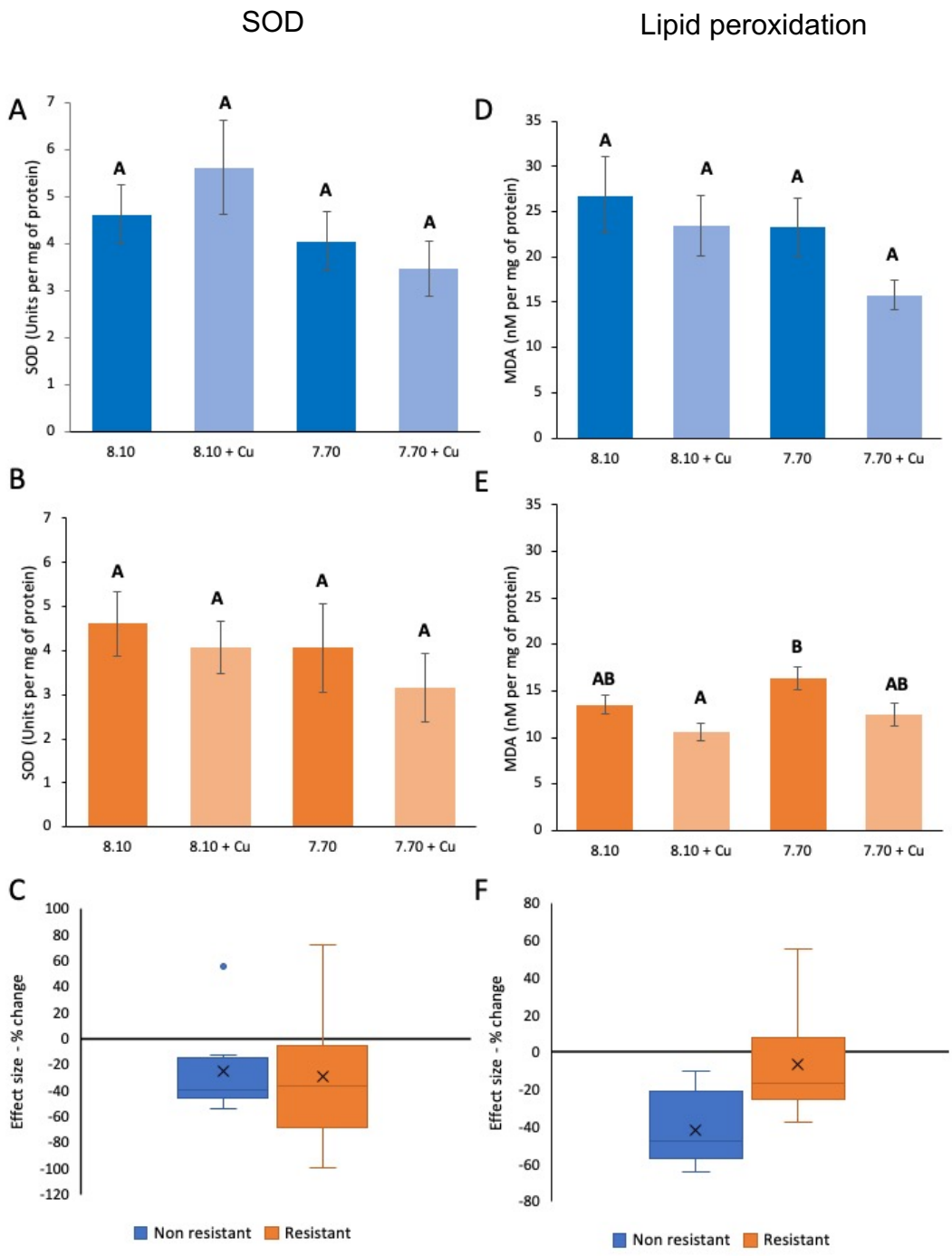


Figure 4.3. Oxidative stress end points in *Hediste diversicolor* measured as the activity of superoxide dismutase (SOD)(A+B) and lipid peroxidation (D+E) using non-resistant and resistant populations in ambient (pH_{NBS} 8.10) and OA (pH_{NBS} 7.70) conditions with or without the addition of copper. Mean (±SE). The effect size (C+F) compares the percentage change in SOD activity and lipid peroxidation between control conditions (pH_{NBS} 8.10 without copper) and the combined stressor condition (pH_{NBS} 7.70 with copper) in both the non-resistant and resistant populations. (X represents the mean with the line representing the median value and blue dot represents an outlier point).

4.3.8. *DNA damage*

The percentage of DNA damage in *Hediste diversicolor* coelomocytes in the non-resistant population under ambient seawater pH_{NBS} of 8.10 was 31.4 ± 4.94%. Worms had lower DNA damage levels when under OA conditions of 21.6 ± 4.93%, the lowest percentage of damage across all treatment groups. However, this decrease was not significant (two-way ANOVA for pH, F = 0.684, P = 0.796). The addition of copper caused a decrease in DNA damage under ambient conditions to 22.7 ± 1.871. However, the opposite was seen under OA conditions were DNA damaged showed the largest increase from 21.6 ± 4.93% to 37.8 ± 6.553% in the combined stressor treatment (pH_{NBS} 7.70 with copper). The addition of copper did not cause significant changes on DNA damage (two-way ANOVA for copper, F = 0.848, P = 0.364). A significant interaction between pH and copper was present (two-way ANOVA for pH*copper, F = 6.439, P = 0.016). (Figure 4.4 A).

In the resistant population levels of damage were fairly equal across all groups, with a slight decrease seen in the double stressor treatment (pH_{NBS} 7.70 with copper). Here the damage fell to 32.44 ± 2.06% from 36.24 ± 3.2% (pH_{NBS} 8.10 with copper). This decrease due to OA was not significant (two-way ANOVA for pH, F = 0.357P = 0.873). There was also no significant impact of copper or any copper*OA interaction effect for this population (two-way ANOVA for copper, F = 0.611, P = 0.263), (two-way ANOVA for pH*copper, F = 0.772, P = 0.086). (Figure 4.4 B).

The effect size of exposure to combined OA and copper for DNA damage was not significantly different between the metal resistant Restronguet population and the non-resistant Exton population (T-test, t = -1.430, p = 0.186). In the non-resistant population the average percentage change was positive at 20.44%, however, in the resistant Restronguet population there was a negative percentage change of -10.53%. Worms from the non-resistant population also had a higher variability response (Figure 4.4 C).

DNA damage

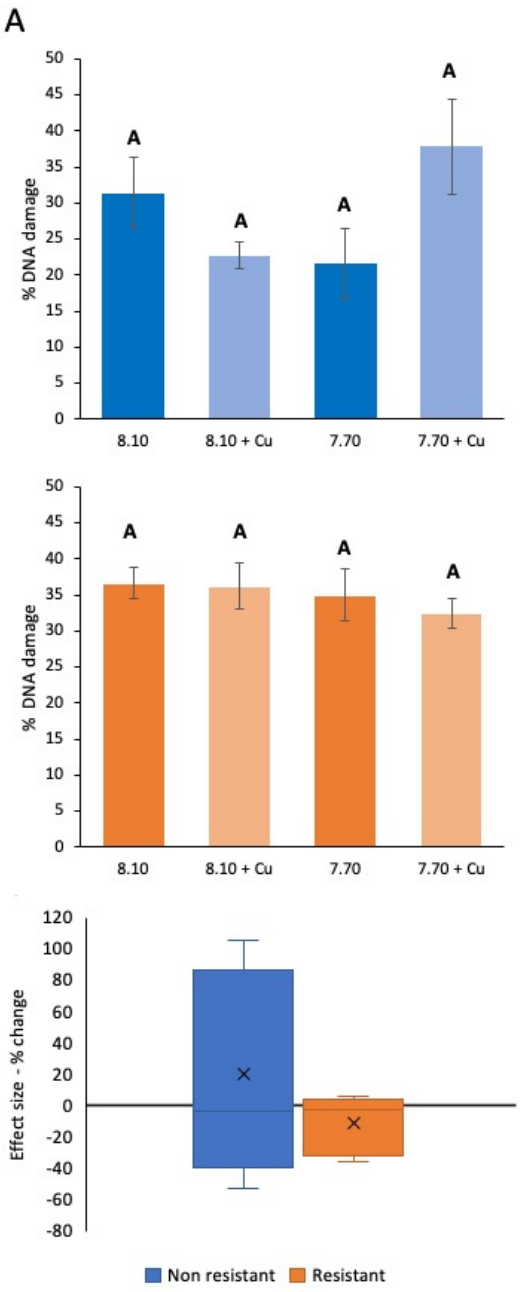


Figure 4.4. Percentage DNA damage in non-resistant (A) and resistant (B) populations of *Hediste diversicolor* in ambient (pH_{NBS} 8.10) and OA (pH_{NBS} 7.70) conditions with or without the addition of copper. Mean (±SE). The effect size (C) compares the percentage change in DNA damage between control conditions (pH_{NBS} 8.10 without copper) and the combined stressor condition (pH_{NBS} 7.70 with copper) in both the non-resistant and resistant populations. (X represents the mean with the line representing the median value).

4.4. Discussion

The data collected for study has revealed a number of interesting differences in the physiological and toxicity responses to combined exposures to copper and ocean acidification conditions in a metal resistant population of the polychaete *Hediste diversicolor* compared to a near-by non-resistant population. The Restronguet Creek *H. diversicolor* population is a well-documented example of adaptation to high metal contamination, with copper LC50s of 2193 μg l^{-1} reported for this population of worms (Pook et al. 2009), approximately double that of near-by but unconnected non-resistant populations. Hence to compare relative responses to OA and elevated copper in combination for these worms, different concentrations of copper that elicited a similar level of toxicity response under exposures at ambient (seawater pH_{NBS} 8.1) were used and then populations compared using a calculated effect size for a number of both physiological and toxicological end points to the combined stressors. These effect size comparisons revealed significant energetic differences with higher metabolic rates in resistant populations and changes in the O:N ratio between the populations, but with OA appearing to alleviate the mild copper toxicity responses for both populations.

Feeding rates tended to be higher in the resistant worms from Restronguet compared to the non-resistant worms across all treatments, ranging from 81 to 70 Artemia consumed per hour across treatments compared to 59-34 in non-resistant worms. This fits with the hypotheses presented by Pook et al (2009) that the resistance to copper pollution in the Restronguet worms comes at an energetic cost to individuals, following their finding that Restronguet worms had a lower scope for growth and fecundity than non-resistant individuals from near-by population. Here, a small but significant increase in feeding rate in the non-resistant worms was observed under OA exposure conditions, and the effect size of OA and copper combined exposures was small but positive (i.e. increased feeding) but this was not present for the Restronguet worms. Whilst there was no significant difference between the two populations in the effect size of combined OA and copper for feeding rate, the variability in response was greater in the non-resistant worms. Despite this increase in feeding rate induced by OA exposure in the non-resistant worms, metabolic rate, measured as oxygen consumption, was relatively unaffected by OA or copper in any of the treatment

conditions. However, the mean effect size of combined OA and copper exposures was in opposing directions for the two populations, with the non-resistant population showing a negative change in metabolic rate under combined OA and copper whilst the resistant worms showed a positive change in metabolic rate on average to the combined stressors. This response was also much more variable across individuals in the resistant population.

Ammonia excretion rate and O:N ratio was influenced by treatment for both *H. diversicolor* populations, but in significantly different ways between the two populations. For the non-resistant Exton worms OA exposure led to a decreased ammonia excretion rate, with a significant interaction term between OA and copper present. This in turn caused an increased O:N ratio in Exton worms exposed to OA. In the resistant Restronguet worms, copper exposure induced a significant 2.8-fold increase in ammonia excretion rate and therefore a decrease of the O:N ratio, with a smaller but still significant OA effect in this population. Elevated O:N ratios and reduced ammonia excretion might indicate that a mixture of proteins and lipids are used as energy substrates (Taboada et al. 1998). This may indicate an energetic advantage, compared to lower O:N ratios, as the transformation of lipids is relatively cheaper compared to the cost of protein degradation to amino acids (Hochachka 1991). Together, these data align with the hypothesis that Restronguet worms have different energetic constraints placed on their resource partitioning than the non-resistant Exton worms, as a result of their metal resistance.

Previous studies looking at whether exposures to OA conditions result in impacts on feeding rate and energetic partitioning have found conflicting results. For example in the limpet *Patella vulgat*a exposure to OA conditions of seawater pH 7.53 (pCO_2 = 2803.8 µatm) for a period of 5 days (short term) revealed no significant effect on feeding rate (Marchant et al. 2010). Corals have been found to exhibit both decreased (*Stylophora pistillata*) (Houlbreque et al. 2015) and increased (*Acropora cervicornis*) (Towle, Enochs and Langdon 2015) feeding rates in response to OA. Interestingly the increased feeding rates seen in the Caribbean coral, *Acropora cervicornis*, enabled them to maintain growth rates and buffer OA-reduced calcification (Towle et al. 2015) but this is not a response found in all coral species indicating different species will be impacted differently. Increased feeding rate in response to OA has also been observed

in the copepod *Centropages tenuiremis*, potentially to balance energy costs (of pumping protons) that OA brings for this species (Li and Gao 2012). Marine invertebrate larvae, generally considered to be the most vulnerable life history stage, show mixed responses as well with decreased feeding rates seen in sand dollars (*Dendraster excentricus*) (Chan et al., 2011) but the northern shrimp (*Pandalus borealis*) showed no significant changes when exposed to both OA and OA in combination with increased temperatures (Arnberg et al. 2013). A decrease in time spent feeding has been seen in gastropods (*Nassarius festivus*) when exposed to the following metals; copper, zinc, cadmium and chromium (Cheung et al. 2002).

The changes in ammonia excretion rates and O:N ratio observed here are also indicative of an energetic stress in the Restronguet population, with these worms moving from using a more lipid based energy substrate to a more protein based one, causing increased energetic costs. These findings for *H. diversicolor* are similar to those found under OA exposures in three species of bivalves; *Pinctada fucata*, *Chlamys nobilis* and *Perna viridis* where ammonia excretion rates decreased as seawater pH did due to lower catabolism of amino acids (Liu and He 2012). However, the opposite has been seen in the mussel *Mytilus edulis* (Thomsen and Melzner 2010) and juveniles of the clam *Ruditapes decussatus* (Fernandez-Reiriz et al. 2011) with an increase in ammonia excretion being found under OA conditions, leading to a decrease in O:N ratio. Higher amino acid metabolism is energetically less effective, however, in mussels this increase of ammonia through protein breakdown and the following exportation of ammonium can serve as an intracellular pH regulatory mechanism (Thomsen et al. 2010).

Small but significant differences were also observed in the effect size of the toxicity endpoints measured between the resistant Restronguet and non-resistant Exton worms. Overall these data fit with the findings of the previous chapter (Chapter 3) that revealed OA acts to buffer the toxicity effects of low sub-lethal copper exposures, with the effect size for combined OA and copper being negative (i.e. reduced compared to ambient no copper exposures) for all endpoints except DNA damage in the non-resistant Exton worms.

Lipid peroxidation (measured as TBARS) was approximately 30% lower in the resistant Restronguet worms across all treatments compared to the non-resistant worms, with a tendency for a decrease in lipid peroxidation with the addition of copper in both populations. This study has also found that OA and copper have no significant effects on SOD activity for both populations of *H. diversicolor*, with activity being lowest in the combined stressor exposure for both populations. A decrease in lipid peroxidation to combined stressors has previously been reported in polychaetes (Nielson et al. 2019). When comparing effect size to the combined stressors the non-resistant worms had a significantly greater reduction in lipid peroxidation than the Restronguet worms, i.e. a stronger response to combined stressors but in the opposite direction to what might be predicted. This reduction in lipid peroxidation in response to the addition of copper, which is known to induce oxidative stress and therefore might be expected to increase lipid damage, has been reported previously in a number of studies where sub-lethal copper exposures are used, often in parallel with an increase in anti-oxidant enzyme activity. Both mussels and sea urchins have shown increases in lipid peroxidation under combined stressors (OA and copper), with mussels showing both increased and decreases in SOD activity in response to this damage. The mussel *Mytilus edulis*, showed no increase in SOD in response, indicating that anti-oxidant defences may be overwhelmed (Lewis et al. 2016) whereas the mussel *Mytilus coruscus* showed an increase in SOD, although this was not enough to prevent lipid damage (Huang et al. 2018) Although sea urchins (*Paracentrotus lividus*) did increase their SOD activity, this was not enough to prevent lipid damage (Lewis et al. 2016).

It is possible that other antioxidant enzymes, including catalase, glutathione-*S*-transferase and glutathione peroxidases, which have all been found in polychaetes (Lv et al. 2016, Yuan et al. 2010, da Rosa et al. 2005) are being used instead to prevent the lack of lipid peroxidation seen in the combined stressor treatments. These findings are in agreement with previous work in chapter 3 on another polychaete, *Alitta virens*, which found that OA buffers the responses to copper. Levels of lipid peroxidation were again lowest (and not significantly different) when exposed to both OA (pH_{NBS} 7.7) and copper pollution, compared to all other treatment groups. No change in SOD activity was seen in the OA – copper treatment compared to the control (pH_{NBS} 8.10) either (Nielson et al. 2019).

One of the key mechanisms of metal adaptation described for the Restronguet population of *H. diversicolor* is the up regulation of Metallothioneins (MTs) like proteins (Pook et al. 2009). MTs are non-enzymatic proteins which play a vital part in an organism's detoxification defence system. They are able to bind to particular heavy metals, including copper, through their thiol groups, to sequester excess copper away from metabolism (Thirumoorthy et al. 2007, Amiard et al. 2006, Suzuki et al. 2002). MT's can limit the effects of hydroxyl and superoxide radicals by scavenging them (Thirumoorthy et al. 2007, Amiard et al. 2006). The role of MT's in *Hediste diversicolor* from Restronguet Creek has been investigated by McQuillan et al., (2014). It is known that these worms secrete mucus in response to enhanced copper exposure, however, this does not act as an adsorption barrier against excessive metal uptake (Geffard et al. 2005), therefore, another defensive system must be in place. It was found that this resistant population of worms displayed a significantly different gene expression profile compared to populations from 'clean' sites with gene transcripts encoding principle copper homeostasis proteins, including copper ion transporters and MT-like proteins being significantly more abundant (McQuillan et al. 2014). Interestingly transcripts encoding antioxidants (i.e. superoxide dismutase (SOD) and catalase (CAT)) and cellular pathways showed no change in gene expression, meaning it is possible that the hyper activation of copper detoxification pathways (including the increased expression of MT-like proteins) are sufficient in mitigating radical formation caused by copper (McQuillan et al. 2014). This could also help to explain why DNA repair pathways are also unaffected by the high levels of copper in Restronguet Creek (McQuillan et al. 2014) and may be why we see no significant changes in DNA damage even in the face of additional copper. The lack of change in SOD activity measured here may be due to the elevated levels of metallothionein-like proteins that the Restronguet have been shown to have as part of their adaptation to copper, hence it is possible that this pathway protected *Hediste diversicolor* from any copper related damage, making increases in SOD activity unnecessary.

DNA damage was relatively high across all treatments for both populations, ranging from 21.6% to 37.8 in the non-resistant population and 32.4% to 36.6% in the resistant population. High levels have been seen in the polychaete *Alitta virens* previously both in contaminated sediments, where levels reached over 35% (Watson et al. 2018) and to the same level of copper (as the non-resistant population; 0.25 µM) where DNA

damage was 29.8% to 34% (Nielson et al. 2019). Although there was no significant impact on effect size, the average effect size went in different directions for the different populations. DNA damage effect size in the resistant population decreased slightly, compared to the highly significant variability seen in the non-resistant population (meaning these worms are more stressed with some individuals showing high levels of damage). This could be due to the fact that these worms prioritise DNA damage repair, an energetically costly process (Blakefield and Harris 1994), in order to survive in environments with very high copper levels. It is possible that the increase seen in metabolic rate might be due to the increase required for DNA damage and lipid peroxidation repair. In the non-resistant population the highest level of damage is seen in the combined stressor treatment, however, the resistant population has least damage in this treatment, with values below that of non-resistant worms. The increase in damage to both copper and OA, compared to single stressor and control treatments, has been seen in both polychaetes (*Alitta virens)* (Nielson et al. 2019) and in mussels (*Mytilus edulis*) and sea urchins (*Paracentrotus lividus*) with mussel DNA damage rising to 27%.

Bioenergetics play a central role in the tolerance to environmental stress with increasing levels of stress causing the progressive decline of the aerobic scope of an organism (Sokolova 2013). Energetic costs in resistant populations to environmental stressors has been previously documented. Pook et al., (2009), found a reduction in scope for growth in *Hediste diversicolor* (formally *Nereis diversicolor*), also from Restronguet Creek. These worms exhibited a reduction in lipids and carbohydrate storage and, although we did not measure this end point, we see an increase in ammonia excretion coupled with a decreased O:N ratio. The decrease in O:N ratio that we see may be due to the fact that there are less lipids available meaning proteins are used as a substrate for metabolism. Hird et al., 2016, also found a reduction in O:N ratio coupled with a decrease in lipid content whilst looking at the effects of fluoxetine in *H. diversicolor* indicating that the depleted lipid stores are substituted by increased protein metabolism (Hird et al. 2016). Further costs of resistance have been seen in other species. A reduction in fecundity and lower population growth rates have been seen in Killifish (*Heterandria Formosa*) that are resistant to cadmium (Xie and Klerks 2004) and copepods (*Tigriopus japonicas*) resistant to copper (Kwok et al., 2009) with the cost of resistance potentially causing changes in resource allocation

from reproduction to energy demanding processes such as metal detoxification and repair of cellular damage (Xie et al., 2009; Kwok et al., 2009). Increased energy demands due to metal toxicity has also been previously documented. Cadmium has been shown to causes increases in oxygen consumption due to protein synthesis increases, in particular elevated metallothioneins and heat shock proteins (Cherkasov et al. 2006, Sokolova and Lannig 2008, Sokolova et al. 2012). Copper showed a similar affect in oysters (*Saccostrea glomerata*) with a higher aerobic metabolism seen in oysters exposed to this metal (Scanes et al. 2018).

Metal pollution can cause energetic costs to marine organisms through toxicological damage and the resulting repair mechanisms. Environmental stress can also affect the optimal allocation of energy by modulating energy demands for survival (Sokolova 2013). Environmental stressors, including changes in salinity and OA, have also been shown to affect energy budgets in a variety of invertebrates. Shrimp (*Neomysis integer*) exhibited changes in cellular energy allocation with less energy allocated to their reserves at lower salinities compared to shrimp kept at higher salinities (Erk et al. 2008). Resource allocation is also clear to see under OA. The upregulation of metabolism and ability to calcify in the brittlestar (*Amphiura filiformis*) came at a substantial cost of muscle wastage (Wood, Spicer and Widdicombe 2008). Sea urchin larvae (*Strongylocentrotus purpuratus*) exhibited an increase in protein synthesis and ion transport which resulted in 84% of available energy being allocated to these processes under acidification (Pan et al., 2015).

By comparing these two different populations we have seen that there are differences in physiological and toxicity response in worms that are resistant to one stressor and those that aren't. Copper adaptation energetically stresses the Restronguet population and OA subtly alters some of the responses to the combination of OA and copper. However, these changes are small, with toxicity responses being minimally affected, and ultimately OA is not adding strong additional pressure on this population. The lack of data regarding the pH conditions of metal contaminated sediments, where these worms inhabit, may mean that this population may already be exposed and adapted to lower pH conditions. This will be tested in my final chapter.

Table 4.1. Seawater carbonate chemistry and copper levels for all experimental treatments over the exposure period for the Exton population (A) and for the Restronguet population (B). Data as mean ± standard error. Temperature, salinity, and pH were measured daily in each replicate tank. DIC (n = 48) and copper concentration (n = 12) were measured every three days. The other carbonate parameters were calculated using CO2sys.

A.

Treatment	Temperature (ºC)	pH_{NBS}	Salinity	Copper (µM)	Copper (µM) Min/max	TA (µmol/kg)	pCO_2 (µatm)	HCO_3^- (µmol/kg)	CO_3^{2-} (µmol/kg)	ΩCa	ΩAr
8.10 + Cu	14.36 ± 0.09	8.13 ± 0.01	22.32 ± 0.13	0.029 ± 0.005	0.020/0.039	2117.8 ± 54.1	467.2 ± 14.3	1881.5 ± 46.3	99.3 ± 4.3	2.6 ± 0.1	1.6 ± 0.07
8.10	14.33 ± 0.13	8.10 ± 0.01	22.11 ± 0.14	0.006 ± 0.008	0.002/0.014	2095.1 ± 54.1	500.6 ± 15.2	1876.5 ± 46.2	91.8 ± 4.0	2.4 ± 0.1	1.4 ± 0.07
7.70 + Cu	14.64 ± 0.13	7.70 ± 0.01	22.41 ± 0.13	0.031 ± 0.020	0.021/0.049	1992.6 ± 10.0	1297.0 ± 15.9	1903.5 ± 46.6	37.0 ± 0.4	1.0 ± 0.1	0.6 ± 0.07
7.70	14.54 ± 0.13	7.69 ± 0.01	22.51 ± 0.13	0.0006± 0.011	0.0002/0.012	1978.9 ± 54.6	1319.5 ± 16.8	1892.4 ± 46.6	35.6 ± 0.5	0.9 ± 0.1	0.6 ± 0.07

B.

Treatment	Temperature (ºC)	pH_{NBS}	Salinity	Copper (µM)	Copper (µM) Min/max	TA (µmol/kg)	pCO_2 (µatm)	HCO_3^- (µmol/kg)	CO_3^{2-} (µmol/kg)	ΩCa	ΩAr
8.10 + Cu	14.27 ± 0.12	8.14 ± 0.02	22.10 ± 0.25	0.053 ± 0.038	0.031/0.091	2224.2± 67.1	489.5 ± 18.1	1978.9 ± 49.8	103.9 ± 8.1	2.7 ± 0.2	1.6 ± 0.13
8.10	14.43 ± 0.17	8.11 ± 0.02	21.93 ± 0.23	0.006 ± 0.011	0.003/0.017	2115.6 ± 76.4	509.8 ± 18.4	1931.7 ± 59.4	95.5 ± 7.9	2.5 ± 0.2	1.5 ± 0.13
7.70 + Cu	14.43 ± 0.09	7.67 ± 0.02	22.53 ± 0.12	0.069 ± 0.011	0.042/0.082	2212.2 ± 95.7	1519.6 ± 86.9	2119.8 ± 91.6	38.9 ± 2.6	1.0 ± 0.7	0.6 ± 0.04
7.70	14.460± 0.10	7.696 ± 0.01	22.40 ± 0.15	0.009 ± 0.028	0.002/0.021	2114.6 ± 76.7	1488.9 ± 18.0	2027.5 ± 71.2	36.5 ± 2.6	0.9 ± 0.6	0.6 ± 0.03

Chapter Five

Sediment dwelling *Alitta virens* are already exposed to reduced pH with sediments buffering against pH changes of overlying seawater and periods of tidal emersion.

5.1. Introduction

Oceans are changing at an unprecedented rate meaning that understanding organism's responses to their environment is increasingly important to making predictions about responses to global change. Predictions made for open ocean pH levels are not an accurate reflection for coastal and estuarine waters, with these waters being exposed to an array of processes, other than OA, that can alter seawater pH. These process include eutrophication, upwellings and long term changes in the geological cycle of CO_2 (Duarte et al. 2013). Coastal and estuarine waters are already exposed to highly variable pHs when compared to open oceans, which maintain a stable pH level of around 8.10. For example, the Elkhorn Slough tidal estuary and Monterey Bay exhibit pH lows of 7.45 and 7.86, with pH levels ranging by 0.99 and 0.50 units respectively (Hofmann et al. 2011). Even lower pH levels have been seen in the Hood Canal (part of the Puget Sound estuary) with deep water (<75 m) having pH levels of 7.39 in the summer time, again this estuary shows variability with the pH increasing to 7.56 in winter time (Feely et al. 2010).

Many organisms, including many ecologically important polychaete species, not only inhabit these waters but the sediment in which these waters flow over, adding another complex dynamic to this environment. The geochemistry of marine sediments is controlled by both the composition of the material initially deposited in the sediments and the chemical, biological or physical processes that affect this material after its deposition (Burdige 2007). These processes are in part effected by the activities of sediment dwelling organisms which continually mix the porewater solutes and sediment particles in a process known as bioturbation (Richter 1952), allowing organic material to be repeatedly exposed to oxygen (Jørgensen and Kasten 2006). The

infauna disproportionally influence biogeochemical reactions through burrowing, feeding, ventilatory and locomotory behaviour which promote the vertical and lateral distribution of sediment particles (Solan et al. 2004) and transport of oxygen, extending the oxic zone deeper into the sediment (Jørgensen and Kasten 2006).

Shallow-water sediments are particularly important as they provide a significant contribution to many biogeochemical processes, including providing a large proportion of nutrients for primary production (Gazeau et al. 2014). In these shallow waters, the chemistry of the coastal ocean is strongly coupled to sediment biogeochemical processes, with the carbonate dissolution at the sediment-water interface buffering the pH of the water column (Rassmann et al. 2018) Organic matter is exported from the ocean surface with 25% of it sinking onto the sediment, although this shows significant regional variability (Lutz et al., 2002) and with this input of organic detritus being one of the main sources of carbon to burrowing fauna (Ravaglioli et al. 2019a). The importance of sediment is highlighted here where 90% of the organic matter that reaches it is demineralised via oxic or anoxic pathways (Burdige 2006). However, this remineralization releases metabolic CO_2 which can lead to local acidification of bottom water (Rassmann et al. 2016), thus changing the pH organisms living here are exposed to. Sediments represent a highly complex environment being governed by many processes. These sediments already naturally experience local changes in pH levels with the impact of anthropogenic changes having the potential to cause further changes.

Anthropogenic stressors, including global warming, oxygen depletion and OA, can alter the structure and functioning of infaunal communities (Ravaglioli et al. 2019a). The distribution of CO_2 and pH within marine sediments is largely controlled by microbially mediated redox reactions that are linked to the mineralization of organic matter as well as abiotic processes. The amount of CO_2 and therefore the sediment pH can vary enormously between different sediments (Widdicombe et al., 2011). Previous studies have shown that OA can significantly alter meiobenthic assemblage structures and enhance organic matter degradation rates in seagrass sediments (Ravaglioli et al. 2019b) as well as increase uptake of algal detritus (Ravaglioli et al. 2019a), leading to excess oxygen consumption in deep water (Oschlies et al. 2008). However, sediments (undisturbed cores) in the Arctic, showed no changes in

mineralization and denitrification when exposed to decreasing pH levels of the overlying water under laboratory conditions (Gazeau et al. 2014). It is worth noting that these sediments were exempt of large dwelling organisms which play key roles in bioturbation.

Alitta virens are found in inter-tidal zones where they face a constantly changing environment as the tide goes in and out, alternating between immersion and emersion for varying amounts of time dependent on the height of the intertidal zone they inhabit. Studies looking at the effect of this on an organism's acid-base physiology have largely focused on sessile bivalves. Oysters and mussels are unable to relocate during emersion but are able to close their shells, which stops the gas exchange between the organism and the external medium (Allen and Burnett 2008). These organisms are also unable to feed during this time and their body temperature is influenced by the external temperature often changing drastically when compared to body temperatures during immersion (Helmuth 1998, Mangan et al. 2019). During emersion, both mussels and oysters experience acidosis, due to an increase in haemolymph pCO_2 (Allen and Burnett 2008, Dwyer and Burnett 1996, Mangan et al. 2019), with the oyster *Crassostrea virginica* showing some buffering capacity from an increase in calcium ions (Dwyer and Burnett 1996). Mangan et al., 2019 found that air temperature and mussel size strongly influenced this acidosis in the mussel *Mytilus edulis*, with larger mussels and higher temperatures resulting in the greatest acidosis. There was also a strong tidal effect with periods of emersion having a greater physiological impact on acid-base balance than OA did, therefore, looking at tidal conditions is paramount to understanding responses to future OA (Mangan et al. 2019).

Seasonal variability of ocean carbonate chemistry is projected to change substantially as atmospheric CO_2 concentrations continue to rise and may worsen the impacts of climate change on marine organisms (Kwiatkowski and Orr 2018). This is particularly relevant to inter-tidal zones as this environment exacerbates the hypercapnic effects of elevated pCO_2. The distribution of some organisms, especially sessile ones, may be impacted with those inhabiting the upper shores being most effected where emersion is greatest. In future conditions the upper vertical limit of the distribution on the shore of the Sydney rock oyster, *Saccostrea glomerata*, may be reduced as the physiological limits of these organisms may be reached, meaning they may not be

able to exist as high on the shore line as they currently do (Scanes et al. 2017). Not all inter-tidal living organisms are sessile. Crustaceans are capable of making rapid transitions between aquatic and aerial environments, with some species remaining inactive upon low tide whilst some remain active regardless of the stage of the tide (Burnett 1988). The porcelain crab, *Petrolisthes cincipes*, is found in the inter-tidal zone and when exposed to pH and temperature extremes experience negative impacts due to reduced energy for behaviour and reproduction (Paganini et al., 2014). Another non sessile group of organisms are polychaetes. Little research has been undertaken into looking at the tidal effects on these burrowing worms. *Arenicola marina*, the lugworm, cannot irrigate its burrow at low tide and it remains isolated in its burrow, depleting the oxygen reserves and relying primarily on anaerobic respiration. During emersion, both a metabolic and respiratory acidosis occur, with blood pH decreasing from 7.48 to 7.35. However, the acidosis is minimized by the Haldane effect, which increases the removal of CO_2 (Burnett 1988, Toulmond 1973).

Sediments are the main sink and source of heavy metals in aquatic habitats, with more than 90% of heavy metal load in these systems being related to suspended particles and sediments (Zhang et al. 2014, Amin et al. 2009). Estuarine systems receive enhanced inputs of terrestrial organic matter which has important metal binding capabilities (Buffle, Tessier and Haerdi 1984). This organic matter, along with texture and geology of the sediment, organism behaviour and the variation of pH all influence the bioavailability of metals (Zhang et al. 2014). Grain size also influences heavy metal concentrations with finer sediments containing more heavy metals than coarser ones due to smaller grain size particles having a larger surface area-to-volume ratio (Amin et al. 2009, Martincic et al., 1990, Salomons and Förstner 1984).

Both pH and salinity are recognized as the key variables that control the bioavailability and toxicity of heavy metals bound to sediments (Riba et al. 2004). With the increase of atmospheric CO_2 resulting in decreasing seawater pH levels, the impact of this on metal availability and toxicity is important to consider. Acidification has been found to increase the bioavailability of metals (Basallote et al. 2020), including the mobility of cadmium, lead, copper and zinc (Riba et al. 2003, Riba et al. 2004, Roberts et al. 2013) with a decrease in pH producing a high percentage of free ions for all four metals. This decrease of pH, coupled with an increase in salinity, increases the toxicity

associated with the heavy metals bound to sediments to the clam *Ruditaps philippinarum* (Riba et al. 2003). Similar effects of OA on contaminated sediments (with heavy metals) have also been seen, where increased pCO_2 increased the flux of metals from sediment bound to labile state, resulting in these sediments becoming more toxic to *Corophium volutator* with a 2.7 fold increase in DNA damage (Roberts et al. 2013). However, this toxicological interaction could not be explained by effects of pH on metal speciation and instead it is the physiological effects of OA and contaminants that will determine the responses of benthos (Roberts et al. 2013).

Given the impact of both tidal cycles and sediments on metal toxicity and acid-base physiology it is important to look at realistic exposure scenarios when looking at inter-tidal benthic organisms. A more complex but environmentally realistic habitat of sediment was used to see how they influence the interactions of stressors as well as looking at the influence of tides, which bring both periods of emersion and immersion, on polychaetes. Here we expose the ragworm *Alitta virens* to OA and copper both singularly and in combination measuring both the acid-base capacity of this polychaete and toxicity end points. The organisms will be in sediment for the duration of the exposure and a tidal cycle will be recreated, mirroring the conditions of emersion and immersion that this population of *Alitta virens* experience in their natural habitat. We test the hypothesis that the pH of the sediment would decrease during low tide resulting in greater physiological and toxicity effects during this time.

5.2. Methods

5.2.1 *Preliminary field measurements of tidal sediment pH range*

Preliminary data on sediment porewater pH for a representative rag worm bed over a period of tidal emersion was collected from Exton, Devon, England (50.6681° N, 3.4381° W) on the 7th August 2017, a spring high tide, where tides were at their maximum. Measurements were taken as soon as the sediments were uncovered and were repeated hourly until sediments were covered again to get the most reflective measurements of a full tidal range. It was not possible to take measurements once the sediments were covered. Sediment interstitial pH_{NBS} was measured using a Mettler Toledo InLab Solids pH probe calibrated using Mettler Toledo pH buffers. The probe

was carefully inserted into the sediment to a depth of approximately 5 cm during the emersion from a time period of 2 hours before low tide to 2 hours after low tide. Measurements were made every 10 meters from the top of the shoreline to a maximum of 150 meters.

5.2.2. *Animal collection and maintenance*

Due to difficulty in obtaining worms from Exton adult *Alitta virens* and sediment were collected from a nearby site at Teignmouth, Devon, England (50.5404° N, 3.5117° W) during March 2019 by carefully digging them from the intertidal mud (mid to high shore) with a fork. Only immature adults were collected, with gravid worms avoided. The worms were maintained in a holding tank in natural sediment, sieved to a size of 1mm, from the same site in a temperature controlled room (15 °C) at a salinity of 34 ppt for 48 hours before the exposure started.

5.2.3. *Preliminary experimental set-up testing*

A preliminary experimental test was set up to look at the sediment pH changes over 7 days in the aquarium in temperature controlled rooms of 15^{o}C. Tanks (5L) were filled with 5 cm of sediment from the field site (Teignmouth) and set up in three differing Seawater pH_{NBS} treatments; 8.10, 7.70 and 7.30, with 10 tanks per treatment. Each tank had its own individual air stone and seawater pH_{NBS} in the two OA treatments (7.70 and 7.30) was maintained using an Aalborg Mass Flow Controller GFC set at the correct ratio of air to CO_2. Per treatment the lay out of the tanks was as follows; 7 contained sediment and 4 worms each, 2 contained just sediment (no worms) and finally one tank was left blank (just seawater) as a control. Seawater pH (SevenGo Duo, pH/conductivity meter SG23) and sediment pH_{NBS} (Mettler Toledo InLab Solids pH probe calibrated using Mettler Toledo pH buffers) samples were taken daily over the course of 7 days.

5.2.4. *Experimental design*

In order to test the effects of OA and copper over a tidal cycle, worms were placed into tanks containing natural sediment from their collection site under simulated tidal

conditions, with the overlying seawater manipulated to one of the four following treatments to be comparable to the work of the previous chapters; (1) ambient seawater pH conditions (pH_{NBS} 8.10) with no added copper, (2) ambient seawater pH conditions (pH_{NBS} 8.01) with 0.25 µM copper sulphate added to the water, (3) near-future OA conditions (pH_{NBS} 7.70, the expected pH under RCP 8.5, IPCC AR5) with no added copper, (4) near-future OA conditions (pH_{NBS} 7.70) with 0.25 µM copper sulphate added. Individuals were kept at 15 °C for 14 days in their tanks. Twelve worms per treatment, maintained 4 per tank (3 tanks per treatment), were then transferred into experimental tanks set up to mimic a tidal cycle.

Tanks were set up to establish a tidal cycle of seawater inundation and then emersion as shown in Figure 5.1. Smaller 5 litre plastic tanks each filled with 5 cm of natural sediment from the collection site were placed inside a bigger 20 litre tank to allow the smaller tank to completely empty during the tidal cycle. To each of the smaller 5 litre tanks 4 worms were added and each tank was set to an angle to allow the water to fully drain through the front of the tanks, where a large hole was made covered by a fine mesh. This mesh was fine enough to prevent sediment being washed away but large enough to allow water to drain. Water was pumped into the larger tank, until the smaller tank was submerged (15 litre), from a holding tank, maintained at either pH 8.10 or pH 7.70, via the bigger tube on the right and exited via the tube on the left using the small pump seen in figure 5.1. At the end of each high tide, water was pumped out of the system into the waste system, with fresh seawater being used the following high tide. The holding tank was kept constantly topped up. The tidal cycle was set using a 24-hour Status Timer Switch, with high tide (water covering) lasting for 5 hours and low tide (sediment exposed) for 7 hours. These hours represent the natural tidal cycle at the location the organisms were collected. Each pump (both in and out) took 15 minutes to fill or to empty the tanks.

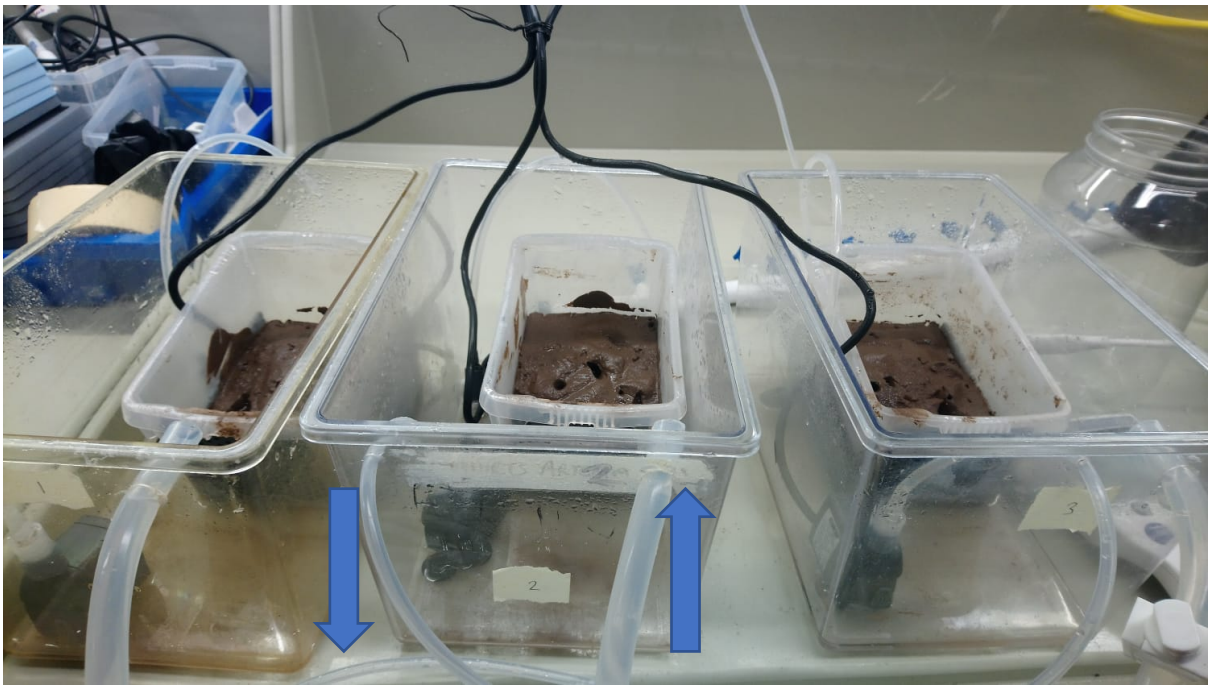


Figure 5.1. The experimental exposure set up, showing the 3 tanks for treatment 1 at low tide. The blue arrows indicate water flowing in from the header tank and water flowing out using the small pump seen inside the larger tank. This experimental system enables a simulated tidal cycle of emersion and immersion for a mud flat, using seawater from a header tanks maintained at either pH 8.10 or pH 7.70.

This experimental design was repeated twice to ensure there was enough repeats. This resulted in a total of 48 worms (12 per treatment) being used for both the acid-base physiology analysis (24 at the start of high tide and 24 at the end) and the Comet assay. There was a total of 96 worms analysed for oxidative stress end points.

5.2.5 *Seawater manipulation*

Artificial seawater (Tropic Marine©) was used to fill the individual tanks, from the main holding tanks (one holding tank per pH treatment) with the seawater in both individual tanks and the main holding tanks being manipulated to the required pH_{NBS}. Seawater pH_{NBS} values of 8.10 (with resulting pCO_2 of 487.9 µatm) and 7.70 (pCO_2 1357.2 µatm) were targeted, representing current and near future OA treatments respectively according to the IPCC WGI AR5 RCP 8.5 scenario (Stocker 2013, Meinshausen et

al. 2011). Full seawater chemistry is provided in Table 5.1. Seawater pH_{NBS} in the OA conditions was maintained at 7.70 using an Aalborg Mass Flow Controller GFC set at the correct ratio of air to CO_2 and bubbled separately into each individual tank as well as the holding tank. For the ambient treatments, each tank, including the holding tank, had an individual air stone to keep them fully aerated and at the correct pH. Seawater pH (SevenGo Duo, pH/conductivity meter SG23), temperature and salinity, maintained at 34ppt, (SevenGo Duo, pH/conductivity meter SG23) were measured daily in the holding tanks and the individual exposure tanks. Sediment pH_{NBS} (Mettler Toledo InLab Solids pH probe, as above) was measured daily at the start of emersion, mid low tide and end low tide.

Water samples were taken every third day throughout the experiment (48 for DIC, 6 for copper in total), from random tanks from each treatment. Samples were taken to make sure they covered both the start and end of high tide in order to best represent the exposure condition experienced by the worms over this period to make sure all exposure conditions were represented. Water samples for DIC analysis were preserved (0.04 % of final volume) with 4 % mercuric chloride for storage prior to analysis (Dickson 2007a) whilst samples for metals analysis were added to acid-washed 50 ml tubes and acidified using 50 µl of concentrated hydrochloric acid. Seawater DIC analysis was carried out using a bespoke system based on that described by Friederich et al. (2002) and using Dickson seawater `standards, as described in detail in Lewis et al. (2013a). Total alkalinity (TA) and pCO_2 were calculated from the measured values of pH_{NBS} and DIC using CO2sys, applying the constants from Mehrbach et al., (1973) and the KSO_4 dissociation constants from Dickson (1990b). The concentrations of copper in the seawater samples were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) using an Agilent 7900 Spectrometer utilising a collision cell with helium as the collision gas to minimise polyatomic interferences. Copper standards, matrix matched to the samples, at concentrations of 1, 10 and 100 mg/l respectively, were used for instrument calibrations. Analysis employed in-line addition of scandium as an internal standard and calibration of the ICP-MS was validated by the use of a quality control standard of 10 mg/l.

5.2.6 *Physiological responses*

After 13 days of exposure to the four seawater treatments worms were removed from their sediment and analysed for acid –base responses to the varying tidal cycle at two time points; 1) at the point at which the sediment became aerially exposed (i.e. following 5 hours of submergence) and 2) at the end of the 'low tide' period, i.e. after 7 hours of the sediment being aerially exposed. At each time point, 6 worms per treatment were analysed for acid-base physiology (below). Worms were removed from their tanks and samples of coelomic fluid were collected from each individual worm using an 18 gauge needle and 1 ml syringe carefully inserted into the coelomic cavity of the organism, working from the anterior region to the posterior region. Coelomic fluid was immediately analysed for acid-base physiology.

Immediately following sample collection, the pH_{NBS} of *A. virens* coelomic fluid samples (taken as above) was measured at 15 °C in microcentrifuge tubes using a Metrohm 826 pH mobile pH electrode and meter calibrated using NBS buffers. After this measurement, 50 µl was stored in a micro capillary tubed which was sealed with paraffin oil at one end and Critoseal® sealant at the other and put immediately in a falcon tube on ice. After all samples were complete they were then analysed for TCO_2 following collection using a Mettler Toledo 965D Carbon Dioxide Analyser. Fifty microlitres of a 10 mM $NaHCO_3$ standard was used between each coelomic fluid sample for calibration purposes. Acid-base parameters were then calculated using a modified version of the Henderson-Hasselbalch equation, as described in Lewis et al., 2016 paper (Lewis et al. 2016), which had used previously calculated constants from Truchot, 1976, based on the crab, *Carcinus maenas* (Truchot 1976).

5.2.7. *Toxicity responses*

The remaining worms were sampled the following day after 14 days in the treatment conditions for their toxicity responses (48 worms (12 per treatment) were analysed for DNA damage using the Comet assay with coelomic fluid being collected as described above.

All worms (96 in total, 24 per treatment) were snap-frozen in liquid nitrogen for later use in the oxidative stress assays. Prior to use, frozen worms were defrosted then

homogenised in PBS buffer using a hand-held homogeniser, centrifuged at 10,000 g for 20 minutes then the supernatant frozen until use at -20 °C.

5.2.8. *DNA damage*

DNA damage was measured as single strand breaks using the comet assay. From the coelomic fluid collected as described above, 100 µl from each individual worm was used immediately for the comet assay. Comet assay was run as described in chapter 2.

5.2.9. *Oxidative stress endpoints*

Superoxide dismutase (SOD), an antioxidant enzyme, was measured using an assay which generates O_2- and uses nitroblue tetrazolium (NBT) which changes colour, from clear to purple, when it comes in contact with a free radical. SOD inhibits this colour change hence levels can be quantified by determining the level of inhibition of this colour change in a sample compared to a standard (Beaucham and Fridovic 1971). The assay was run as per methods described in chapter 2.

The thiobarbituric acid reactive substances (TBARS) assay (Camejo 1998) was used to determine lipid peroxidation which quantifies malondialdehyde, a secondary product of lipid peroxidation, via its reaction with thiobarbituric acid (Lewis et al. 2016). The assay was run as per chapter 2.

5.2.10. *Statistical analysis*

All data was analysed for normality using the Shapiro-Wilk test. Preliminary laboratory data was analysed using a 2-way analysis of variance (ANOVA) general linear model with ‘pH’, ‘worm presence’ and pH x worm presence’ as fixed factors. Data for pCO_2, bicarbonate and TBARS was first normalised using the log transformation and DNA damage data was normalised using the arcsine transformation. Normal data (DNA damage, TBARS pCO_2, bicarbonate and pH) was analysed using a 2-way analysis of variance (ANOVA) general linear model with ‘pH’, ‘copper’ and ‘pH x copper’ as fixed factors. Non-normal data (SOD) was analysed using Scheirer-Ray-Hare test (a non-

parametric alternative to the 2-way ANOVA), an extension of the Kruskal-Wallis test. Tukey's post-hoc test was carried out on all data. All statistical analysis was performed using SPSS software.

A t-test (independent-sample t test) was used to check there was no difference between part 1 and 2. All end points measured showed no significant difference between each part of the exposure. T-tests were also used to compare all acid-base physiology data from the start of low tide to the end of low tide.

5.3. Results

5.3.1. *Preliminary field measurements of tidal sediment pH range*

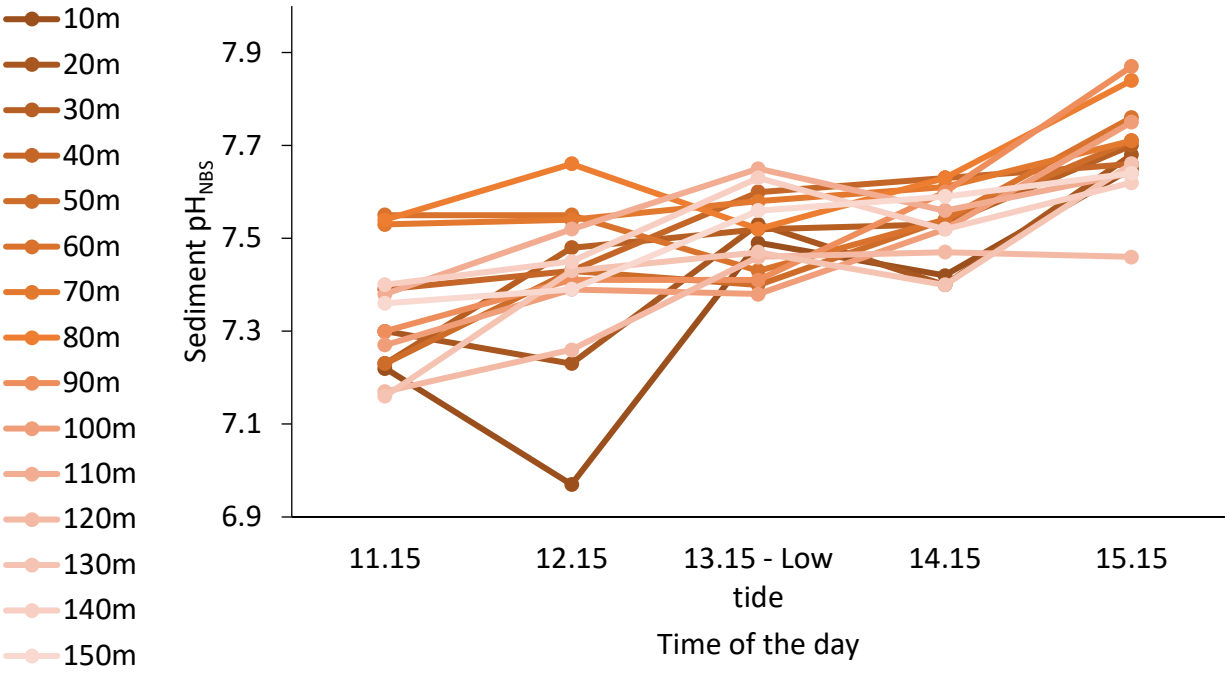


Figure 5.2. The field sediment pH_{NBS} measurements taken on a spring high tide at hourly intervals from two hours before low tide to two hours after low tide at Exton, Devon, England. Measurements were taken every 10 m from the top of the shoreline down to 150 m (lower shore) as soon as the sediments were uncovered, up until re-immersion occurred. Low tide was at 13.15 hrs.

5.3.2. *Preliminary experimental set-up testing*

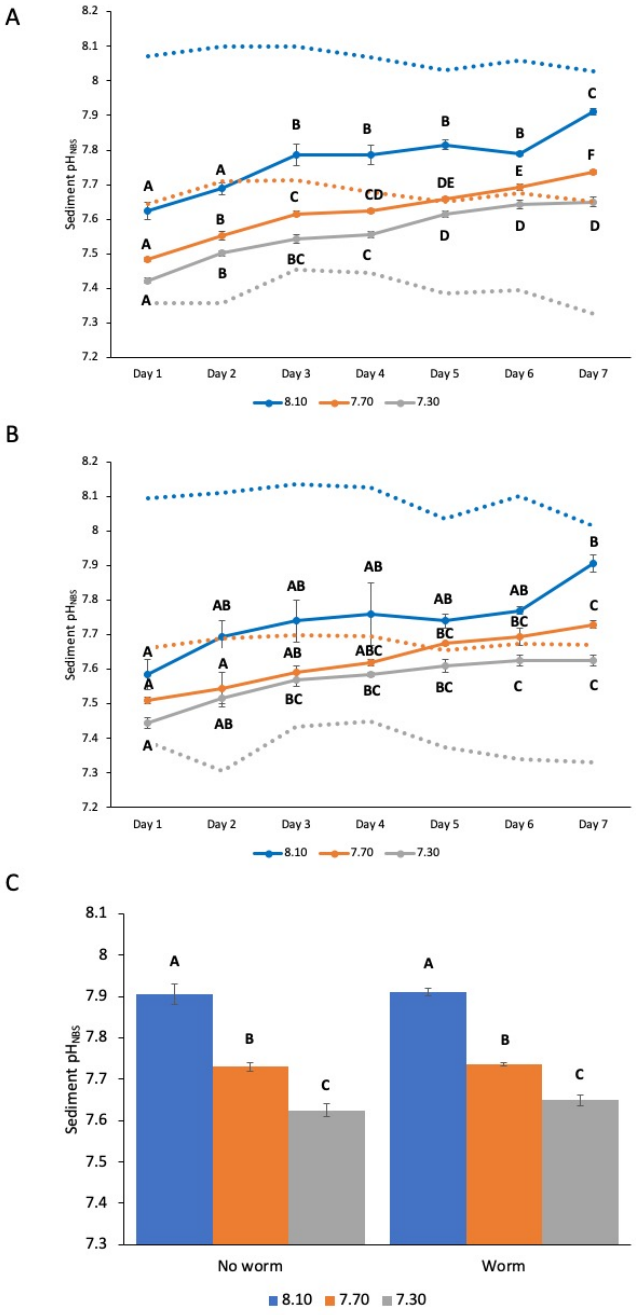


Figure 5.3. The pH of the sediment exposed to 3 different overlying static (no tidal cycle) seawater pH_{NBS} levels; 8.10, 7.70 and 7.30 over a 7-day interval (data as mean ±SE); (A) in the presence of *Alitta virens* and (B) without the presence of *Alitta virens*. Seawater pH is represented with dashed lines for each treatment; (C) shows the sediment pH in both treatments (with and without *Alitta virens*) after 7 days. Differing letters represent significant differences between the four treatments based on Tukey's post-hoc ($p<0.05$).

The sediment pH of all treatments increased over the duration of the exposure for exposures with and without worms. The sediment pH in the ambient pH 8.10 seawater treatment remained below seawater pH for the entire exposure, reaching a high of pH 7.90 by day 7, i.e. a relative pH difference between seawater and sediment pH of –0.2 pH units. The sediment pH in the near-future OA treatment (pH 7.70) stayed below that of the overlying water until day 7 where it reached 7.73 in sediment both with and without worms, i.e. a relative difference of +0.03 pH_{NBS} units between seawater and sediment. In the final seawater pH treatment of 7.3, the sediment pH remained above this value throughout the exposure, increasing from 7.42 (with worms)/7.44 (without worms) to 7.65/7.63, i.e. a relative difference at day 7 of = +0.35/0.33 pH units. There were significant differences between each day for all seawater pH's in both treatments with or without worms except treatment 8.10 without worms (one-way ANOVA, $F = 3.771$, $p = 0.053$). The remaining pH treatments without worms were highly significant (one-way ANOVA pH 7.7, $F = 13.580$, $p = 0.002$), (one-way ANOVA pH 7.3, $F = 14.545$, $p = 0.001$). All pH treatments with worms had significant differences between days (one-way ANOVA pH 8.1, $F = 19.771$, $p < 0.001$), (one-way ANOVA pH 7.7, $F = 108.871$, $p < 0.001$), (one-way ANOVA pH 7.3, $F = 52.975$, $p < 0.001$). A Tukey's post hoc reveals which days were different from each other as pH increased over time.

There was a significant difference on day 7 between pH groups in both sediments without worms (one-way ANOVA, $F = 63.18$, $p = 0.004$) and with worms (one-way ANOVA, $F = 152.68$, $p < 0.001$). A Tukey's post hoc reveals that pH 8.10 was significantly different to pH 7.70 both without and with worms ($p = 0.012$/$p < 0.001$) and pH 7.3 ($p = 0.003$/$p < 0.001$) and that pH 7.7 was significantly different to pH 7.30 ($p = 0.050$/$p < 0.001$). (Figure 5.3. A and B).

At day 7 there was a significant difference in pH (two-way ANOVA for pH, $F = 148.8$, $p = <.001$), there was however, no significance between sediments containing worms and those not containing worms (two-way ANOVA for worm presence, $F = 0.907$, $p = 0.352$) with both exposures showing very similar pH levels to the different seawater pH treatments. There was no significant interaction between pH and the presence of worms (two-way ANOVA for pH*worm presence, $F = 0.236$, $p = 0.792$). A Tukey's post hoc reveals that both ambient treatments were significantly different to both pH 7.70

treatments, which were both significantly different to both treatments at a pH of 7.30. (Figure 5.3. C).

5.3.3. *Seawater chemistry*

The seawater carbonate chemistry from every treatment, along with copper levels are summarised in Table 1.

5.3.4. *Sediment pH*

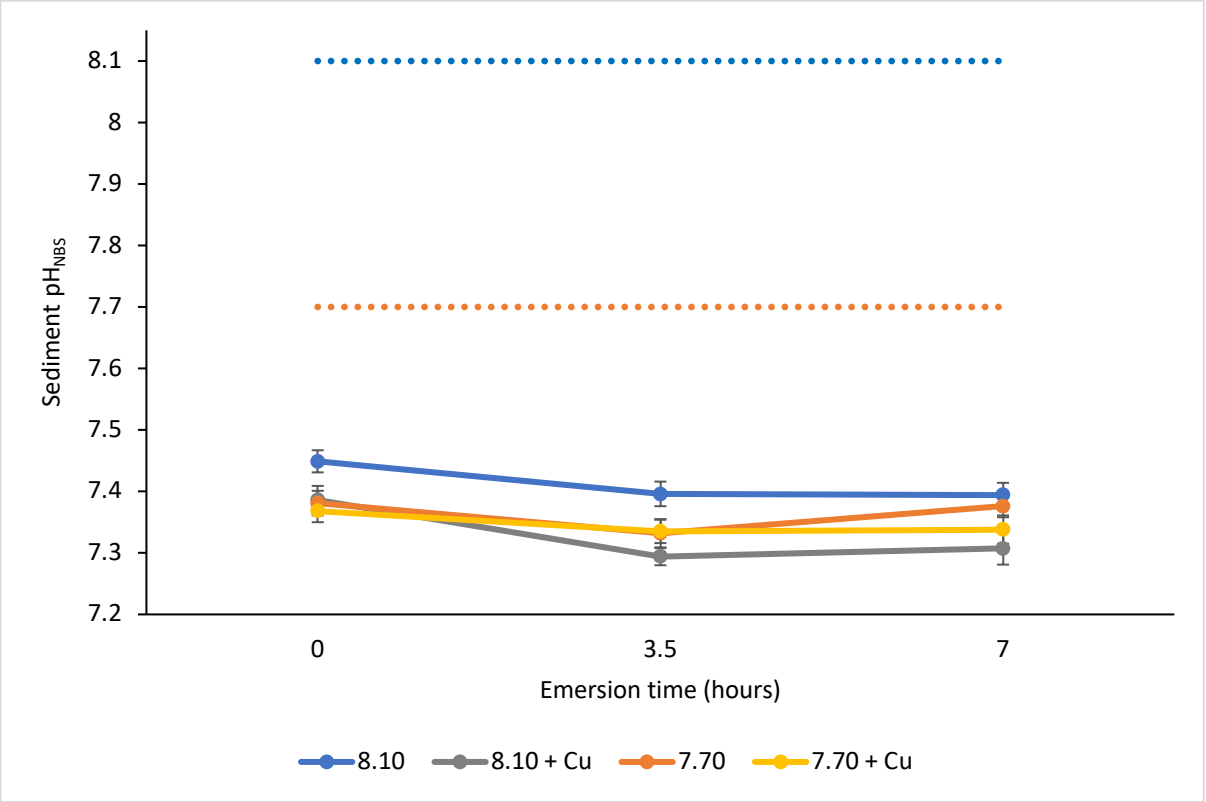


Figure 5.4. The change in sediment pH_{NBS} over a 7-hour emersion period from the start of low tide (emersion) in four different treatment groups; ambient pH_{NBS} (8.10), ambient pH and 0.25 µM copper, near future OA (pH_{NBS} 7.70), OA and 0.25 µM copper over a 14-day exposure period. Data as mean (±SE). Seawater pH is represented with dashed lines for each treatment.

In contrast to the pattern revealed from the *in situ* field measurements, in all treatment groups, sediment pH_{NBS} in the aquarium exposure set up decreased between the start of emersion (low tide) and the end of emersion, with the ambient treatment and copper

showing the biggest decrease from a pH_{NBS} of 7.39 to 7.31. This decrease was significantly different to the start of emersion for both ambient treatments, with (independent sample t-test, t = 2.273, p = 0.027) and without copper (independent sample t-test, t = 2.079, p = 0.043) but no significant decrease was seen in the OA treatments, with (independent sample t-test, t = 1.026, p = 0.310) and without copper (independent sample t-test, t = 0.239, p = 0.812.). (Figure 5.4)

After 7 hours of emersion sediment pH_{NBS} with overlying ambient seawater pH_{NBS} (8.10, without copper) was 7.39, with the OA treatment (without copper) falling slightly to 7.38. In copper treatments the opposite trend was seen with OA seawater having a slightly higher sediment pH_{NBS} of 7.34, compared to ambient seawater where the sediment pH_{NBS} was 7.31. The changes in sediment pH_{NBS} due to seawater pH_{NBS} was not significant (two-way ANOVA for pH, F = 0.0093, p = 0.761). The addition of copper did however, cause a significant decrease (two-way ANOVA for copper, F = 8.162, p = 0.005) in sediment pH_{NBS}, with the biggest effect being seen in ambient conditions where sediment pH_{NBS} fell from 7.39 to 7.31. There was no significant pH*copper interaction (two-way ANOVA for pH*copper, F = 1.263, p = 0.264).

The difference between treatment groups relative to the overlying seawater pH_{NBS} is much smaller within the sediments, with sediment pH_{NBS} in the ambient seawater (8.10) treatments being 7.39 (no copper) and 7.34 (with copper) after 7 hours of emersion, a difference of -0.71 and -0.76 pH units respectively. The difference between seawater pH_{NBS} and sediment pH_{NBS} was significantly different for ambient conditions with (independent sample t-test, t = 39.647, p < 0.001) and without copper (independent sample t-test, t = 41.985, p < 0.001). For OA treatments the difference was also significant with (independent sample t-test, t = 19.309, p < 0.001) and without copper (independent sample t-test, t = 20.105, p < 0.001).

In the OA treatments, the pH_{NBS} of the sediment at the end of emersion was 7.38 (no copper) and 7.34 (with copper), a difference of -0.32 and -0.36 pH units. The ambient with copper treatment actually had the lowest sediment pH_{NBS}.

5.3.5. *Acid-base physiology*

For all treatments, both at the start and end of low tide, coelomic fluid pCO_2 stayed below 9 mmHg except in the combined OA and copper treatment at the end of emersion where coelomic fluid pCO_2 levels rose to 16.28 ± 6.08 mmHg. There was, however, no significant difference between the start of low tide and the end of low tide (independent-sample t test) in any of the four treatment group for worm coelomic fluid pCO_2.At the start of low tide all coelomic fluid pCO_2 levels were between 3.90 ± 0.91 mmHg and 8.77 ± 2.20 mmHg and there was no significant effect of seawater pH (two-way ANOVA for pH, F = 0.932, P = 0.346). Copper caused a small decrease in coelomic fluid pCO_2 levels from 7.03 ± 1.93 mmHg to 3.90 ± 0.91 mmHg in ambient seawater conditions and from 8.77 ± 2.20 mmHg to 5.46 ± 1.52 mmHg, this decrease was not significant (two-way ANOVA for copper, F = 3.546, P = 0.074). There was also no significant interaction effect (two-way ANOVA for pH*copper, F = 0.003, P = 0.957). (Figure 5.5 A).

At the end of low tide copper caused a large, but not significant, increase in the OA treatment with worm coelomic fluid pCO_2 levels rising to 16.28 ± 6.08 mmHg (OA with copper) from 4.16 ± 1.35 mmHg (two-way ANOVA for copper, F = 3.253, P = 0.086). There was no significant effect of OA (two-way ANOVA for pH, F = 1.166, P = 0.293) or an interaction effect (two-way ANOVA for pH*copper, F = 3.433, P = 0.079). (Figure 5.5 A).

Worm coelomic fluid bicarbonate levels were higher in all treatments bar OA (without copper) at the end of low tide compared to the start of low tide with the biggest difference seen in the combined stressor treatment where levels more than doubled over the duration of emersion. Despite this there was no significant difference between the start of low tide and the end of low tide (independent-sample t test) in any of the four treatment groups for bicarbonate levels.

At the start of low tide, copper caused a decrease in bicarbonate levels in both the ambient, from 7.28 ± 2.72 mmol to 3.53 ± 0.59 mmol, and OA treatments, from 7.65 ± 1.92 mmol to 6.72 ± 0.88 mmol. This effect was not significant (two-way ANOVA for copper, F = 1.799, P = 0.195). There was also no significant effect on either OA (two-

way ANOVA for pH, F = 1.038, P = 0.320) or the interaction between copper and OA (two-way ANOVA for pH*copper, F = 0.652, P = 0.429). (Figure 5.5 B).

At the end of low tide bicarbonate levels rose to their highest level of 14.41 ± 3.54 mmol under OA conditions, with the addition of copper from 5.07 ± 1.93 mmol (OA, without copper). The opposite was seen in the ambient treatments where copper decreased bicarbonate levels from 9.68 ± 3.82 mmol to 5.06 ± 0.73 mmol. Neither copper (two-way ANOVA for copper, F = 0.708, P = 0.410) or OA (two-way ANOVA for pH, F = 0.715, P = 0.408) caused significant effects, however, there was a significant interaction between copper and OA (two-way ANOVA for pH*copper, F = 6.203, P = 0.022). (Figure 5.5 B).

Coelomic fluid pH_{NBS} stayed between 7.27 and 7.50 across all treatment groups. The combined stressor pH_{NBS} decreased over emersion time from 7.50 to 7.42, whereas, in all other treatments coelomic fluid pH_{NBS} increased during this time. Again, there was no significant difference between the start of low tide and the end of low tide (independent-sample t test) in any of the four treatment group for coelomic fluid pH_{NBS}.

The addition of copper caused an increase in pH in the OA treatment, rising from 7.28 ± 0.13 (OA, no copper) to 7.51 ± 0.07 (OA with copper) at the start of low tide. However, this increase was not significant (two-way ANOVA for copper, F = 1.986, P = 0.174). At the end of low tide, copper caused a slight decrease in both ambient and OA treatments, again, this was not significant (two-way ANOVA for copper, F = 0.404, P = 0.532). OA had no significant effect on coelomic fluid pH at either the start of low tide (two-way ANOVA for pH, F = 0.823, P = 0.375) or at the end of low tide (two-way ANOVA for pH, F = 0.254, P = 0.620). There was no significant interaction effect at either the start or end of low tide (two-way ANOVA for pH*copper, F = 0.630, P = 0.437), (two-way ANOVA for pH*copper, F = 0047, P = 0.831). (Figure 5.5 C).

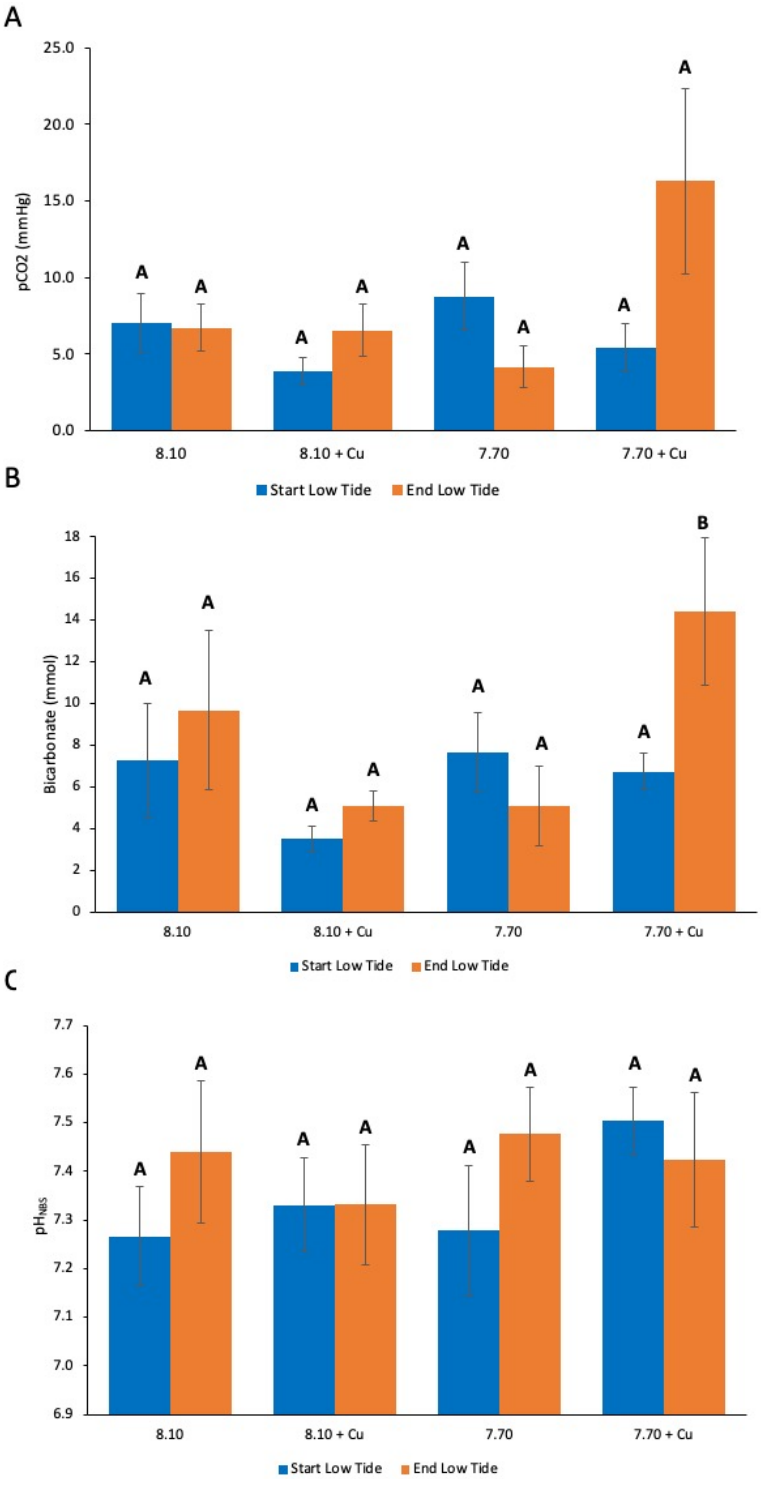


Figure 5.5. Acid-base measurements, taken at the start and end of low tide (7 hour emersion) in the coelomic fluid of *Alitta virens* after a 14 day exposure to four different treatments, ambient and near future OA scenarios with and without 0.25 µM copper on a tidal cycle. Data expressed as mean (±SE). (A) Coelomic fluid pCO_2, (B) Coelomic fluid bicarbonate levels, (C) Coelomic fluid pH_{NBS} levels.

5.3.6 *DNA damage*

Exposure to copper led to an increase in DNA damage with damage rising from 18.6 ± 2.7 % (ambient pH, no copper) to 24.9 ± 2.5 % (ambient pH with copper) and 24.4 ± 2.9 % (OA with copper), however, this increase was not significant (two-way ANOVA for copper, F = 3.777, P = 0.060). There was also no significant effect of OA on the level of damage (two-way ANOVA for pH, F = 0.239, P = 0.628) with levels rising slightly to 21.5 ± 1.8 % (OA, no copper) compared to the control. There was no significant interaction term between OA and copper (two-way ANOVA for pH*copper, F = 0.500, P = 0.484). (Figure 5.6 A).

5.3.7 *Oxidative stress*

There was no significant effect of OA on levels of lipid peroxidation, measured as the amount of malondialdehyde (two-way ANOVA for pH, F = 0.171, P = 0.680). The addition of copper caused an increase in both pH treatments, from 3.17 ± 0.29 (ambient pH, no copper) to 3.79 ± 0.40 (ambient pH with copper) at pH 8.1 and from 3.29 ± 0.31 (OA, no copper) to 3.62 ± 0.55 (OA with copper). This small increase was not significant (two-way ANOVA for copper, F = 0.498, P = 0.482) and neither was the interaction term between OA and copper (two-way ANOVA for pH*copper, F = 0.291, P = 0.591). (Figure 5.6 B).

SOD activity was similar across all treatments, with copper causing no significant difference (two-way ANOVA for copper, H = 0.095, P = 0.758) in both the ambient and OA treatments. OA caused no significant difference (two-way ANOVA for pH, H = 0.029, P = 0.865). There was also no significant interaction effect (two-way ANOVA for pH*copper, H = 0.0003, P = 0.987). (Figure 5.6 C).

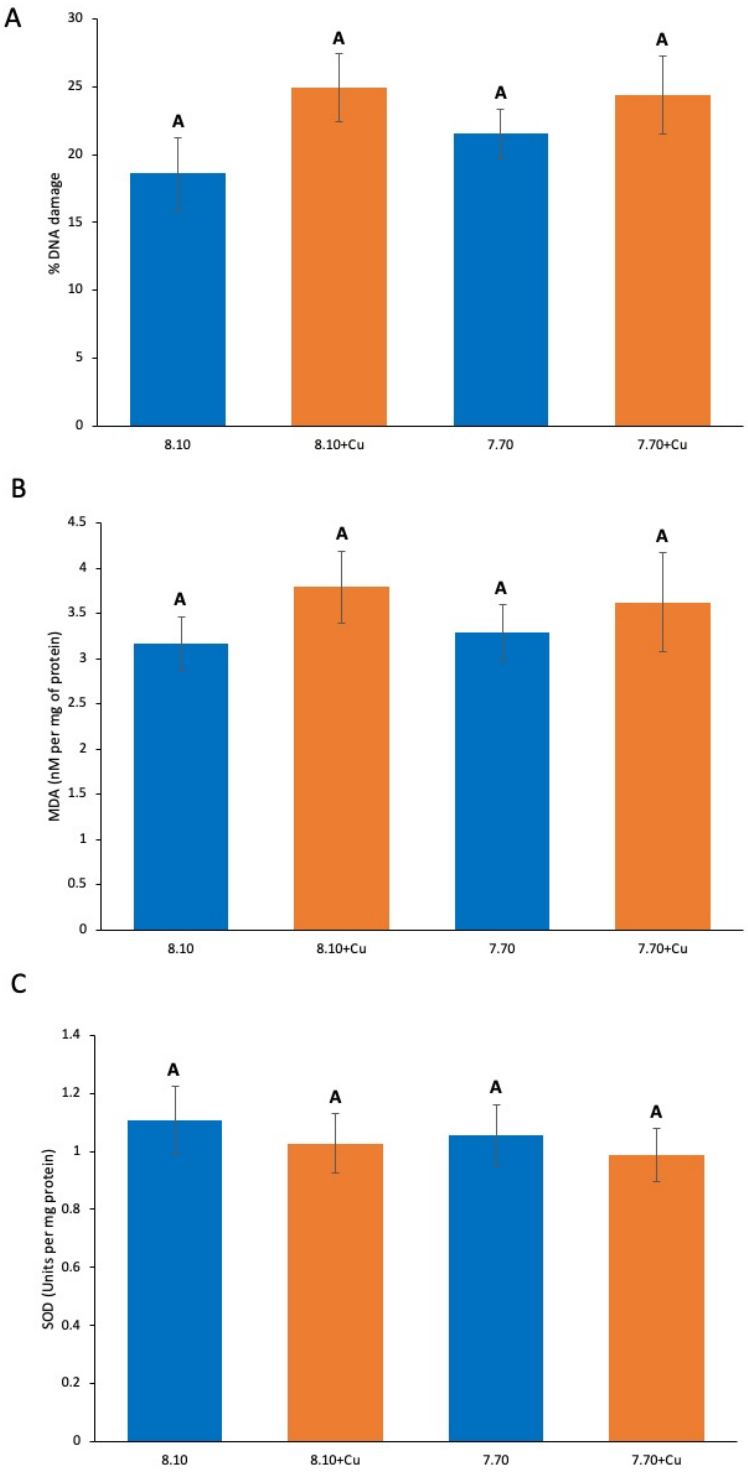


Figure 5.6. Indicators of oxidative stress in *Alitta virens* after a 14-day exposure to four different treatments, ambient (pH_{NBS} 8.1) and near future OA (pH_{NBS} 7.7) with and without 0.25 µM copper, on a tidal cycle. Data expressed as mean (±SE). (A) The percentage DNA damaged, (B) The level of lipid peroxidation measured as MDA (nM per mg protein), (C) The activity of SOD measured as units per mg protein.

5.4. Discussion

This data reveals the importance of including the influence of tides and sediments when investigating the response of intertidal sediment dwelling biota to future climate stressors. The field data collected in this chapter shows sediment pH in intertidal mud flats where this species resides can be already reach pH levels as low as 6.97 *in situ*. Hence these sediments can already reach significantly lower pH values than that of the overlying seawater both currently and that are predicted under near future climate change scenarios. In aquarium based manipulations sediments reached lows of 7.29 during a 7-hour emersion period with significant decreases in sediment pH between the start and end of low tide in the ambient seawater treatment conditions. Organisms inhabiting this sediment are hence already experiencing pHs lower than predicted for pelagic and epibenthic species under OA and are additionally being exposed to changing pH's throughout the tidal cycle. Interestingly our field data and laboratory data looking at sediment pH differ with pH increasing over time *in situ* but decreasing over emersion in the laboratory. It is possible that microbial processes make a difference in sediment pH with sediments being one of the most complex microbial habitats. Preliminary sediment pH data also shows an increase over the duration of the exposure, however, there was no tidal cycle in place here so conditions are likely to be different to those experienced in the main experiment.

The exposures to overlying seawater with manipulated seawater of higher pCO_2 and lower pH, to simulate various OA scenarios, revealed that whilst there was still a significant difference between sediment pH_{NBS} in the pH_{NBS} 8.10 and pH_{NBS} 7.70 treatments after 7 days, the relative difference between seawater pH and sediment pH was smaller in the pH_{NBS} 7.70 treatment, falling from a difference of 0.19 units in the ambient 8.10 treatment to just 0.03 in the pH_{NBS} 7.70 treatment with the presence of worms. In the lowest pH_{NBS} treatment (7.30) sediment pH was actually higher than the overlying seawater pH and the relative difference between the sediment and seawater here was highest at 0.35 units suggesting that sediment processes dominate over seawater pH/pCO_2 processes as sediment pH levels are stable and are buffering the OA effects. In mesocosm experiments using sediment cores collected from The Bay of Villefranche, France, maintained in the laboratory under both ambient seawater and OA conditions, Rassmann et al. (2018) also found that sediments buffered porewater

pH changes from reduced overlying seawater pH levels (7.40). They suggested this was most likely via carbonate dissolution which could explain the merging of pH profiles between OA treatments and the control seawater treatments (pH 8.12) (Rassmann et al. 2018). In both the ambient and OA treatments in the Rassmann et al study, pH reached its lowest point at the bottom of the oxic sediment layer at 5 mm in depth, after which there was a slight pH increase to between 7.35 and 7.55 in the anoxic parts of the sediment (Rassmann et al. 2018). Data taken from the end of emersion shows that OA doesn't significantly affect sediment pH, with no significant differences in the treatments having an overlying seawater pH_{NBS} of 7.70, compared to those with ambient seawater of 8.10. The addition of copper to the overlying water did significantly decrease sediment pH in the tidal exposure. This copper effect has been previously seen in estuarine sediments where the addition of copper and zinc produced lower pH due to hydrolysis of free metal species (Simpson et al., 2004), with the extent of this pH decrease also being dependent on the type of metal and sediment (Hutchins et al. 2009). Tidal activity has been shown to influence sediment pH in coastal areas off Canada with interstitial water pH being higher on a flood (incoming) tide rather than an ebb tide suggesting that incoming seawater was acting as a buffer. (Bendell et al. 2014). Respiration during ebb tides would result in the accumulation of CO_2 thus causing decreases in sediment pH during this time (Bendell et al. 2014).

It is worth noting that we did not use a microprobe to measure our sediment pH nor was it possible to take sediment readings from inside the burrows. It has been previously reported that the pH in polychaetes burrows can differ compared to both the overlying water and to the overall sediment by as much as 2 pH units, over distances of mm (Zhu et al. 2006) with active ventilation of the burrows causing a plume of acidified water to enter the overlying water (Stahl et al. 2006). Minimum zones of pH formed along the burrow walls of both *Hediste diversiocolor* and *Allita* (formally *Nereis*) *succinea* with pH levels reaching as low as 6.20 (Zhu et al. 2006, Stahl et al. 2006).

Studies looking at the impacts of carbon capture storage leaks, where a simulated *p*CO2 leak led to seawater pH values dropping to near pH 7.00 (Taylor et al. 2015) observed decreases sediment pH coincided with this more extreme reduced seawater pH (Queiros et al. 2015, Taylor et al. 2015). Here pore water pH in shallow sediments

dropped by 0.80 pH units under the simulated CO_2 leakage exposure (Taylor et al. 2015). However, impacts of decreased pH has been found to vary greatly between sediment types, depending upon baseline pH and sediment permeability with fine sand sediment being more stable with an even progression of gradient with depth (Queiros et al. 2015). Courser, sandy mud sediments were more variable, exhibiting a higher pH than expected, which the authors possibly attributed to fauna moving away from the sediment surface, reducing bioturbation in this area making it less permeable to low pH (Queiros et al. 2015).

During the tidal emersion period, worms within the sediment showed no evidence of a CO_2-induced acidosis in all but the OA and copper combined treatment, where coelomic fluid pCO_2 increased, along with a rise bicarbonate levels. This is in contrast to what happens for epibenthic species, where previous studies looking at intertidal mussels (*Mytilus edulis*) in a similar estuarine setting revealed a strong acidosis during tidal emersion, greater than that induced by OA exposures (Mangan et al. 2019). During tidal emersion mussel haemolymph pH significantly decreased with mussels from the estuary showing the greatest change with haemolymph pH dropping 0.71 pH units to a low of 6.87 (Mangan et al. 2019). Interestingly, in this study there was an increase in both the worms' coelomic fluid pCO_2 and bicarbonate levels where a significant OA and copper interaction was seen suggesting that these organisms are physiologically stressed by combined stressors, on top of emersion. However, the worms were able to maintain a stable coelomic fluid pH across all treatments, including after emersion, with the increase in bicarbonates under the combined stressors treatment likely to aid this compensation. It is worth noting that the sediment pH for both of the copper treatments were similar at pH_{NBS} 7.34 under OA seawater conditions and 7.31 under ambient at the end of emersion, hence this physiological response is not simply being driven by the pH experienced by the worm, and must be an interaction driven by combined copper and OA responses. In chapter 2, *Alitta virens* (not in sediment) also showed pH stability in their coelomic fluid with an increase pCO_2 being coupled with an increase in bicarbonate ions, adding further weight to the evidence that this species is able to acid-base regulate (Nielson et al. 2019).

A number of previous studies looking at the impacts of tidal emersion on acid base physiology of intertidal invertebrates have been conducted, though these have tended

to use epibenthic or rocky shore species. Increases in pCO_2 of organisms coelomic fluid has been recorded in both sea urchins (*Psammechinus miliaris* and *Echinus esculentus*) and crabs (*Scylla serrata* and *Necora puber*) in response to emersion (Rastrick et al. 2014, Varley and Greenaway 1992, Spicer et al., 1988). Sea urchins were able to completely compensate for this change, due to emersion, with no significant differences being shown in the pH of their coelomic fluid (Spicer et al. 1988). The crab *Necora puber* exhibited significant acidosis under OA conditions, a haemolymph pH decrease by 0.30-0.60 units, in response to the increase in pCO_2 and its recovery, via removal of extracellular pCO_2 and lactate, was slower after exposure to increased temperature and pCO_2 (Rastrick et al. 2014). Future climate conditions may therefore reduce this crabs physiological capacity to remain in intertidal zones, affecting the range of distribution for this species (Rastrick et al. 2014). The impact of tidal periods is further shown in the mussel *Mytilus edulis* where the emersion induced acidosis was greater by 0.70 pH units than acidosis induced by OA (Mangan et al. 2019).

The differences between the physiological responses of *Alitta virens* to periods of emersion measured here and those of the mussel *Mytilus edulis* observed by Mangan et al., (2019) may in part be explained by the fact that *Alitta virens* inhabit the sediment whereas the mussels sit on top of it and are totally exposed during low tide. Organisms living in sediment may be exposed to far less pH changes due to OA than those inhabiting the overlying water with the potential for sediments to act as buffers to changing pH levels. The data from this chapter shows in the sediment the pH change between ambient and OA conditions was minimal compared to the 0.40-unit pH decrease seen in the overlying water. Inhabiting the sediment will also serve to prevent the worms from drying out and allow them to maintain respiratory CO_2/O_2 exchange during low tides. Conversely, mussels close shut during this time and will therefore experience a build-up of metabolic CO_2 that will further exacerbate acidosis.

Alitta virens showed slight, but not significant, increases in DNA damage and lipid peroxidation to the addition of copper in both pH treatments. This is different to data found in chapter 2, where copper showed a significant increase in DNA damage to the copper treatments (Nielson et al. 2019). DNA damage was on the whole lower in the sediment exposure and lipid peroxidation was dramatically lower with all

treatments showing less damage than worms which were not in sediment. This difference may be explained by the addition of sediment as copper is known to have reduced reactivity, and therefore toxicity, when bound to sediments and organic complexes (Gardner and Ravenscroft 1991).

Ocean acidification also has no significant impact on both DNA damage and lipid peroxidation with the combination of both stressors (OA and copper) causing no additional damage to either DNA or lipids. This finding is consistent with chapter 2 where there was no combined stressor effect on DNA damage or lipid peroxidation, also in *Alitta virens* (Nielson et al. 2019). Polychaetes (*Hediste diversicolor*) exposed to OA and mercury, both as single stressors and combined, showed increased anti-oxidant defences (SOD/CAT/GST) compared to the control, however, there was no increase in lipid peroxidation suggesting the defence systems were enough to prevent damage (Freitas et al. 2017). The authors found no combined stressor effect which might be due to the fact that mercury accumulation wasn't influenced by pH (Freitas et al. 2017). Polychaetes seem to be able to cope well with combined stressors in terms of their oxidative stress response. However, this has not been the case for all species. The mussel, *Mytilus edulis* and the sea urchin, *Paracentrotus lividus* both saw a significant increase in DNA damage to copper under OA conditions as well as significant increases in lipid peroxidation compared to the control, this is despite sea urchins increasing SOD activity (Lewis et al. 2016). Contaminated sediments have also been show to become acutely more toxic to the amphipod *Corophium volutator* under elevated pCO_2 resulting in a 2.7-fold increase in DNA damage at 750 µatm pCO_2. The lack of significant changes in this study of lipid peroxidation to both copper and OA is likely to be why SOD activity didn't change, as activity levels had no need to be increased.

This experimental work did not reveal any OA effect across the end points measured. A study by Widdicombe and Needham on the burrowing behaviour of *A. virens* under OA scenarios found that OA had no impact on the size and structure of worm burrows (Widdicombe and Needham 2007b), which fits with the suggestion that sediment conditions do not differ between treatments despite the CO_2 manipulation of the overlying seawater. However, this is not always the case. Impacts of OA seawater conditions have been reported on the toxicity response of other sediment dwelling

organisms with increased toxicity responses in *Corophium volutator* being recorded following OA exposures to natural sediments contaminated with metals (Roberts et al. 2013). A decrease in abundance of nematodes (99 species in total found in the sediment samples) was found under OA exposures (pH 7.50) in natural sediments which were brought into the lab, potentially due to changes in behaviour of the burrowing sea urchin (*Echinocardium cordatum*) which were present in these exposures (Dashfield et al. 2008). However, meiofaunal abundances were unaffected in natural sediments (brought into the lab), from both France and Japan exposed to seawater pH's of 7.40 (Rassmann et al. 2018, Kurihara et al., 2007).

It is clear from this study that sediment pH is considerably different to that of the overlying water, resulting in dwelling organisms inhabiting different, and more variable conditions. Long emersion times due to tidal cycles may add further pressure with a significant interaction between OA and copper causing an increase in bicarbonate ions due to increasing pCO_2. However, the polychaetes *Alitta virens*, was able to maintain stable internal pH as well as showing no increases in oxidative stress damage as this species looks to be robust to tidal changes in future climate conditions.

Table 5.1. Seawater carbonate chemistry and copper levels for all experimental treatments over the exposure period. Data expressed as mean ± standard error. Temperature, salinity, and pH were measured daily in each replicate tank. DIC (n = 48) and copper concentration (n = 6) were measured every three days. The other carbonate parameters were calculated using CO2sys (Lewis and Wallace 1998).

Treatment	Temperature (°C)	pH_{NBS}	Salinity	Copper (µM)	Copper (µM) Min/max	TA (µmol/kg)	pCO_2 (µatm)	HCO_3^- (µmol/kg)	CO_3^{2-} (µmol/kg)	ΩCa	ΩAr
8.10 + Cu	15.22 ± 0.06	8.28 ± 0.004	34.20 ± 0.12	0.090		2312.8 ± 12.1	2.66.9 ± 9.97	1792.2 ± 10.7	211.2 ± 6.6	5.1 ± 0.2	3.3 ± 0.1
8.10	15.51 ± 0.05	8.25 ± 0.007	34.24 ± 0.04	0.004	0.006/0.002	2274.2 ± 7.98	314.8 ± 15.2	1816.4 ± 12.8	185.1 ± 6.1	4.4 ± 0.1	2.9 ± 0.1
7.70 + Cu	14.99 ± 0.07	7.78 ± 0.007	34.54 ± 0.06	0.083		2252.9 ± 11.6	996.9 ± 19.9	2070.2 ± 12.3	73.7 ± 0.6	1.8 ± 0.0	1.1 ± 0.0
7.70	15.00 ± 0.06	7.76 ± 0.007	34.51 ± 0.11	0.001	0.002/0.0005	2242.5 ± 8.78	1152.2 ± 29.2	2081.8 ± 7.6	64.8 ± 1.9	1.6 ± 0.0	0.9 ± 0.0

Chapter Six

Discussion

Understanding organism's responses to rapidly changing ocean chemistry is key to predicting their survival. With multiple anthropogenic impacts affecting our oceans it is impossible to test all combinations, but scenario testing enables us to look at those that are likely to occur together and interact (Boyd et al. 2018). Coastal pH/pCO_2 is very variable and extremes are predicted to increase as climate change progresses. Coastal sediments are also still suffering from historic contamination from pollutants such as metals. The fact that the speciation and bioavailability of copper changes with pH makes it a really interesting combination of stressors to study added to the fact that they are also highly likely to be experienced by marine species in combination. There is now evidence for a number of species that OA acts to increase the toxicity of the metal copper (Campbell et al. 2014, Lewis et al. 2016, Siddiqui and Bielmyer-Fraser 2015), however, prior to the work of this thesis there was a knowledge gap for sediment dwelling species. In this thesis I therefore focussed on OA – copper interactions in polychaetes, a keystone species inhabiting sediments often polluted with elevated levels of copper (Bryan and Gibbs 1983a).

I first conducted a meta-analysis of the literature to determine the number of studies that have looked for OA-contaminant interactions and ascertain what interactions have been observed to date. This revealed that, the majority of studies looking at OA – contaminant interactions have indeed focused on the metals and in particular copper. The biological end points measured, across a range of taxa, included growth, contaminant accumulation, immune response, oxidative stress markers, DNA damage and mortality. However, none of these endpoints show any clear trends in direction or magnitude of pH-induced effect size for any of the contaminants studied. Changes in toxicity of any given contaminant, including for the metals, when experienced under a CO_2-induced lower pH's were not consistent across species, life history stage or studies. One factor that may account for between-species differences in pH-induced response might be the acid-base physiology of an organism, since the regulation of internal pH is key to determine the extent to which seawater pH levels affects them.

Polychaetes live in benthic sediment, where high levels of contaminants can occur, however, there is a lack of data around their ability to acid-base regulate in the face of a changing ocean. Therefore, acid base parameters were measured in experimental chapters.

For the experimental chapters in this thesis I looked at the physiological and ecotoxicological responses of two polychaetes to combined exposures of ocean acidification and the pH sensitive metal, copper, under a range of different scenarios with increasing environmental relevance. This work built on a number of previous studies demonstrating that OA can increase the toxicity of copper to organisms (Lewis et al. 2013a, Lewis et al. 2016, Siddiqui and Bielmyer-Fraser 2015). For example, both DNA damage and lipid peroxidation increased under OA conditions (pH_{NBS} 7.70) in both mussels (*Mytilus edulis*) and sea urchins (*Paracentrotus lividus*) compared to ambient conditions (pH_{NBS} 8.10) in the face of copper pollution. Since the majority of these previous studies were performed as water borne exposures, I first re-created these exposure scenarios to look at species-specific responses related purely to species physiology. This work revealed that both *Alitta virens* and *Hediste diversicolor* exhibit very different responses to combined OA –copper exposures than observed for mussels and sea urchins. No significant increases in copper toxicity to DNA damage or lipid peroxidation were observed for either species when exposed under OA conditions compared to control ambient pH_{NBS} 8.10 conditions. In fact, for *Alitta virens* the addition of 0.25 µM copper appeared to dampen the effects of OA with a reduction in haemolymph pCO_2 levels as well as reduced lipid peroxidation and SOD activity under OA and copper treatments compared to OA alone.

What was interesting in these exposures was the lack of a pCO_2 response in the coelomic fluid of the polychaetes, despite increases in the seawater pCO_2 surrounding them. *Alitta virens* coelomic fluid pCO_2 levels did not significantly increase despite seawater pCO_2 increasing from 453 µatm to 1309 µatm, a rise I would have expected to drive up coelomic fluid pCO_2. This is different to the responses reported in other invertebrates, including crabs (Spicer et al. 2007), mussels and sea urchins (Lewis et al. 2016, Moulin et al. 2014, Miles et al. 2007) where an increase in seawater pCO_2 leads to a concurrent increase in internal pCO_2 regardless of their ability to acid-base regulate. For example, the sea urchin, *Echinometra mathaei* showed an increase in

coelomic fluid pCO_2 of the same magnitude as the increase in the seawater pCO_2 (Moulin et al. 2014). It is unclear why this change in coelomic fluid pCO_2 doesn't occur in the polychaetes in these exposure scenarios.

The ability to acid base regulate has widely been discussed as important in determining a species' sensitivity to OA and their persistence in high CO_2 environments (Portner 2008, Calosi et al. 2013a, Wittmann and Portner 2013) with differences not only seen between taxa but also between species (Portner 2008, Melzner et al. 2009, Widdicombe and Spicer 2008). The importance of maintaining a stable pH is key as enzymes needed for energy and growth have an optimum, narrow, pH range, which differ according to body temperature and must be kept in order to allow enzymes to function effectively and efficiently (Somero 1986). The internal fluid pH also determines the affinity in which oxygen binds to the respiratory pigment (Newbatt 2015). Acid base maintenance and ionic homeostasis is a key physiological target for the interactive effects of OA and metal exposure (Ivanina and Sokolova 2015) and regulation must occur as uncompensated acidosis has the potential to disrupt metabolism and also effect the performance of oxygen binding pigments (Gutowska et al. 2010). Differing effects of copper contamination have been seen amongst marine invertebrates. The crab, *Carcinus maenas*, show acidosis in response to sub-lethal copper concentrations, although this was partly compensated for (Boitel and Truchot 1989). Additionally, mussels (*Mytilus edulis*) have shown an acidosis response to copper, with the addition of OA causing a further drop in coelomic pH (Lewis et al. 2016). Conversely, copper caused an alkalosis of coelomic fluid pH in sea urchins (*Paracentrotus lividus*) with no additional changes seen under OA conditions (Lewis et al. 2016).

For some of the sub-lethal toxicity end points used in these experiments, e.g. lipid peroxidation, the internal coelomic fluid pH will be the pH level at which the copper taken up by an individual exerts its toxicity, hence, in organisms able to maintain internal pH the difference for toxicity effects between ambient and OA treatment might be reduced since the speciation of copper at the point of toxicity is no so different between treatments. The levels of copper taken up by an individual in the first place (bioavailability) is more likely to be influenced by the external conditions, i.e. seawater pH however, so is not straight forward. Polychaetes were able to maintain a stable

coelomic fluid pH across all treatments potentially meaning copper within the coelomic fluid is similar across treatments, possibly explaining why no increase in oxidative stress was seen. DNA damage happens within the cell. Whilst intra-cellular pH was not measured here, it is has been shown in other marine invertebrates that are poor acid base regulators (e.g. mussels) that intra-cellular pH can be regulated close to control levels under OA exposures (Michaelidis et al. 2005, Zange et al., 1990), but at an energetic cost (Deigweiher et al. 2010). The polychaete data differs again from that seen in mussels and sea urchins (Lewis et al. 2016) and is in line with the lack of consistent patterns in responses revealed by the meta-analysis. This supports the paradigm that acid base physiology plays a role in species responses with interactions being more complex than just contamination speciation under differing pH's.

One paradigm that has been proposed for explaining sub-lethal responses to multiple stressors is the 'energy limited tolerance to stressors' hypothesis (Sokolova 2013), which proposes that bioenergetics plays a central role in the tolerance to environmental stress. Previous work on a metal resistant population of *Hediste diversicolor*, which are genetically adapted to survive is sediments with a high copper and zinc concentrations, found this metal resistance comes at an energetic cost (Pook et al. 2009). This population therefore provides an opportunity to look at how adaptation to one stressor (copper) and the energetic cost of that adaption, might influence response to a second stressor (OA) and how this species may cope in future ocean conditions.

Two different populations of the polychaete *Hediste diversicolor*, one metal resistant and one non-resistant, were exposed to a combination of OA and copper with feeding rate, metabolic rate and toxicity end points measured. My data reveals differences in the physiological and toxicity responses to combined OA and copper exposure in the metal-resistant population compared to the non-resistant population. The resistant Restronguet population showed increased metabolic rate as well as feeding rate during the exposure resulting in a significantly higher metabolic rate treatment effect size. This population also saw a decreased treatment effect size on O:N ratio, compared to the non-resistant population, a potential energetic disadvantage as lower O:N point to an increase in protein degradation over lipids, with lipid transformation being relatively cheaper (Hochachka 1991). These findings point to organisms in the

resistant population being more energetically stressed as well as altering their metabolic substrate, hence data are in line with the Sokolova (2013) hypothesis and the previous work on resistant *H. diversicolor* showing these polychaetes are energetically stressed, being smaller in size with reduced lipid reserves compared to non-resistant populations (Pook et al. 2009). As with the initial experiment of Chapter 3, in both populations of *H. diversicolor*, the toxicity end points, DNA damage, lipid peroxidation and SOD activity, remained relatively unaffected in all exposure scenarios with the combination of OA and copper not seeming to cause any adverse effects and in the case of lipid peroxidation, the combined treatment had lower levels of damage compared to OA alone.

Whilst these results provide insight into some of the physiological mechanisms involved in driving responses to combined OA and copper exposures, water borne exposures do not represent particularly environmentally relevant conditions for these polychaetes worms. Hence in the final chapter, I created a sediment exposure set on a tidal cycle, mirroring conditions that this organism experiences in their natural habitat. When comparing results to *Alitta virens* seen in chapter 3 to those in sediment-based exposures from chapter 5 the biggest difference was that the OA exposure effect had effectively disappeared with the sediment pH for all treatments being within a similar range of 7.31 and 7.45, which acted to reduce the relative difference in pH between the two 'pH' treatments. This resulted in a decrease in DNA damage across all treatments with the highest damage being 24.9%, whilst those polychaetes without sediment, in the water-borne exposure from chapter 3, had DNA damage of up to 34%. Lipid peroxidation was also much lower when the worms were in sediment, thus exposed to different pH levels, with all treatments having levels of under 4 nM per mg of protein, in contrast those worms without sediment all had levels between 4 and 7.45 nM per mg of protein. This could be due to the fact that the sediment is buffering the effect of both copper pollution and OA to the worms. It is worth noting that the polychaetes inhabiting the sediment showed, on average, over four times higher levels of SOD activity compared to those without sediment which may account for the much lower levels of lipid peroxidation seen in these worms. Those exposed in sediment also showed less variable responses with all treatments having similar values, with the exception of a small increase in both DNA damage and lipid peroxidation under copper treatments. The lack of an OA effect is likely to be

because the sediments pH does not differ nearly as much between ambient and OA treatments compared to the 0.40-unit drop seen in the overlying seawater conditions. The sediment looks to mitigate this effect and the pH is actually lower in the ambient copper treatment than the OA treatments.

The worms inhabiting the sediment are clearly exposed to differing pH levels than that of the overlying seawater, with pH levels being much lower than the overlying water. This clearly demonstrates the need for making ecotoxicology studies more environmentally relevant. As a result of this lower pH, the coelomic fluid pCO_2 of these worms are much higher than those not in sediment, with levels being over double across all treatments leading to higher levels of bicarbonate in order to maintain a stable pH. The ability for this species to acid-base regulate, via increases of bicarbonate, is clear with worms in both sediment and without sediment showing no significant differences across treatments, despite the coelomic fluid pH being lower in those from the sediment scenario. Toxicity testing in sediments is much more complex than open water due to the binding of contaminants to the sediment particles and complex microbial processes that act in sediments. It is worth noting that copper levels were tested in the water and not the sediment that our worms were exposed to so there may be differences between these two mediums.

The effect of tidal emersion has also been demonstrated here, which is an important aspect to include for inter tidal species. During the course of emersion, the pCO_2 levels and consequently bicarbonate levels increased compared to levels at the start of low tide meaning these organisms are physiologically stressed on a daily basis. However, as previously mentioned the increase in bicarbonate levels were sufficient to maintain a stable coelomic fluid pH. The opposite effect has been seen in mussels during tidal emersion where acidosis occurred as this species was unable to acid-base regulate (Mangan et al. 2019).

Overall, the work of my PhD highlights that it is not only organism's physiology alone that influences stressor interaction, but also their habitat and ecology. Sediment dwelling organisms are already experiencing very different pH conditions, and are living in a complex habitat where microbial processes dominate, with sediments probably representing some of the most complex microbial habitats on Earth (Urakawa

et al., 1999) which may all contribute to uptake of contaminants and their toxicity effects. In continental shelf sediments microbial activity depletes high energy acceptors such as oxygen and nitrogen within a few millimetres to centimetres below the surface sediment (Durbin and Teske 2011) thus, altering the sediment chemistry to any organisms inhabiting it. Organisms are also able to alter their behaviour including movement to more favourable environments, changes in burrowing and altered ventilation rates. For example, bivalves have shown increased gaping under decreased pH conditions (Bamber 1990). However, the time of shells spent open in *Mytilus galloprovincialis* in response to metal pollution (mercury, copper, zinc and cadmium) decreased with complete closure being seen at high levels of mercury and copper (Fdil et al. 2006).

There are multiple global and local stressors which have the potential to affect the fitness of marine biota and it is impossible to test all combinations of possible interactions. Therefore, scenario testing of likely stressor combinations is a sensible approach for exposures to understand what additive or synergistic interactions may occur. Benthic marine invertebrates are exposed to a multi-stressor environment where stressor levels will continue to be exacerbated by environmental changes such as global warming and increased atmospheric carbon dioxide (Byrne and Przeslawski 2013). Interactions between different stressors may change the conditions in our oceans thus exposing organisms to different environments. Biological responses to both OA and warming varies across taxonomic groups and life history stages with the combine in stressors generally exhibiting a stronger biological effect (either positive or negative) (Harvey et al., 2013) showing the importance of combined exposures as opposed to single stressors experiments. Harvey, Gwynn-Jones and Moore's (2013) meta-analysis found that four out of the five biological end points measured (calcification, photosynthesis, reproduction and survival) showed synergistic interactions to OA and warming combined (Harvey et al. 2013).

Coastal ecosystems are also progressively affected by hypoxia (Laffoley and Baxter 2019) which is only predicted to increase in the future (Conley et al. 2009). The worldwide spread of coastal hypoxic zones also simultaneously spread CO_2 – enriched zones as degradation of organic material is related to the production of CO_2 (Melzner et al. 2013). The combination of hypoxia and OA (pH between 7.72-7.80) in

a laboratory experiment resulted in reduced respiration compared to a single stressor to a range of invertebrates (anthozoans, molluscs, crustaceans and echinoderms), off the Chilean coast (Steckbauer et al. 2015). There were significant differences between species with echinoderms showing the lowest respiration rates. No increase in mortality was seen which is not overly surprising seeing as these environmental conditions already exist in waters off Chile (Steckbauer et al. 2015).

Work into the potential for hypoxia to interact with aquatic contaminants has also tended to use copper as the test contaminant. Copper interactions with hypoxia has been seen in a number of fish species with copper causing stronger hypoxic effects. The three-spined stickleback (*Gasterosteus aculeatus*) was able to acclimate to hypoxic conditions by decreasing its critical oxygen level, however, the addition of copper prevented this response alongside increasing ventilation rate (Fitzgerald et al. 2019). Hypoxia also was found to cause significant changes in copper toxicity throughout development in both the three-spined stickleback and the zebrafish (*Danio rerio*) with hypoxia reducing toxicity during early development but increasing toxicity after hatching (Fitzgerald et al. 2016, Fitzgerald et al., 2017), highlighting the importance of looking at a range of life history stages.

Overall the two species of polychaetes looked at in this body of work has shown them to be robust to near future conditions compared to other species and highlights the importance of looking at species physiology and interaction with their environment which may be key to driving OA and contaminant interactions, rather than focusing on chemical speciation changes alone. This poses certain challenges to ecotoxicologists tasked with protecting coastal habitats as global ocean change progresses, in trying to understand all these interactions. Making robust predictions for the future will require understanding of different species physiology, life history stages, environments, behaviour, multiple stressor interactions and potential for adaptations.

Bibliography

Addadi, L., S. Raz & Weiner S. (2003) Taking advantage of disorder: Amorphous calcium carbonate and its roles in biomineralization. *Advanced Materials,* 15**,** 959-970.

Agostini, S., Harvey B., Wada S., Kon K., Milazzo M., Inaba K. &. Hall-Spencer J. M (2018) Ocean acidification drives community shifts towards simplified non-calcified habitats in a subtropical-temperate transition zone. *Scientific Reports,* 8, article number 11354.

Alenius, B. & Munguia P. (2012) Effects of pH variability on the intertidal isopod, *Paradella dianae*. *Marine and Freshwater Behaviour and Physiology,* 45**,** 245-259.

Allen, H. E., Hall, R. H. & Brisbin, T. D. (1980) Metal speciation - Effects on aquatic toxicity. *Environmental Science & Technology,* 14**,** 441-443.

Allen, S. M. & Burnett L. E. (2008) The effects of intertidal air exposure on the respiratory physiology and the killing activity of hemocytes in the pacific oyster, *Crassostrea gigas* (Thunberg). *Journal of Experimental Marine Biology and Ecology,* 357**,** 165-171.

Amiard, J. C., Amiard-Triquet C., Barka S., Pellerin J. & Rainbow P. S. (2006) Metallothioneins in aquatic invertebrates: Their role in metal detoxification and their use as biomarkers. *Aquatic Toxicology,* 76**,** 160-202.

Amin, BA. Ismail A., Arshad A., Yap C. K. & Kamarudin M. S. (2009) Anthropogenic impacts on heavy metal concentrations in the coastal sediments of Dumai, Indonesia. *Environmental Monitoring and Assessment,* 148**,** 291-305.

Arnberg, M., Calosi P., Spicer J. I., Tandberg A. H. S., Nilsen M., Westerlund S. & Bechmann R. K. (2013) Elevated temperature elicits greater effects than decreased pH on the development, feeding and metabolism of northern shrimp (*Pandalus borealis*) larvae. *Marine Biology,* 160**,** 2037-2048.

Atkinson, C. A., Jolley D. F. & Simpson S. L. (2007) Effect of overlying water pH, dissolved oxygen, salinity and sediment disturbances on metal release and sequestration from metal contaminated marine sediments. *Chemosphere,* 69**,** 1428-1437.

Aufdenkampe, A. Mayorga K., E., Raymond P. A., Melack J. M., Doney S. C., Alin S. R., Aalto R. E. & Yoo K (2011) Riverine coupling of biogeochemical cycles between land, oceans, and atmosphere. *Frontiers in Ecology and the Environment,* 9**,** 53-60.

Azetsu-Scott, K., Starr M., Mei Z. P. & Granskog M (2014) Low calcium carbonate saturation state in an Arctic inland sea having large and varying fluvial inputs: The Hudson Bay system. *Journal of Geophysical Research-Oceans,* 119**,** 6210-6220.

Bamber, R. N. (1990) The effects of acidic seawater on three species of lamellibranch mollusc. *Journal of Experimental Marine Biology and Ecology: Elsevier* 181-191..

Bao, Y., Qiao F. L & Song Z. Y (2012) Historical simulation and twenty-first century prediction of oceanic CO_2 sink and pH change. *Acta Oceanologica Sinica,* 31**,** 87-97.

Barker, S. & Ridgwell A (2012) *Ocean Acidification*. Nature Education Knowledge.

Basallote, M. D., Borrero-Santiago, A. R., Canovas, C. R., Hammer, K. M. , Olsen A. J. & Ardelan, M. V (2020) Trace metal mobility in sub-seabed sediments by CO_2 seepage under high-pressure conditions. *Science of the Total Environment,* 700, 134761.

Bates, N. R., Mathis J. T. & Cooper L. W (2009) Ocean acidification and biologically induced seasonality of carbonate mineral saturation states in the western Arctic Ocean. *Journal of Geophysical Research-Oceans,* 114, C11007.

Baumann, H., Wallace, R. B, TTagliaferri T & Gobler C. J (2015) Large natural pH, CO_2 and O_2 fluctuations in a temperate tidal salt marsh on diel, seasonal, andiInterannual time Scales. *Estuaries and Coasts,* 38**,** 220-231.

Bayne, B. L. (1999) Physiological components of growth differences between individual oysters (*Crassostrea gigas*) and a comparison with *Saccostrea commercialis*. *Physiological and Biochemical Zoology,* 72**,** 705-713.

Beaucham, C. & Fridovic, I (1971) Superoxide dismutase - Improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry,* 44**,** 276-287.

Beaumont, N. J., Aanesen, M., Austen, M. C., Borger, T. , Clark, J. R., Cole, M. , Hooper, T., Lindeque, P.K,. Pasco C & Wyles K.J (2019) Global ecological, social and economic impacts of marine plastic. *Marine Pollution Bulletin,* 142**,** 189-195.

Bednarsek, N., Feely, R. A., Reum, J. C. P., Peterson, B, Menkel, J, Alin, S. R. & Hales B (2014) *Limacina helicina* shell dissolution as an indicator of declining habitat suitability owing to ocean acidification in the California Current Ecosystem. *Proceedings of the Royal Society B-Biological Sciences,* 281:20140123.

Bednaršek, N., Tarling, G, Bakker, D, Fielding, S. Cohen, A., Kuzirian, A, McCorkle, D, Leze, B & Montagna R (2012) Description and quantification of pteropod shell dissolution: A

sensitive bioindicator of ocean acidification. *Global Change Biology* 18 (7) 2378-2388.

Beman, J., Arrigo K, & Matson P (2005) Agricultural runoff fuels large phytoplankton blooms in vulnerable areas of the ocean. *Nature,* 434**,** 211-214.

Bendell, L. I., Chan, K, Crevecoeur S & Prigent C (2014) Changes in ammonium and pH within intertidal sediments in relation to temperature and the occurrence of non-indigenous bivalves. *Open Journal of Marine Science: Scientific Research*, 4, 151-162..

Beniash, E., Aizenberg, J, Addadi L & Weiner S (1997) Amorphous calcium carbonate transforms into calcite during sea urchin larval spicule growth. *Proceedings of the Royal Society B-Biological Sciences,* 264**,** 461-465.

Bijma, J., Pörtner, H.-O. Yesson C & Rogers A.D (2013) Climate change and the oceans – What does the future hold? *Marine Pollution Bulletin: Elsevier* , 47, 2, 495-505..

Bindoff, N. L., Cheung, W.W.L, Kairo, .G, Arístegui, J, Guinder, V.A, Hallberg, R Hilmi, N. Jiao, N, Karim, M.S, Levin, L, O'Donoghue, S, Purca Cuicapusa, S.R Rinkevich, B, Suga, T, Tagliabue, A and Williamson, P (2019) Changing ocean, marine ecosystems, and dependent communities. In: I*PCC Special Report on the Ocean and Cryosphere in a Changing Climate*. *IPCC Special Report on the Ocean and Cryosphere in a Changing Climate*: IPCC.

Birchenough, S. N. R., Reiss, H, Degraer, S, Mieszkowska, N, Borja ,A, Buhl-Mortensen, L, Braeckman, U, Craeymeersch, J, De Mesel, I, Kerckhof, F, Kroncke, I, Parra, S, Rabaut, M, Schroder, A, Van Colen, C, Van Hoey, G, Vincx, M & Watjen, K (2015) Climate change and marine benthos: a review of existing research and future directions in the North Atlantic. *Wiley Interdisciplinary Reviews-Climate Change,* 6**,** 203-223.

Blackford, J. C. & Gilbert, F. J (2007) pH variability and CO_2 induced acidification in the North Sea. *Journal of Marine Systems,* 64**,** 229-241.

Blakefield, M. K. & Harris, D. O (1994) Delay of cell-differentiation in *Anabaen-Aequalis* caused by UV-B radiation and the role of photo reactivation and excision-repair. *Photochemistry and Photobiology,* 59**,** 204-208.

Boitel, F. & Truchot, J. P (1989) Effects of sublethal and lethal coppe levels on hemolyph acid-base-balance and ion concentration in the shore crab *Carcinus-maenas* kept in undiluted sea-water. *Marine Biology,* 103**,** 495-501.

Bollmann, M., Bosch, T, Colijn, F, Ebinghaus, R, Froese, R & Güssow, K (2010) World Ocean Review: Living with the oceans. *Maribus Gemeinnutzige GmbH,* 15582, 236.

Bonnard, M., Romeo, M & Amiard-Triquet, C (2009) Effects of copper on the burrowing behavior of estuarine and coastal invertebrates, the polychaete *Nereis diversicolor* and the bivalve *Scrobicularia plana*. *Human and Ecological Risk Assessment,* 15**,** 11-26.

Boxall, A. B. A., Comber, S. D, Conrad, A. U, Howcroft J & Zaman, N (2000) Inputs, monitoring and fate modelling of antifouling biocides in UK estuaries. *Marine Pollution Bulletin,* 40**,** 898-905.

Boyd, P. W., Collins, S, Dupont, S, Fabricius, K, Gattuso, J.P, Havenhand, J, Hutchins, D. A., Riebesell, U, Rintoul, M. S, Vichi, M, Biswas,H Ciotti, A, Gao, K, Gehlen, M, Hurd,C.L, Kurihara, H,. McGraw, C.M, Navarro, J.M, Nilsson,G.E, Passow, U & Portner H.O (2018) Experimental strategies to assess the biological ramifications of multiple drivers of global ocean change-A review. *Global Change Biology,* 24**,** 2239-2261.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytic Biochemistry: Elsevier,* 72, 1-2, 248-254.

Braeckman, U., Van Colen, C, Guilini, K, Van Gansbeke, D, Soetaert, K, Vincx, M & Vanaverbeke, J (2014) Empirical evidence reveals seasonally dependent reduction in nitrification in coastal sediments subjected to near future ocean acidification. *Plos One,* 9(10), e108153.

Breitbarth, E., Bellerby, R.J, Neill, C.C, Ardelan, M.V, Meyerhofer,M, Zollner,E, Croot, P.L & Riebesell, U (2010) Ocean acidification affects iron speciation during a coastal seawater mesocosm experiment. *Biogeosciences,* 7**,** 1065-1073.

Breitburg, D., Levin, L.A, Oschlies, M. Gregoire,,M Chavez, F.P, Conley, D.J, Garcon, V, Gilbert, D, Gutierrez, D, Isensee, K, Jacinto, G.S, Limburg, K.E Montes, I, Naqvi, S.W.A Pitcher,C.G Rabalais, N.MRoman, M.R, Rose, K.A, Seibel, B.A ,Telszewski, K, Yasuhara, M & Zhang, J (2018) Declining oxygen in the global ocean and coastal waters. *Science,* 359**,** 6371, eaam7240.

Breton, T. S. & Prentiss, N.K (2019) Metal stress-related gene expression patterns in two marine invertebrates, *Hediste diversicolor* (Annelida, Polychaeta) and *Littorina*

littorea (Mollusca, Gastropoda), at a former mining site. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology,* 225, 108588.

Brito, R., Chimal, M.E, Gaxiola, G & Rosas, C (2000) Growth, metabolic rate, and digestive enzyme activity in the white shrimp *Litopenaeus setiferus* early postlarvae fed different diets. *Journal of Experimental Marine Biology and Ecology,* 255**,** 21-36.

Brown , A., Budge, S, Kaloriti D, Tillmann A, Jacobsen M, Yin Z, Ene I, Bohovych I, Sandai D, Kastora S, Potrykus J, Ballou E, Childers D, Shahana S & L. M (2014) Stress adaptation in a pathogenic fungus. *Journal of Experimental Biology* 271:144-155.

Brown, R. J., Galloway T. S., Lowe D., Browne M. A., Dissanayake A., Jones M. B. & Depledge M. H. (2004) Differential sensitivity of three marine invertebrates to copper assessed using multiple biomarkers. *Aquatic Toxicology,* 66**,** 267-278.

Bryan, G. & Gibbs P. (1983a) *Heavy metals in the Fal estuary, Cornwall: A study of long term contamination by mining waste and its effects on estuarine organisms*. Plymouth: Marine Biological Association of the United Kingdom.

Bryan, G. W. & Langston, W.J (1992) Bioavailability, accumulation and effects on heavy metals in sediments with special reference to United Kingdom estuaries. *Environmental Pollution,* 76**,** 89-131.

Buffle, J., A. Tessier & Haerdi, W (1984) *Interpretation of Trace Metal Complexation by Aquatic Organic Matter*. Complexation of trace metals in natural waters. Developments in Biogeochemistry: Springer, Dordrecht.

Bugg, T. D. H (2003) Dioxygenase enzymes: catalytic mechanisms and chemical models. *Tetrahedron,* 59**,** 7075-7101.

Burdige, D (2006) *Geochemistry of Marine Sediments*. *OEAS Faculty Books*: Princeton University Press.

Burdige, D. J (2007) Preservation of organic matter in marine sediments: Controls, mechanisms, and an imbalance in sediment organic carbon budgets? *Chemical Reviews,* 107**,** 467-485.

Burnett, L. E (1988) Physiological-responses to air exposure - acid-base balance and the role of branchial water stores. *American Zoologist,* 28**,** 125-135.

Byrne, M. & Przeslawski, R (2013) Multistressor Impacts of Warming and Acidification of the Ocean on Marine Invertebrates' Life Histories. *Integrative and Comparative Biology,* 53**,** 582-596.

Byrne, R. H (2002) Inorganic speciation of dissolved elements in seawater: the influence of pH on concentration ratios. *Geochemical Transactions,* 3**,** 11-16.

C.M.G. van den Berg (2000) Determination of Organic Complexation. *Environmental Science: Springer, Berlin, Heidelberg*, 175-187.

Calosi, P., Rastrick, S. P. S, Graziano, M, Thomas, S.C. Baggini, C, Carter, H.A, Hall-Spencer, J.M, Milazzo, J & Spicer J.I (2013a) Distribution of sea urchins living near shallow water CO2 vents is dependent upon species acid-base and ion-regulatory abilities. *Marine Pollution Bulletin,* 73**,** 470-484.

Calosi, P., Rastrick, S. P. S, Lombardi, C, de Guzman, H.J, Davidson, L, Jahnke, M, Giangrande, A, Hardege, J. D, Schulze, A, Spicer, J.I, & Gambi, M.C (2013b) Adaptation and acclimatization to ocean acidification in marine ectotherms: an in situ transplant experiment with polychaetes at a shallow CO_2 vent system. *Philosophical Transactions of the Royal Society B-Biological Sciences,* 368, 1627.

Camejo. G, Wallin, B & Enojavi, M (1998) Analysis of oxidation and antioxidants using microtiter plates. *Free radical and antioxidant protocols*, 108, 377-387.

Campbell, A. L., Mangan, S, Ellis R. P & Lewis, C (2014) Ocean acidification increases copper toxicity to the early life history stages of the polychaete *Arenicola marina* in artificial seawater. *Environmental Science & Technology,* 48**,** 9745-9753.

Canadell, J. G., Le Quere, C, Raupach, M. R, Field, C. B, Buitenhuis, E. T,. Ciais, P, Conway, T. J, Gillett, N. P, Houghton R.A & Marland , G(2007) Contributions to accelerating atmospheric CO_2 growth from economic activity, carbon intensity, and efficiency of natural sinks. *Proceedings of the National Academy of Sciences of the United States of America,* 104**,** 18866-18870.

Cao, R. W., Wang, Q, Yang, D. L, Liu, Y. L, Ran, W, Qu, Y, Wu, H.F, Cong, M, Li, T, Ji, L & Zhao , J.M (2018) CO2-induced ocean acidification impairs the immune function of the Pacific oyster against Vibrio splendidus challenge: An integrated study from a cellular and proteomic perspective. *Science of the Total Environment,* 625**,** 1574-1583.

Chan, K. Y. K., Grunbaum, D & O'Donnell, M.J (2011) Effects of ocean-acidification-induced morphological changes on larval swimming and feeding. *Journal of Experimental Biology,* 214**,** 3857-3867.

Chan, N. C. S. & Connolly, S.R (2013) Sensitivity of coral calcification to ocean acidification: a meta-analysis. *Global Change Biology,* 19**,** 282-290.

Chapman, P. M., Wang, F.Y, Germano, J.D, & Batley, G (2002) Pore water testing and analysis: the good, the bad, and the ugly. *Marine Pollution Bulletin,* 44**,** 359-366.

Cherkasov, A. S., Biswas, P.K,. Ridings, D.M, Ringwood, A.H & Sokolova I.M (2006) Effects of acclimation temperature and cadmium exposure on cellular energy budgets in the marine mollusk Crassostrea virginica: linking cellular and mitochondrial responses. *Journal of Experimental Biology,* 209**,** 1274-1284.

Cheung, S. G., Tai, K. K, Leung, C.K & Siu, Y.M (2002) Effects of heavy metals on the survival and feeding behaviour of the sandy shore scavenging gastropod Nassarius festivus (Powys). *Marine Pollution Bulletin,* 45**,** 107-113.

Cigliano, M., Gambi, M.C, Rodolfo-Metalpa, R, Patti, F. P & Hall-Spencer, J.M (2010) Effects of ocean acidification on invertebrate settlement at volcanic CO2 vents. *Marine Biology,* 157**,** 2489-2502.

Claiborne, J. B., Edwards, S.L, & Morrison-Shetlar, A.I (2002) Acid-base regulation in fishes: Cellular and molecular mechanisms. *Journal of Experimental Zoology,* 293**,** 302-319.

Cole, J. (2013) *Fundamentals of Ecosystem Science: Chapter 6 The Carbon Cycle With a Brief Introduction to Global Biogeochemistry*. Elsevier.

Collard, M., Laitat, K, Moulin, L, Catarino, A.I, Grosjean, P & Dubois, P (2013) Buffer capacity of the coelomic fluid in echinoderms. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology,* 166**,** 199-206.

Comber, S. & Gunn, A (1995) Behaviour of trace-metals in sewage-sludge after dispersal in seawater. *Environmental Technology,* 16**,** 419-432.

Comeau, S., Gorsky, G, Jeffree, R, Teyssie, J.L & Gattuso, J. P (2009) Impact of ocean acidification on a key Arctic pelagic mollusc (Limacina helicina). *Biogeosciences,* 6**,** 1877-1882.

Conley, D. J., Carstensen, J, Vaquer-Sunyer, R & Duarte, C. M (2009) Ecosystem thresholds with hypoxia. *Hydrobiologia,* 629**,** 21-29.

Cooper, T. F., De 'Ath, G, Fabricius, K.E & Lough, J.M (2008) Declining coral calcification in massive Porites in two nearshore regions of the northern Great Barrier Reef. *Global Change Biology,* 14**,** 529-538.

Costanza, R. (1999) The ecological, economic, and social importance of the oceans. *Ecological Economics,* 31**,** 199-213.

Council, National Research (2010) *Ocean Acidification: A National Strategy to Meet the Challenges of a Changing Ocean*. The National Academies Press.

Cozar, A., Echevarria, F, Gonzalez-Gordillo, J, Irigoien,X, UbedaB, Hernandez-Leon, S, Palma, P, Navarro, S, Garcia-de-Lomas, J, Ruiz, A, Fernandez-de-Puelles, M & Duarte, C (2014) Plastic debris in the open ocean. *Proceedings of the National Academy of Sciences of the United States of America,* 111**,** 10239-10244.

Crain, C. M., Kroeker, K & Halpern, B.S (2008) Interactive and cumulative effects of multiple human stressors in marine systems. *Ecology Letters,* 11**,** 1304-1315.

Crowe , T. P., Thompson, R.C, Bray, S & Hawkins, S.J (2000) Impacts of anthropogenic stress on rocky intertidal communities. *Journal of Aquatic Ecosystem Stress and Recovery: Kluwer Academic Publishers*, 7, 273–297.

Cyronak, T., Santos, I.R,. Erler, D.V, Maher, D.T & Eyre, B.D (2014) Drivers of pCO_2 variability in two contrasting coral reef lagoons: The influence of submarine groundwater discharge. *Global Biogeochemical Cycles,* 28**,** 398-414.

da Rosa, C. E., Iurman, M.G, Abreu, P.C, Geracitano, L.A & Monserrat, J.M (2005) Antioxidant mechanisms of the nereidid Laeonereis acuta (Anelida : Polychaeta) to cope with environmental hydrogen peroxide. *Physiological and Biochemical Zoology,* 78**,** 641-649.

Das, K. & Roychoudhury, A (2014) Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers in Environmental Science,* 2, 53.

Dashfield, S. L., Somerfield, P.J, Widdicombe, S, Austen, M.C & Nimmo, M (2008) Impacts of ocean acidification and burrowing urchins on within-sediment pH profiles and subtidal nematode communities. *Journal of Experimental Marine Biology and Ecology,* 365**,** 46-52.

Davenport, H. W. (1974) The ABC of acid-base chemistry. *Yale Journal of Biology and Medicine,* Chicago: The University of Chicago Press. 23(2): 162

de Orte, M. R., Sarmiento, A.M, Basallote, M.D, Rodriguez-Romero,A, Riba, I & delValls, A (2014) Effects on the mobility of metals from acidification caused by possible CO_2 leakage from sub-seabed geological formations. *Science of the Total Environment,* 470**,** 356-363.

Deigweiher, K., Hirse, T, Bock, C, Lucassen, M & Portner, H.O (2010) Hypercapnia induced shifts in gill energy budgets of Antarctic notothenioids. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology,* 180**,** 347-359.

der Beek, T. A., Weber, F.A, Bergmann, A Hickmann, S, Ebert, I, Hein, A &. Kuster, A (2016) Pharmaceuticals in the environment - Global occurrences and perspectives. *Environmental Toxicology and Chemistry,* 35**,** 823-835.

Dickson, A (1990a) Standard potential of the reaction: AgCl(s) + 1/2 H2(g) = Ag(s) + HCl(aq), and the standard acidity constant of the ion HSO4− in synthetic sea water from 273.15 to 318.15 K. *Journal of Chemical Thermodynamics*, 22, 113-127.

Dickson, A. G., Sabine, C.L. and Christian, J.R. (Eds.) (2007) Guide to Best Practices for Ocean CO_2 Measurements. *North Pacific Marine Science Organization*, 191.

Dizdaroglu, M (1993) Chemistry of free radical damage to DNA and nucleoproteins. *DNA and Free Radicals*, 19-39.

Doney, S., Fabry, V, Feely, R & Kleypas, J (2009) Ocean acidification: The other CO_2 problem. *Annual Review of Marine Science,* 1**,** 169-192.

Doney , S., Ruckelshaus, M, Emmett Duffy, J, Barry, J, Chan, F, English, C, Galindo, H, Grebmeier, J, Hollowed, A, Knowlton, N, Polovina, J, Rabalais, N, Sydeman, W & Talley, L (2012) Climate Change Impacts on Marine Ecosystems. *Annual Review of Marine Science*, 4:1, 11-37.

Donnachie, R. L., Johnson, A.C, Moeckel, C, Pereira, M.J & Sumpter, J.P (2014) Using risk-ranking of metals to identify which poses the greatest threat to freshwater organisms in the UK. *Environmental Pollution,* 194**,** 17-23.

Dorey, N., Maboloc, E, & Chan, K.Y.K (2018) Development of the sea urchin *Heliocidaris crassispina* from Hong Kong is robust to ocean acidification and copper contamination. *Aquatic Toxicology,* 205**,** 1-10.

Duarte, C. M., Hendriks, I.E, Moore, T.S, Olsen, Y.S, Steckbauer, A,. Ramajo, L Carstensen, J, Trotter, J.A, & McCulloch, M (2013) Is ocean acidification an open-ocean syndrome? Understanding anthropogenic impacts on seawater pH. *Estuaries and Coasts,* 36**,** 221-236.

Dufault, A. M., Cumbo, V.R, Fan, T.Y &. Edmunds, P.J (2012) Effects of diurnally oscillating *p*CO(2) on the calcification and survival of coral recruits. *Proceedings of the Royal Society B-Biological Sciences,* 279**,** 2951-2958.

Dupont, S., Dorey, N, & Thorndyke M (2010a) What meta-analysis can tell us about vulnerability of marine biodiversity to ocean acidification? *Estuarine Coastal and Shelf Science,* 89**,** 182-185.

Dupont, S., Lundve, B & Thorndyke, M (2010b) Near future ocean acidification increases growth rate of the lecithotrophic larvae and juveniles of the sea star *Crossaster papposus*. *Journal of Experimental Zoology (Molecular and Developmental Evolution),* 314(5): 382–389.

Dupont, S. & Portner, H.O (2013) Get ready for ocean acidification. *Nature,* 498**,** 429-429.

Dupont, S. & Thorndyke, M.C (2008) Ocean acidification and its impact on the early life-history stages of marine animals. *No. 36 Impacts of acidification on biological, chemical and physical systems in the Mediterranean and Black Seas - Menton, 1: CIESM Workshop Monographs n°36.*

Dupont, S. & Thorndyke, M.C (2009) Impact of CO_2 driven ocean acidification on invertebrates early life-history – What we know, what we need to know and what we can do. Biogeosciences Discussions: Copernicus Publications, 6, 3109-3131.

Durbin, A. M. & Teske, A (2011) Microbial diversity and stratification of South Pacific abyssal marine sediments. *Environmental Microbiology,* 13**,** 3219-3234.

Dutilleul, M., Reale, D, Goussen, B, Lecomte, C, Galas, S & Bonzom, J.M (2017) Adaptation costs to constant and alternating polluted environments. *Evolutionary Applications,* 10**,** 839-851.

Dwyer, J. J. & Burnett, L.E (1996) Acid-base status of the oyster *Crassostrea virginica* in response to air exposure and to infections by Perkinsus marinus. *Biological Bulletin,* 190**,** 139-147.

Eisler, R. (1998) Copper hazards to fish, wildlife and invertebrates: a synoptic review. In *Contaminant Hazard Reviews*. Laurel, MD, 5, 99.

Eriander, L., Wrange, A.L & Havenhand, J.N (2016) Simulated diurnal pH fluctuations radically increase variance in-but not the mean of-growth in the barnacle *Balanus improvisus*. *Ices Journal of Marine Science,* 73**,** 596-603.

Erickson, R. J., McKim, J.M, Lien, G.J, Hoffman, A.D & Batterman, S.L (2006) Uptake and elimination of ionizable organic chemicals at fish gills: I. Model formulation, parameterization, and behavior. *Environmental Toxicology and Chemistry,* 25**,** 1512-1521.

Erk, M., Muyssen, B.T.A, Ghekiere, A & Janssen, C.R (2008) Metallothionein and cellular energy allocation in the estuarine mysid shrimp *Neomysis integer* exposed to cadmium at different salinities. *Journal of Experimental Marine Biology and Ecology,* 357**,** 172-180.

Evans, T. G., Pespeni, M.H, Hofmann, G.E, Palumbi, S.R & Sanford, E (2017) Transcriptomic responses to seawater acidification among sea urchin populations inhabiting a natural pH mosaic. *Molecular Ecology,* 26**,** 2257-2275.

Fabry, V. J., Seibel, B.A, Feely, R.A & Orr, J.C (2008) Impacts of ocean acidification on marine fauna and ecosystem processes. *Ices Journal of Marine Science,* 65**,** 414-432.

Fdil, M. A., Mouabad, A, Outzourhit, A, Benhra, A, Maarouf, A & Pihan, J.C (2006) Valve movement response of the mussel *Mytilus galloprovincialis* to metals (Cu, Hg, Cd and Zn) and phosphate industry effluents from Moroccan Atlantic coast. *Ecotoxicology,* 15**,** 477-486.

Feely, R. A., Alin, S.R, Newton, J, Sabine, C.L, Warner, M, Devol, A, Krembs, C & Maloy, C (2010) The combined effects of ocean acidification, mixing, and respiration on pH and carbonate saturation in an urbanized estuary. *Estuarine Coastal and Shelf Science,* 88**,** 442-449.

Feely, R. A., Sabine,C.L, Hernandez-Ayon, J.M, Ianson D, & Hales, B (2008) Evidence for upwelling of corrosive "acidified" water onto the continental shelf. *Science,* 320**,** 1490-1492.

Feely, R. A., Sabine, C.L, Lee, K, Berelson, W, Kleypas, J, Fabry, V.J & Millero, F.J (2004) Impact of anthropogenic CO2 on the CaCO3 system in the oceans. *Science,* 305**,** 362-366.

Fernandez-Reiriz, M. J., Range, P,. Alvarez-Salgado, X.A & Labarta, U (2011) Physiological energetics of juvenile clams *Ruditapes decussatus* in a high CO_2 coastal ocean. *Marine Ecology Progress Series,* 433**,** 97-105.

Fitzer, S. C., Chung, P, Maccherozzi, F, Dhesi, .S, Kamenos, N.A, Phoenix, V.R & Cusack, M (2016) Biomineral shell formation under ocean acidification: a shift from order to chaos. *Scientific Reports,* 6, 21076.

Fitzer, S. C., Phoenix, V.R, Cusack, M & Kamenos, N.A (2014) Ocean acidification impacts mussel control on biomineralisation. *Scientific Reports,* 4, 6218.

Fitzgerald, J. A., Jameson, H.M, Fowler, V.H.D, Bond, G.L, Bickley, L.K, Webster, T.M.U, Bury, N.R, Wilson, R.J & Santos, E.M (2016) Hypoxia suppressed copper toxicity during early development in zebrafish embryos in a process mediated by the activation of the HIF signaling pathway. *Environmental Science & Technology,* 50**,** 4502-4512.

Fitzgerald, J. A., Katsiadaki, I & Santos, E.M (2017) Contrasting effects of hypoxia on copper toxicity during development in the three-spined stickleback (*Gasterosteus aculeatus*). *Environmental Pollution,* 222**,** 433-443.

Fitzgerald, J. A., Urbina, M.G,. Rogers,N.J,. Bury, N.R, Katsiadaki, I, Wilson, R & Santos, E.M (2019) Sublethal exposure to copper supresses the ability to acclimate to hypoxia in a model fish species. *Aquatic Toxicology,* 217, 105325.

Foo, S. A. & Byrne, M (2017) Marine gametes in a changing ocean: Impacts of climate change stressors on fecundity and the egg. *Marine Environmental Research,* 128**,** 12-24.

Foo, S. A., Dworjanyn, S.A, Poore, A.G.B & Byrne , M (2012) Adaptive capacity of the habitat modifying sea urchin *Centrostephanus rodgersii* to ocean warming and ocean acidification: Performance of Early Embryos. *Plos One,* 7(8): e42497.

Freitas, R., de Marchi, L, Moreira, A, Pestana, J, Wrona, F, Figueira, E, & Soares, A (2017) Physiological and biochemical impacts induced by mercury pollution and seawater acidification in *Hediste diversicolor*. *Science of the Total Environment,* 595**,** 691-701.

Freitas, R., Pires, A, Moreira, A, Wrona, F.J, Figueira, E & Soares, A.M.V.M (2016a) Biochemical alterations induced in *Hediste diversicolor* under seawater acidification conditions. *Marine Environmental Research,* 117**,** 75-84.

Freitas, R., Pires, A, Velez,C, Almeida, A, Moreira, A, Wrona, F.J, Soares, A & Figueira, E (2016b) Effects of seawater acidification on *Diopatra neapolitana* (Polychaete, *Onuphidae*): Biochemical and regenerative capacity responses. *Ecological Indicators,* 60**,** 152-161.

Frieder, C. A., Gonzalez, J.P, Bockmon, E.E Navarro, M.O & Levin, L.A (2014) Can variable pH and low oxygen moderate ocean acidification outcomes for mussel larvae? *Global Change Biology,* 20**,** 754-764.

Frieder, C. A., Nam, S.H, Martz, T.R & Levin, L.A (2012) High temporal and spatial variability of dissolved oxygen and pH in a nearshore California kelp forest. *Biogeosciences,* 9**,** 3917-3930.

Friederich, G. E., Walz, P.M, Burczynski, M.G & Chavez, F.P (2002) Inorganic carbon in the central California upwelling system during the 1997-1999 El Nino-La Nina event. *Progress in Oceanography,* 54**,** 185-203.

Galloway, T. S., Cole, M & Lewis, C (2017) Interactions of microplastic debris throughout the marine ecosystem. *Nature Ecology & Evolution,* 1, 0116.

Gambi, M. C., Musco, L, Giangrande, A,. Badalamenti, F, Micheli, F & Kroeker, K.J (2016) Distribution and functional traits of polychaetes in a CO_2 vent system: winners and losers among closely related species. *Marine Ecology Progress Series,* 550**,** 121-134.

Gardner, M. J. & Ravenscrof, J. E (1991) The range of copper-complexing ligands in the Tweed estuary. *Chemical Speciation & Bioavailability*, 3, 1, 22-29.

Gattuso, J. P. & Hansson, L (2011) *Ocean acidification*. United States: Oxford University Press Inc., New York.

Gattuso, J. P.,. Magnan, A, Bille, R, Cheung,W, Howes, E, Joos, F, Allemand, D, Bopp, L, Cooley, S, Eakin, C, Hoegh-Guldberg, O, Kelly, R, Portner, H, Rogers, A, Baxter, J, Laffoley, D, Osborn, D, Rankovic, A, Rochette, J, Sumaila, U, Treyer, S & Turley, C (2015) Contrasting futures for ocean and society from different anthropogenic CO_2 emissions scenarios. *Science,* 349, 6243.

Gaw, S., Thomas, K & Hutchinson, T (2014) Sources, impacts and trends of pharmaceuticals in the marine and coastal environment. *Philosophical Transactions of the Royal Society B-Biological Sciences,* 369, 1656.

Gazeau, F., Parker, L.M, Comeau, S, Gattuso, J.P, O'Connor, W.A, Martin, S, Portner, H.O & Ross, P.M (2013) Impacts of ocean acidification on marine shelled molluscs. *Marine Biology,* 160**,** 2207-2245.

Gazeau, F., van Rijswijk, P, Pozzato, L & Middelburg, J.J (2014) Impacts of ocean acidification on sediment processes in shallow waters of the arctic ocean. *Plos One,* 9(4): e94068.

Geffard, A., Smith, B.C, Amiard-Triquet, D, Jeantet, A.Y & Rainbow, P.S (2005) Kinetics of trace metal accumulation and excretion in the polychaete *Nereis diversicolor. Marine Biology,* 147**,** 1291-1304.

Geracitano, L. A., Bocchetti, R,. Monserrat, J.M, Regoli, F & Bianchini, A (2004) Oxidative stress responses in two populations of *Laeonereis acuta* (Polychaeta, Nereididae) after acute and chronic exposure to copper. *Marine Environmental Research,* 58**,** 1-17.

Godbold, J. A. & Solan, M (2013) Long-term effects of warming and ocean acidification are modified by seasonal variation in species responses and environmental conditions. *Philosophical Transactions of the Royal Society B-Biological Sciences,* 368**,** 11.

Gomme, J (1984) *Biology of the integument 1 invertebrates*. Berlin, Heidelberg, New York, Tokyo: Springer-Verlag.

Gopalakrishnan, S., Thilagam, H & Raja, P.V (2007) Toxicity of heavy metals on embryogenesis and larvae of the marine sedentary polychaete *Hydroides elegans*. *Archives of Environmental Contamination and Toxicology,* 52**,** 171-178.

Grosell, M., Blanchard, J, Brix, K.V & Gerdes, R (2007) Physiology is pivotal for interactions between salinity and acute copper toxicity to fish and invertebrates. *Aquatic Toxicology,* 84**,** 162-172.

Gruber, N. (2011) Warming up, turning sour, losing breath: ocean biogeochemistry under global change. *Philosophical Transactions of the Royal Society a-Mathematical Physical and Engineering Sciences,* 369**,** 1980-1996.

Gruber, N., Clement, D, Carter, B.R, Feely, R.A, van Heuven, S,Hoppema, M, Ishii, M, Key, R.A, Kozyr, A, Lauvset, S.K, Lo Monaco, C, Mathis, J.T, Murata, A, Olsen, A, Perez, F.F, Sabine, C.L, Tanhua, T & Wanninkhof, R (2019) The oceanic sink for anthropogenic CO2 from 1994 to 2007. *Science,* 363**,** 1193.

Gunderson, A. R., Armstrong, E. J & Stillman, J. H (2016) Multiple stressors in a changing world: The need for an improved perspective on physiological responses to the dynamic marine environment. *Annual Review of Marine Science, Vol 8,* 8**,** 357.

Gutowska, M. A., Melzner, F, Langenbuch, M, Bock, C, Claireaux, G & Portner, H.O (2010) Acid-base regulatory ability of the cephalopod (*Sepia officinalis*) in response to environmental hypercapnia. *Journal of Comparative Physiology B-Biochemical Systems and Environmental Physiology,* 180**,** 323-335.

Hale, R., Calosi, P, McNeill, L, Mieszkowska, N & Widdicombe, S (2011) Predicted levels of future ocean acidification and temperature rise could alter community structure and biodiversity in marine benthic communities. *Oikos,* 120**,** 661-674.

Hall-Spencer, J. M., Rodolfo-Metalpa, R, Martin, S, Ransome, E, Fine, M, Turner, S.M, Rowley, S.J, Tedesco, R & Buia, M.C (2008) Volcanic carbon dioxide vents show ecosystem effects of ocean acidification. *Nature,* 454**,** 96-99.

Halliwell, B. & Gutteridge, J. M. C (1999) *Free Radicals in Biology and Medicine.* Free Radicals in Biology and Medicine: Oxford University Press.

Halpern, B. S., Walbridge, S, Selkoe, K.A, Kappel, C.V, Micheli, F, D'Agrosa, C, Bruno, J.F, Casey, K.S, Ebert, C,. Fox, H.E, Fujita, R, Heinemann, D, Lenihan,H.A,. Madin, E.M.P, Perry, M.P, Selig, E.R, Spalding, M, Steneck, R & Watson, R (2008) A global map of human impact on marine ecosystems. *Science,* 319**,** 948-952.

Harley, C. D. G., Hughes A.R,. Hultgren,K.M, Miner, B.G, Sorte,C.J.B, Thornber,C.S, Rodriguez, L.F, Tomanek, L & Williams, S.L (2006) The impacts of climate change in coastal marine systems (vol 9, pg 228, 2006). *Ecology Letters,* 9**,** 500-500.

Harris, E. D. (2001) Copper homeostasis: The role of cellular transporters. *Nutrition Reviews,* 59**,** 281-285.

Harvell, D., Altizer, S, Cattadori, I.M, Harrington, L & Weil, E (2009) Climate change and wildlife diseases: When does the host matter the most? *Ecology,* 90**,** 912-920.

Harvey, B. P., Gwynn-Jones, D & Moore, P.J (2013) Meta-analysis reveals complex marine biological responses to the interactive effects of ocean acidification and warming. *Ecology and Evolution,* 3**,** 1016-1030.

Helmuth, B. S. T (1998) Intertidal mussel microclimates: Predicting the body temperature of a sessile invertebrate. *Ecological Monographs,* 68**,** 51-74.

Hendriks, I. E., Duarte, C.M & Alvarez, M (2010) Vulnerability of marine biodiversity to ocean acidification: a meta-analysis. *Estuarine, Coastal and Shelf Science*. 82 (2), 157–164.

Henry, R. P. & Wheatly, M. G (1992) Interaction of respiration, ion regulation, and acid-base-balance in the everyday life of aquatic crustaceans. *American Zoologist,* 32**,** 407-416.

Herman, P. M. J., Middelburg, J. J, Van de Koppel, J & Heip, C. H. R (1999) Ecology of estuarine macrobenthos. *Advances in Ecological Research, Vol 29: Estuaries,* 29**,** 195-240.

Hird, C. M., Urbina, M.A, Lewis, C.N, Snape, J.R & Galloway, T.S (2016) Fluoxetine exhibits pharmacological effects and trait-based sensitivity in a marine worm. *Environmental Science & Technology,* 50**,** 8344-8352.

Hochachka, P. W (1991) *Design of energy metabolism. In: Comparative animal physiology*. New York: Wiley-Liss.

Hoegh-Guldberg, O. & Bruno, J. F (2010) The impact of climate change on the world's marine ecosystems. *Science,* 328**,** 1523-1528.

Hoegh-Guldberg, O., Mumby, P.J, Hooten, A.J, Steneck, R.S, Greenfield, P, Gomez, E, Harvell, C.D, Sale, P.F, Edwards, A.J, Caldeira, K, Knowlton, N, Eakin, C.M, Iglesias-Prieto,R, Muthiga, N, Bradbury,R.H, Dubi, A & Hatziolos, M.E (2007) Coral reefs under rapid climate change and ocean acidification. *Science,* 318**,** 1737-1742.

Hofmann, G. E., Smith, J.E, Johnson,K.S, Send, U, Levin, L.A, Micheli, F, Paytan, A, Price, N.N, Peterson, B, Takeshita, Y, Matson, P.G, Crook, E.G, Kroeker, K.J, Gambi,M.C, Rivest, E.B, Frieder, C.A, Yu, P.C & Martz, T.R (2011) High-Frequency Dynamics of Ocean pH: A Multi-Ecosystem Comparison. *Plos One,* 6(12).

Hofmann, L. C., Straub, S, & Bischof, K (2012) Competition between calcifying and noncalcifying temperate marine macroalgae under elevated CO_2 levels. *Marine Ecology Progress Series,* 464**,** 89-105.

Houlbreque, F., Reynaud, S, Godinot, C, Oberhansli, F, Rodolfo-Metalpa, R & Ferrier-Pages, C (2015) Ocean acidification reduces feeding rates in the scleractinian coral *Stylophora pistillata*. *Limnology and Oceanography,* 60**,** 89-99.

Hu, M. R. Y., Hwang, P. P. & Tseng, Y. C (2015) Recent advances in understanding trans-epithelial acid-base regulation and excretion mechanisms in cephalopods. *Tissue Barriers,* 3(4): e1064196.

Huang, X. Z., Liu, Y.M, Liu, Z.K, Zhao, Z.H, Dupont, S, Wu, F.L, Huang, W, Chen, J.F, Hu, M.H,. Lu, W.Q & Wang, Y.J (2018) Impact of zinc oxide nanoparticles and ocean acidification on antioxidant responses of *Mytilus coruscus*. *Chemosphere,* 196**,** 182-195.

Hutchins, C. M., Teasdale, P.R, Lee, S.Y & Simpson, S.L (2009) The effect of sediment type and pH-adjustment on the porewater chemistry of copper- and zinc-spiked sediments. *Soil & Sediment Contamination,* 18**,** 55-73.

Ihli, J., Wong, W.C, Noel, E.H, Kim, Y.Y, Kulak, C, Christenson, H.K, Duer, M.J & Meldrum, F.C (2014) Dehydration and crystallization of amorphous calcium carbonate in solution and in air. *Nature Communications,* 5, 3169.

Imlay, J. A. & Linn, S (1988) DNA damage and oxygen radical toxicity. *Science: American Association for the Advancement of Science*, 240, 4857, 1302-1309.

IPCC. 2019. *IPCC, 2019: IPCC Special Report on the Ocean and Cryosphere in a Changing Climate.*

Ishimatsu, A., Hayashi, M, Lee, K.S, Kikkawa, T & Kita, J (2005) Physiological effects on fishes in a high-CO_2 world. *Journal of Geophysical Research-Oceans,* 110, C09S09.

Islam, M. S. & Tanaka, M (2004) Impacts of pollution on coastal and marine ecosystems including coastal and marine fisheries and approach for management: a review and synthesis. *Marine Pollution Bulletin,* 48**,** 624-649.

Ivancic, I. & Degobbis, D (1984) An optimal manual procedure for ammonia analysis in natural-waters by the Indophenol blue method. *Water Research,* 18**,** 1143-1147.

Ivanina, A. V. & Sokolova, I. M (2015) Interactive effects of metal pollution and ocean acidification on physiology of marine organisms. *Current Zoology,* 61**,** 653-668.

Jokiel, P. L., Rodgers, K.S, Kuffner, I.B,. Andersson, A.J, Cox, E.F & Mackenzie, F.T (2008) Ocean acidification and calcifying reef organisms: a mesocosm investigation. *Coral Reefs,* 27**,** 473-483.

Jones, B. & Bolam, T (2007) Copper speciation survey from UK marinas, harbours and estuaries. *Marine Pollution Bulletin,* 54**,** 1127-1138.

Jørgensen, B. & Kasten, S (2006) *Sulfur Cycling and Methane Oxidation*. Springer, Berlin, Heidelberg: Marine Geochemistry.

Kapsenberg, L., Alliouane, S, Gazeau, F, Mousseau, L & Gattuso, J.P (2017) Coastal ocean acidification and increasing total alkalinity in the northwestern Mediterranean Sea. *Ocean Science,* 13**,** 411-426.

Kapsenberg, L. & Cyronak, T (2019) Ocean acidification refugia in variable environments. *Global Change Biology,* 25**,** 3201-3214.

Kapsenberg, L. & Hofmann, G. E (2016) Ocean pH time-series and drivers of variability along the northern Channel Islands, California, USA. *Limnology and Oceanography,* 61**,** 953-968.

Kapsenberg, L., Kelley, A, Francis, L & Raskin, S (2015a) Exploring the complexity of ocean acidification: An ecosystem comparison of coastal pH variability. Science Scope, 39.

Kapsenberg, L., Kelley, A.L, Shaw, E.C, Martz, T.R & Hofmann, G.E (2015b) Near-shore Antarctic pH variability has implications for the design of ocean acidification experiments. *Scientific Reports,* 5, 9638.

Karlsson, M. V., Carter, L.J, Agatz, A & Boxall, A.B.A (2017) Novel Approach for Characterizing pH-Dependent Uptake of Ionizable Chemicals in Aquatic Organisms. *Environmental Science & Technology,* 51**,** 6965-6971.

Kawecki, T. J. & Ebert, D (2004) Conceptual issues in local adaptation. *Ecology Letters,* 7**,** 1225-1241.

Kelly, M. W. & Hofmann, G. E (2013) Adaptation and the physiology of ocean acidification. *Functional Ecology,* 27**,** 980-990.

Kimbrough, K. L., Johnson,W.E, Lauenstein, G.G, Christensen, J.D & Apeti, D.A (2008) An assessment of two decades of contaminant monitoring in the nation's coastal zone. *NOAA Technical Memorandum NOS NCCOS 74: Silver Spring.*105pp.

Kristensen, E., Jensen, M.H & Andersen, T.K (1985) The impact of polychaete (*Nereis-virens sars*) burrows on nitrification and nitrate reduction in esturine sediments. *Journal of Experimental Marine Biology and Ecology,* 85**,** 75-91.

Kroeker, K. J., Kordas, R.L, Crim, R, Hendriks, I.E, Ramajo, L, Singh, G.S, Duarte, C.M & Gattuso, J.P (2013) Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming. *Global Change Biology,* 19**,** 1884-1896.

Kroeker, K. J., Kordas, R.L, Crim, R.N & Singh, G.G (2010) Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. *Ecology Letters,* 13**,** 1419-1434.

Kurihara, H (2008) Effects of CO_2-driven ocean acidification on the early developmental stages of invertebrates. *Marine Ecology Progress Series,* 373**,** 275-284.

Kurihara, H., Asai, T, Kato, S & Ishimatsu, A (2009) Effects of elevated $pCO(_2)$ on early development in the mussel *Mytilus galloprovincialis*. *Aquatic Biology,* 4**,** 225-233.

Kurihara, H., Ishimatsu, A & Shirayama, Y (2007) Effects of elevated seawater CO_2 concentration on the meiofauna. *Journal of Marine Science and Technology-Taiwan,* 15**,** 17-22.

Kurihara, H., Kato, S & Ishimatsu, A (2007) Effects of increased seawater $pCO(_2)$ on early development of the oyster *Crassostrea gigas*. *Aquatic Biology,* 1**,** 91-98.

Kwiatkowski, L., Gaylord, B, Hill,T, Hosfelt, J, Kroeker, K.J, Nebuchina, Y, Ninokawa, A, Russell, A.D, Rivest, E.D, Sesboue, M & Caldeira, K (2016) Nighttime dissolution in a temperate coastal ocean ecosystem increases under acidification. *Scientific Reports,* 6.

Kwiatkowski, L. & Orr, J. C (2018) Diverging seasonal extremes for ocean acidification during the twenty-first century. *Nature Climate Change,* 8**,** 141.

Kwok, K. W. H., Grist, E. P. M, & Leung, K. M. Y (2009) Acclimation effect and fitness cost of copper resistance in the marine copepod *Tigriopus japonicus*. *Ecotoxicology and Environmental Safety,* 72**,** 358-364.

Lacoue-Labarthe, T., Martin, S, Oberhansli, F, Teyssie, J.L, Markich, S, Ross, J & Bustamante, P (2009) Effects of increased pCO_2 and temperature on trace element (Ag, Cd and Zn) bioaccumulation in the eggs of the common cuttlefish, *Sepia officinalis*. *Biogeosciences,* 6**,** 2561-2573.

Lacoue-Labarthe, T., Reveillac, E, Oberhansli, F, Teyssie, J.L, Jeffree & Gattuso, J.P (2011) Effects of ocean acidification on trace element accumulation in the early-life stages of squid *Loligo vulgaris*. *Aquatic Toxicology,* 105**,** 166-176.

Laffoley, D. & Baxter, J. M (2019) Ocean deoxygenation: everyone's problem. Causes, impacts, consequences and solutions. International Union for Conservation of Nature, xxii, 562p.

Lange, R. & Marshall, D (2017) Ecologically relevant levels of multiple, common marine stressors suggest antagonistic effects. *Scientific Reports,* 7, 6281.

Langston, W. J., Chesman, B.S,. Burt, J.R, Hawkins, S.J, Readman, J & Worsfold, P (2003) Site characterisation of the South West European marine sites. The Exe Estuary special protection area. *Marine Biological Association Occasional Publication* 10.

Lannig, G., Eilers, S, Portner, H.O, Sokolova, I.M, & Bock, C (2010) Impact of ocean acidification on energy metabolism of oyster, *crassostrea giga*s-changes in metabolic pathways and thermal response. *Marine Drugs,* 8**,** 2318-2339.

Larsen, B. K., Portner, H.O, & Jensen, F.B, (1997) Extra- and intracellular acid-base balance and ionic regulation in cod (*Gadus morhua*) during combined and isolated exposures to hypercapnia and copper. *Marine Biology,* 128**,** 337-346.

Laurent, A., Fennel, K, Ko, D.S, & Lehrter, J (2018) Climate Change Projected to Exacerbate Impacts of Coastal Eutrophication in the Northern Gulf of Mexico. *Journal of Geophysical Research-Oceans,* 123**,** 3408-3426.

Laverock, B., Kitidis, V, Tait, K, Gilbert, K.A, Osborn, K.M & Widdicombe, S (2013) Bioturbation determines the response of benthic ammonia-oxidizing microorganisms to ocean acidification. *Philosophical Transactions of the Royal Society B-Biological Sciences,* 368: 20120441.

Lee, S. & Cundy, A (2001) Heavy metal contamination and mixing processes in sediments from the Humber Estuary, Eastern England. *Estuarine Coastal and Shelf Science,* 53**,** 619-636.

Lee, S. Y (2008) Mangrove macrobenthos: Assemblages, services, and linkages. *Journal of Sea Research,* 59**,** 16-29.

Lewis, A. G. 1995. Copper in water and aquatic environments. *New York: NY : International Copper Association.* 1-2pp.

Lewis, C., Clemow, K & Holt, W.V (2013a) Metal contamination increases the sensitivity of larvae but not gametes to ocean acidification in the polychaete *Pomatoceros lamarckii* (Quatrefages). *Marine Biology,* 160**,** 2089-2101.

Lewis, C., Ellis, R.P, Vernon, E, Elliot, K, Newbatt, S & Wilson, R.W (2016) Ocean acidification increases copper toxicity differentially in two key marine invertebrates with distinct acid-base responses. *Scientific Reports,* 6.

Lewis, C. & Galloway, T (2008) Genotoxic damage in polychaetes: A study of species and cell-type sensitivities. *Mutation Research-Genetic Toxicology and Environmental Mutagenesis,* 654**,** 69-75.

Lewis, C. N., Brown,K.A, Edwards, L.A, Cooper, G & Findlay, H.S (2013b) Sensitivity to ocean acidification parallels natural $pCO(_2)$ gradients experienced by Arctic copepods under winter sea ice. *Proceedings of the National Academy of Sciences of the United States of America,* 110**,** E4960-E4967.

Lewis, E. & Wallace, D (1998) Program developed for CO_2 system calculations. Carbon dioxide information analysis centre. *Atmospheric Sciences Division, New York*.

Li, W. & Gao, K. S (2012) A marine secondary producer respires and feeds more in a high CO2 ocean. *Marine Pollution Bulletin,* 64**,** 699-703.

Linares, C., Vidal, M, Canals, M, Kersting, D.K, Amblas, D, Aspillaga, E Cebrian, E, Delgado-Huertas, A, Diaz, D, Garrabou, J, Hereu, B, Navarro, L, Teixido, N & Ballesteros, E (2015) Persistent natural acidification drives major distribution shifts in marine benthic ecosystems. *Proceedings of the Royal Society B-Biological Sciences,* 282: 20150587

Liu, W. G. & He, M. X (2012) Effects of ocean acidification on the metabolic rates of three species of bivalve from southern coast of China. *Chinese Journal of Oceanology and Limnology,* 30**,** 206-211.

Lopez-Mendilaharsu, M., Gardner, S.C, Seminoff, J.A & Riosmena-Rodriguez, R (2005) Identifying critical foraging habitats of the green turtle (*Chelonia mydas*) along the Pacific coast of the Baja California peninsula, Mexico. *Aquatic Conservation-Marine and Freshwater Ecosystems,* 15**,** 259-269.

Lovett, D. & Felder, D (1990) Ontogenetic Change in Digestive Enzyme Activity of Larval and Postlarval White Shrimp *Penaeus setiferus* (Crustacea, Decapoda, Penaeidae). The Biological Bulletin, 178(2), 144-159.

Lucu, C. & Towle, D. W (2003) Na++K+-ATPase in gills of aquatic crustacea. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology,* 135**,** 195-214.

Lutz, M., Dunbar, R & Caldeira, K (2002) Regional variability in the vertical flux of particulate organic carbon in the ocean interior. *Global Biogeochemical Cycles,* 16 (3).

Lv, L. L., Dong, X.X, Lv, F, Yu, Y.B, Zhao, W.H & Liu, F (2016) Antioxidant enzymes responses of polychaete *Perinereis aibuhitensis* following chronic exposure to 17 beta-estradiol. *Italian Journal of Animal Science,* 15**,** 552-557.

Manallack, D. T (2009) The acid-base profile of a contemporary set of drugs: implications for drug discovery. *Sar and Qsar in Environmental Research,* 20**,** 611-655.

Mangan, S., Urbina, M.A, Findlay, H.S, Wilson, R.W & Lewis, C (2017) Fluctuating seawater pH/pCO$_2$) regimes are more energetically expensive than static pH/pCO$_2$) levels in the mussel *Mytilus edulis*. *Proceedings of the Royal Society B-Biological Sciences,* 284 (1865).

Mangan, S., Wilson, R.W, Findlay, H.S & Lewis, C (2019) Acid - base physiology over tidal periods in the mussel *Mytilus edulis*: size and temperature are more influential than seawater pH. *Proceedings of the Royal Society B-Biological Sciences,* 286: 20182863.

Manzello, D. P. (2010) Coral growth with thermal stress and ocean acidification: lessons from the eastern tropical Pacific. *Coral Reefs,* 29**,** 749-758.

Marchant, H. K., Calosi, P & Spicer, J.I (2010) Short-term exposure to hypercapnia does not compromise feeding, acid-base balance or respiration of *Patella vulgata* but surprisingly is accompanied by radula damage. *Journal of the Marine Biological Association of the United Kingdom,* 90**,** 1379-1384.

Maria, V. L. & Bebianno, M. J. (2011) Antioxidant and lipid peroxidation responses in *Mytilus galloprovincialis* exposed to mixtures of benzo(a)pyrene and copper. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology,* 154**,** 56-63.

Martincic, D., Kwokal, Z & Branica, M (1990) Distribution of zinc, lead, cadmium and copper between different size fractions of sediments. 1. The Limski Kanal (North Adriatic Sea). *Science of the Total Environment,* 95**,** 201-215.

Martinez, M., Intralawan, A, Vazquez, G, Perez-Maqueo, O, Sutton, P & Landgrave, R (2007) The coasts of our world: Ecological, economic and social importance. *Ecological Economics,* 63**,** 254-272.

Matthiessen, P., Reed, J & Johnson, M (1999) Sources and potential effects of copper and zinc concentrations in the estuarine waters of Essex and Suffolk, United Kingdom. *Marine Pollution Bulletin,* 38**,** 908-920.

McCord, J. M., Keele, B.B & Fridovich, I (1971) An enzyme-based theory of obligate anaerobiosis: The physiological function of superoxide dismutase. *Proceedings of the National Academy of Sciences of the United States of America*, 68(5), 1024-1027.

McCoy, S. J., Kamenos ,N.A, Chung, P, Wootton, T.J & Pfister, C.A (2018) A mineralogical record of ocean change: Decadal and centennial patterns in the California mussel. *Global Change Biology,* 24**,** 2554-2562.

McQuillan, J. S., Kille, P, Powell, K & Galloway, T.S (2014) The regulation of copper stress response genes in the polychaete *Nereis diversicolor* during prolonged extreme copper contamination. *Environmental Science & Technology,* 48**,** 13085-13092.

Mee, L. (2012) Between the devil and the deep blue sea: The coastal zone in an era of globalisation. *Estuarine Coastal and Shelf Science,* 96**,** 1-8.

Mehrbach., Culberson, C. H., Hawley, J. E & Pytokowicx, R. M (1973) Measurements of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure 1. *Limnology and Oceanography*, 18, 6, 897-907.

Meinshausen, M., Smith, S.J, Calvin, K, Daniel, J.S, Kainuma, M.L.T, Lamarque, J.F, Matsumoto, K, Montzka, S.A Raper, S.C.B, Riahi, K, Thomson, A, Velders & G.J.M, van Vuuren, D.P.P (2011) The RCP greenhouse gas concentrations and their extensions from 1765 to 2300. *Climatic Change,* 109**,** 213-241.

Melzner, F., Gutowska, M.A, Langenbuch, M, Dupont, S, Lucassen, M, Thorndyke, M.C, Bleich, M & Portner, H.O (2009) Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? *Biogeosciences,* 6**,** 2313-2331.

Melzner, F., Thomsen, J, Koeve, W, Oschlies, A, Gutowska, M.A, Bange, H.W, Hansen, H.P & Kortzinger, A (2013) Future ocean acidification will be amplified by hypoxia in coastal habitats. *Marine Biology,* 160**,** 1875-1888.

Michaelidis, B., Ouzounis, C, Paleras, A & Portner, H.O (2005) Effects of long-term moderate hypercapnia on acid-base balance and growth rate in marine mussels *Mytilus galloprovincialis. Marine Ecology Progress Series,* 293**,** 109-118.

Middelburg, J. J (2019) Biogeochemical Processes and Inorganic Carbon Dynamics*. In: Marine Carbon Biogeochemistry.* Springer Briefs in Earth System Sciences: Springer, Cham.

Mieszkowska, N., Kendall, M.A, Hawkins, S.J, Leaper, R, Williamson, P, Hardman-Mountford, N.J & Southward, A.J (2006) Changes in the range of some common rocky shore species in Britain - a response to climate change? *Hydrobiologia,* 555**,** 241-251.

Miles, H., Widdicombe, S, Spicer, J.I & Hall-Spencer, J (2007) Effects of anthropogenic seawater acidification on acid-base balance in the sea urchin *Psammechinus miliaris*. *Marine Pollution Bulletin,* 54**,** 89-96.

Millero, F. J., Pierrot, D, Lee, K, Wanninkhof, R, Feely, R, Sabine, C.L, Key, R.M & Takahashi, T (2002) Dissociation constants for carbonic acid determined from field measurements. *Deep-Sea Research Part I-Oceanographic Research Papers,* 49**,** 1705-1723.

Millero, F. J., Woosley, R, Ditrolio, B & Waters, J (2009) Effect of ocean acidification on the speciation of metals in seawater. *Oceanography,* 22**,** 72-85.

Moreira, S. M., Moreira-Santos,M, Guilhermino, L & Ribeiro, R (2005) Short-term sublethal in situ toxicity assay with *Hediste diversicolor* (polychaeta) for estuarine sediments based on postexposure feeding. *Environmental Toxicology and Chemistry,* 24**,** 2010-2018.

Morth, J. P., Pedersen, B.P, Buch-Pedersen, M.J, Andersen, J.P, Vilsen, B, Palmgren, M.G & Nissen, P (2011) A structural overview of the plasma membrane Na+, K+-ATPase and H+-ATPase ion pumps. *Nature Reviews Molecular Cell Biology,* 12**,** 60-70.

Moss, R., Babiker, M, Brinkman, S, Calvo, E, Carter, T.R, Edmonds, J, Elgizouli, I, Emori, S, Erda, L, Hibbard, K, Jones, R, Kainuma,M, Kelleher, J, Lamarque, J.F, Manning, M.R, Matthews, B, Meehl,J, Meyer, L, Mitchell, J,.F.B, Nakicenovic, N, O'Neill, B, Pichs, R, Riahi, K, Rose, S.K, Runci, P, Stouffer, R.J, van Vuuren, D.P, Weyant, J.P, Wilbanks, T.J,

van Ypersele, J & Zurek, M (2008) Towards new scenarios for analysis of emissions, climate change, impacts, and response strategies: IPCC Expert Meeting Report, Noordwijkerhout, The Netherlands, 155.

Moss, R. H., Edmonds, J.A,. Hibbard, K.A, Manning, M.R, Rose, S.K, van Vuuren, D.P, Carter, T.R, Emori, S, Kainuma, M, Kram, T,. Meehl, G.A, Mitchell, J.F.B, Nakicenovic, N, Riahi, K, Smith, S.J, Stouffer, R.J, Thomson, A.M, Weyant, J.P & Wilbanks, T.J (2010) The next generation of scenarios for climate change research and assessment. *Nature,* 463**,** 747-756.

Moulin, L., Grosjean,P, Leblud, J, Batigny, A & Dubois, P (2014) Impact of elevated pCO_2 on acid-base regulation of the sea urchin *Echinometra mathaei* and its relation to resistance to ocean acidification: A study in mesocosms. *Journal of Experimental Marine Biology and Ecology,* 457**,** 97-104.

Mouneyrac, C., Mastain, O, Amiard, J.C, Amiard-Triquet, C, Beaunier, P, Jeantet, A.Y, Smith, B.D & Rainbow, P.S (2003) Trace-metal detoxification and tolerance of the estuarine worm *Hediste diversicolor* chronically exposed in their environment. *Marine Biology,* 143**,** 731-744.

Newbatt, S (2015) The combined impacts of ocean acidification and copper on the physiology of European sea bass (*Dicentrarchus labrax*) and shore crabs (maenas). PhD Thesis, School of Biological Sciences, University of Exeter, Exeter.

Nicolaus, E., Law, R, Wright, S & Lyons, B (2015) Spatial and temporal analysis of the risks posed by polycyclic aromatic hydrocarbon, polychlorinated biphenyl and metal contaminants in sediments in UK estuaries and coastal waters. *Marine Pollution Bulletin,* 95**,** 469-479.

Nielson, C., Hird, C & Lewis, C (2019) Ocean acidification buffers the physiological responses of the king ragworm Alitta virens to the common pollutant copper. *Aquatic Toxicology,* 212**,** 120-127.

Omar, A. M., Thomas, H, Olsen, A, Becker, M, Skjelvan, I & Reverdin, G (2019) Trends of ocean acidification and $pCO_{(2)}$ in the Northern North Sea, 2003-2015. *Journal of Geophysical Research-Biogeosciences,* 124**,** 3088-3103.

Oppenheimer, M., Glavovic, B.C, Hinkel, J,van de Wal, R, Magnan, A.K, Abd-Elgawad, A, Cai, R, Cifuentes-Jara, M, DeConto, R.M, Ghosh, T, Hay, J, Isla, F, Marzeion, B, Meyssignac, B & Sebesvari, Z (2019) Sea level rise and implications for low-lying

islands, coasts and communities. In: I*PCC Special Report on the Ocean and Cryosphere in a Changing Climate*.

Orr, J. C., Fabry, V.J, Aumont, O, Bopp, L, Doney, S.C, Feely, R.A, Gnanadesikan, A, Gruber, N, Ishida, A, Joos, F, Key, R.M, Lindsay, K, Maier-Reimer, E, Matear, R, Monfray, P, Mouchet, A,. Najjar, R.G, Plattner, G,K Rodgers, K.B, Sabine, C.L, Sarmiento, J.L, Schlitzer, R, Slater, R, Totterdell, I.J, Weirig,M.F, Yamanaka, Y & Yool, A (2005) Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature,* 437**,** 681-686.

Oschlies, A., Schulz, K.G, Riebesell, U & Schmittner, A (2008) Simulated 21st century's increase in oceanic suboxia by CO_2-enhanced biotic carbon export. *Global Biogeochemical Cycles,* 22, GB4008.

OSPAR (2017) Summary Status of the OSPAR Network of Contaminants Intermediate Assessment 2017.

Pachauri, R. K. & Meyer, L. A (2014) IPCC, 2014: Climate change 2014: Synthesis report. Contribution of working groups i, ii and iii to the fifth assessment report of the intergovernmental panel on climate change. IPCC, Geneva, Switzerland.

Pachauri, R. K. & Reisinger, A (2007) IPCC, 2007: Climate change 2007: Synthesis report. Contribution of working groups i, ii and iii to the fourth assess- ment report of the intergovernmental panel on climate change. IPCC, Geneva, Switzerland, 104pp.

Paganini, A. W., Miller, N.A & Stillman, J.H (2014) Temperature and acidification variability reduce physiological performance in the intertidal zone porcelain crab *Petrolisthes cinctipes*. *Journal of Experimental Biology,* 217**,** 3974-3980.

Pan, T. C. F., Applebaum, S.L & Manahan, D.T (2015) Experimental ocean acidification alters the allocation of metabolic energy. *Proceedings of the National Academy of Sciences of the United States of America,* 112**,** 4696-4701.

Pansch, C., Schaub, I, Havenhand, J & Wahl, M (2014) Habitat traits and food availability determine the response of marine invertebrates to ocean acidification. *Global Change Biology,* 20**,** 765-777.

Pascal, P. Y., Fleeger, J.W, Galvez, F & Carman, K.R (2010) The toxicological interaction between ocean acidity and metals in coastal meiobenthic copepods. *Marine Pollution Bulletin,* 60**,** 2201-2208.

Pespeni, M. H., Sanford, E, Gaylord, B, Hill, T.M, Hosfelt, J.D, Jaris, H.K, LaVigne, M, Lenz, E.A, Russell, A, Young, M.K & Palumbi, S.R (2013) Evolutionary change during experimental ocean acidification. *Proceedings of the National Academy of Sciences of the United States of America,* 110**,** 6937-6942.

Phillips, D. J. H (1980) *Quantitative aquatic biological indicators : their use to monitor trace metal and organochlorine pollution*. London: Applied Science Publishers.

Pini, J. M., Richir, J & Watson, G. J (2015) Metal bioavailability and bioaccumulation in the polychaete *Nereis (Alitta) virens* (Sars): The effects of site-specific sediment characteristics. *Marine Pollution Bulletin,* 95**,** 565-575.

Pires, A., Figueira,E, Moreira, A,Soares, A & Freitas, R (2015) The effects of water acidification, temperature and salinity on the regenerative capacity of the polychaete *Diopatra neapolitana*. *Marine Environmental Research,* 106**,** 30-41.

Pocklington, P. & Wells, P. G (1992) Polychaetes - Key text for marine environmental-quality monitoring. *Marine Pollution Bulletin,* 24**,** 593-598.

Pook, C., Lewis, C & Galloway, T (2009) The metabolic and fitness costs associated with metal resistance in *Nereis diversicolor*. *Marine Pollution Bulletin,* 58**,** 1063-1071.

Portner, H. O (2008) Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. *Marine Ecology Progress Series,* 373**,** 203-217.

Portner, H. O., Langenbuch, M & Reipschlager, A (2004) Biological impact of elevated ocean CO_2 concentrations: Lessons from animal physiology and earth history. *Journal of Oceanography,* 60**,** 705-718.

Przeslawski, R., Byrne, M & Mellin, C (2015) A review and meta-analysis of the effects of multiple abiotic stressors on marine embryos and larvae. *Global Change Biology,* 21**,** 2122-2140.

Queiros, A. M., Taylor, P, Cowles, A, Reynolds, A, Widdicombe, S & Stahl, H (2015) Optical assessment of impact and recovery of sedimentary pH profiles in ocean acidification and carbon capture and storage research. *International Journal of Greenhouse Gas Control,* 38**,** 110-120.

Radha, A. V., Forbes, T.Z, Killian, C.E, Gilbert, P & Navrotsky, A (2010) Transformation and crystallization energetics of synthetic and biogenic amorphous calcium carbonate. *Proceedings of the National Academy of Sciences of the United States of America,* 107**,** 16438-16443.

Ragnarsson, S. A. & Raffaelli, D (1999) Effects of the mussel *Mytilus edulis L*. on the invertebrate fauna of sediments. *Journal of Experimental Marine Biology and Ecology,* 241**,** 31-43.

Rainbow, P. S (1997) Trace metal accumulation in marine invertebrates: Marine biology or marine chemistry? *Journal of the Marine Biological Association of the United Kingdom,* 77**,** 195-210.

Rassmann, J., Lansard, B, Gazeau, F, Guidi-Guilvard, L, Pozzato, L, Alliouane, S, Grenz, C & Rabouille, C (2018) Impact of ocean acidification on the biogeochemistry and meiofaunal assemblage of carbonate-rich sediments: Results from core incubations (Bay of Villefranche, NW Mediterranean Sea). *Marine Chemistry,* 203**,** 102-119.

Rassmann, J., Lansard, B, Pozzato, L & Rabouille, C (2016) Carbonate chemistry in sediment porewaters of the Rhone River delta driven by early diagenesis (northwestern Mediterranean). *Biogeosciences,* 13**,** 5379-5394.

Rastrick, S. P. S., Calosi, P, Calder-Potts, R, Foggo, A, Nightingale, G, Widdicombe, S & Spicer, J.I (2014) Living in warmer, more acidic oceans retards physiological recovery from tidal emersion in the velvet swimming crab, *Necora puber*. *Journal of Experimental Biology,* 217**,** 2499-2508.

Ravaglioli, C., Bulleri, F, Ruhl, S, McCoy, S.J, Findlay, H.S Widdicombe, S & Queiros, A.M (2019a) Ocean acidification and hypoxia alter organic carbon fluxes in marine soft sediments. *Global Change Biology,* 25**,** 4165-4178.

Ravaglioli, C., Lardicci, C, Pusceddu, A, Arpe, E, Bianchelli, S, Buschi, E & Bulleri, F (2019b) Ocean acidification alters meiobenthic assemblage composition and organic matter degradation rates in seagrass sediments. *Limnology and Oceanography*.

Raven, J., Caldeira, K, Elderfield, H, Hoegh-Guldberg, O, Liss, P,. Riebesell,U, Sheperd, J, Turley, C & Watson, A (2005) *Ocean acidification due to increasing atmospheric carbon dioxide*. Royal Society Policy Document.

Regoli, F., Nigro, M & Orlando, E (1998) Lysosomal and antioxidant responses to metals in the Antarctic scallop *Adamussium colbecki*. *Aquatic Toxicology,* 40**,** 375-392.

Rendal, C., Kusk, K.O & Trapp, S (2011) Optiman choice of pH for toxicity and bioaccumulation studies of ionizing organic chemicals. *Environmental Toxicology and Chemistry,* 30**,** 2395-2406.

Reum, J. C. P., Alin, S.R, Feely, R.A, Newton, J, Warner, M & McElhany, P (2014) Seasonal carbonate chemistry covariation with temperature, oxygen, and salinity in a Fjord estuary: Implications for the design of ocean acidification experiments. *Plos One,* 9(2): e89619

Riba, I., Del Valls, T.A, Forja, J.M & Gomez-Parra, A (2004) The influence of pH and salinity on the toxicity of heavy metals in sediment to the estuarine clam *Ruditapes philippinarum*. *Environmental Toxicology and Chemistry,* 23**,** 1100-1107.

Riba, I., Garcia-Luque, E, Blasco, J & Delvalls, T.A (2003) Bioavailability of heavy metals bound to estuarine sediments as a function of pH and salinity values. *Chemical Speciation and Bioavailability,* 15**,** 101-114.

Ricevuto, E., Kroeker, K.J, Ferrigno, F, Micheli, F & Gambi, M.C (2014) Spatio-temporal variability of polychaete colonization at volcanic CO_2 vents indicates high tolerance to ocean acidification. *Marine Biology,* 161**,** 2909-2919.

Richards, R., Chaloupka, M, Sano, M & Tomlinson, R (2011) Modelling the effects of 'coastal' acidification on copper speciation. *Ecological Modelling,* 222**,** 3559-3567.

Richter, R (1952) Fluidal-texture in Sediment-Gesteinen und ober Sedifluktion überhaupt. Notizbl Hess Landesamtes Bodenforsch Wiesbaden, 68-81.

Ridgway, J. & Shimmield, G (2002) Estuaries as repositories of historical contamination and their impact on shelf seas. *Estuarine Coastal and Shelf Science,* 55**,** 903-928.

Riebesell, U., Zondervan, I, Rost, B, Tortell, P.D, Zeebe, R.E & Morel, F.M.M (2000) Reduced calcification of marine plankton in response to increased atmospheric CO_2. *Nature,* 407**,** 364-367.

Robbins, L. L., Wynn, J.G, Lisle,J.T, Yates, K.K, Knorr, P.O, Byrne,R.H,. Liu, X.W, Patsavas, M.C, Azetsu-Scott, K & Takahashi, T (2013) Baseline monitoring of the Western Arctic Ocean estimates 20% of Canadian Basin surface waters are undersaturated with respect to aragonite. *Plos One,* 8(9): e73796.

Roberts, D. A., Birchenough, S.N.R, Lewis, C, Sanders, M.B, Bolam, T & Sheahan, D (2013) Ocean acidification increases the toxicity of contaminated sediments. *Global Change Biology,* 19**,** 340-351.

Rodriguez-Romero, A., Jarrold, M.D, Massamba-N'Siala, D, Spicer, J.I & Calosi, P (2016) Multi-generational responses of a marine polychaete to a rapid change in seawater $pCO(_2)$. *Evolutionary Applications,* 9**,** 1082-1095.

Rogell, B., Hofman, M, Eklund, M, Laurila, A & Hoglund, J (2009) The interaction of multiple environmental stressors affects adaptation to a novel habitat in the natterjack toad *Bufo calamita*. *Journal of Evolutionary Biology,* 22**,** 2267-2277.

Rogers, A. D. & Laffoley, D (2013) Introduction to the special issue: The global state of the ocean; interactions between stresses, impacts and some potential solutions. Synthesis papers from the International Programme on the State of the Ocean 2011 and 2012 workshops. *Marine Pollution Bulletin,* 74**,** 491-494.

Roggatz, C. C., Fletcher, N, Benoit, D.M, Algar, A.C, Doroff, A, Wright,B, Valero, K.C.W & Hardege, J.D (2019) Saxitoxin and tetrodotoxin bioavailability increases in future oceans. *Nature Climate Change,* 9**,** 840-+.

Rohatgi, A (2012) WebPlotDigitalizer: HTML5 based online tool to extract numerical data from plot images.

Ronnback, P (1999) The ecological basis for economic value of seafood production supported by mangrove ecosystems. *Ecological Economics,* 29**,** 235-252.

Roose, P., Albaigés, J, Bebianno, M.J, Camphuysen, C, Cronin, M, de Leeuw, J, Gabrielsen, G, Hutchinson, T, Hylland, K, Jansson, B, Jenssen, B.M, Schulz-Bull, D, Szefer, P, Webster,L, Bakke, T & Janssen., C (2011) Chemical pollution in Europe's seas: Programmes, practices and priorities for research, *Marine Board-ESF, Ostend, Belgium*. Marine Board Position Paper 16.

Ross, P. M., Parker, L, O'Connor, W.A & Bailey, E.A (2011) The impact of ocean acidification on reproduction, early development and settlement of marine organisms. *Water,* 3**,** 1005-1030.

Ruttkay-Nedecky, B., Nejdl, L, Gumulec, J, Zitka, O, Masarik, M, Eckschlager, T, Stiborova, M, Adam, V & Kizek, R (2013) The Role of metallothionein in oxidative stress. *The International Journal of Molecular Sciences*, 14, 6044-6066.

Saarikoski, J. & Viluksela, M (1982) Relation between physicochemical properties of phenols and their toxicity and accumulation in fish. *Ecotoxicology and Environmental Safety,* 6**,** 501-512.

Sabine, C. L., Feely,R.A, Gruber, N, Key, R.M, Lee, K, Bullister, J.L, Wanninkhof, R,Wong, C.S, Wallace, D.W.R, Tilbrook, B, Millero, F.J, Peng, T.H, Kozyr, A, Ono, T & Rios, A.F (2004) The oceanic sink for anthropogenic CO_2. *Science,* 305**,** 367-371.

Sainz, A., Grande , J.A & de la Torre, M.L (2004) Characterisation of heavy metal discharge into the Ria of Huelva. *Environment International,* 30**,** 557-566.

Salomons , W. & Förstner, U (1984) *Metals in the Hydrocycle*. Springer-Verlag Berlin Heidelberg.

Scanes, E., Parker, L.M, O'Connor, W.A, Gibbs, M.C & Ross, P.M (2018) Copper and ocean acidification interact to lower maternal investment, but have little effect on adult physiology of the Sydney rock oyster *Saccostrea glomerata*. *Aquatic Toxicology,* 203**,** 51-60.

Scanes, E., Parker, L.M, O'Connor, W.A,. Stapp, L.S & Ross, P.M (2017) Intertidal oysters reach their physiological limit in a future high-CO_2 world. *Journal of Experimental Biology,* 220**,** 765-774.

Schiedek, D., Sundelin,B, Readman, J & Macdonald, R (2007) Interactions between climate change and contaminants. *Marine Pollution Bulletin,* 54**,** 1845-1856.

Schlegel, P., Havenhand, J.N, Obadia, N & Williamson, J.E (2014) Sperm swimming in the polychaete *Galeolaria caespitosa* shows substantial inter-individual variability in response to future ocean acidification. *Marine Pollution Bulletin,* 78**,** 213-217.

Schnoor, J. L. (2014) Ocean Acidification: The Other Problem with CO_2. *Environmental Science & Technology,* 48**,** 10529-10530.

Serra-Compte, A., Maulvault, A.L, Camacho, C, Alvarez-Munoz, D, Barcelo,D, Rodriguez-Mozaz, S & Marques, A (2018) Effects of water warming and acidification on bioconcentration, metabolization and depuration of pharmaceuticals and endocrine disrupting compounds in marine mussels (*Mytilus galloprovincialis*). *Environmental Pollution,* 236**,** 824-834.

Sharma, P., Jha, A.B, Dubey, R.M & Pessarakli, M (2012) Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Journal of Botany: Hindawi Publishing Corporation*, 217037.

Shi, D. L., Xu, Y, Hopkinson, B.M & Morel, F.M.M (2010) Effect of ocean acidification on iron availability to marine phytoplankton. *Science,* 327**,** 676-679.

Siddiqui, S. & Bielmyer-Fraser, G.K (2015) Responses of the sea anemone, *Exaiptasia pallida*, to ocean acidification conditions and copper exposure. *Aquatic Toxicology,* 167**,** 228-239.

Simpson, S. L., Angel, B.M & Jolley, D.F (2004) Metal equilibration in laboratory-contaminated (spiked) sediments used for the development of whole-sediment toxicity tests. *Chemosphere,* 54**,** 597-609.

Sinha, P. R., Kondo, Y, Koike, M, Ogren, J.A, Jefferson, A, Barrett, T.E, Sheesley,R.J, Ohata, S, Moteki, N, Coe, H, Liu, D, Irwin,M, Tunved, P, Quinn, P.K & Zhao, Y (2017) Evaluation of ground-based black carbon measurements by filter-based photometers at two Arctic sites. *Journal of Geophysical Research-Atmospheres,* 122**,** 3544-3572.

Smirnoff, H (1995) *Antioxidant systems and plant response to the environment*. Environment and plant metabolism.

Sokolova, I. M. (2013) Energy-limited tolerance to stress as a conceptual framework to integrate the effects of multiple stressors. *Integrative and Comparative Biology,* 53**,** 597-608.

Sokolova, I. M., Frederich, M, Bagwe, R, Lannig, G & Sukhotin, A.A (2012) Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. *Marine Environmental Research,* 79**,** 1-15.

Sokolova, I. M. & Lannig, G (2008) Interactive effects of metal pollution and temperature on metabolism in aquatic ectotherms: implications of global climate change. *Climate Research,* 37**,** 181-201.

Solan, M.,. Wigham, B.D, Hudson,I.R, Kennedy, R, Coulon,C.H, Norling, K, Nilsson, H.C & Rosenberg, R (2004) In situ quantification of bioturbation using time-lapse fluorescent sediment profile imaging (f-SPI), luminophore tracers and model simulation. *Marine Ecology Progress Series,* 271**,** 1-12.

Somero, G. N (1986) Protons, osmolytes, and fitness of internal milieu for protein function. *American Journal of Physiology,* 251**,** R197-R213.

Southward, A. J., Hiscock, K, Kerckhof, F, Moyse, J & Elfimov, A.S (2004) Habitat and distribution of the warm-water barnacle *Solidobalanus fallax* (Crustacea : Cirripedia). *Journal of the Marine Biological Association of the United Kingdom,* 84**,** 1169-1177.

Spicer, J. I., Raffo, A & Widdicombe, S (2007) Influence of CO_2-related seawater acidification on extracellular acid-base balance in the velvet swimming crab *Necora puber*. *Marine Biology,* 151**,** 1117-1125.

Spicer, J. I., Taylor, A.C & Hill, A.D (1988) Acid-base status in the sea-urchins *Psammechinus-miliaris* and *Echinus-esculentus* (Echinodermata, Echinoidea) during emersion. *Marine Biology,* 99**,** 527-534.

Spicer, J. I., Widdicombe,S, Needham, H.R & Berge, J.A (2011) Impact of CO_2-acidified seawater on the extracellular acid-base balance of the northern sea urchin Strongylocentrotus droebachiensis. *Journal of Experimental Marine Biology and Ecology,* 407**,** 19-25.

Stachowicz, J. J., Terwin, J.R, Whitlatch, R.B & Osman , R.W(2002) Linking climate change and biological invasions: Ocean warming facilitates nonindigenous species invasions. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 15497-15500.

Stahl, H., Glud, A, Schroder,C.R, Klimant, I, Tengberg, A & Glud, R.N (2006) Time-resolved pH imaging in marine sediments with a luminescent planar optode. *Limnology and Oceanography-Methods,* 4**,** 336-345.

Steckbauer, A., Ramajo,L, Hendriks, I.E, Fernandez, M, Lagos, N.A, Prado, L & Duarte, C.M (2015) Synergistic effects of hypoxia and increasing CO_2 on benthic invertebrates of the central Chilean coast. *Frontiers in Marine Science,* 2, 49.

Stiff, M. J (1971) Copper/bicarbonate equilibria in solution of bicarbonate ion at concentrations similar to those found in natural water. *Water Research,* 5**,** 171.

Stockdale, A., Tipping, E, Lofts, S & Mortimer, R.J.G (2016) Effect of ocean acidification on organic and inorganic speciation of trace metals. *Environmental Science & Technology,* 50**,** 1906-1913.

Stocker, T. F (2013) IPCC, 2013: climate change 2013: The physical science basis. Contribution of working group i to the fifth assessment report of the intergovernmental panel on climate change. eds. Stocker, T.F, Qin, T, Plattner, G.K, Tiger, M,. Allen, S.K, Böschung, J, Navels, A, Tia, Y, Bex, V & Midgley, P.M. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 1535 pp.

Stocks, M (2013) *Chapter 3 - The small molecule drug discovery process – from target selection to candidate selection. Introduction to Biological and Small Molecule Drug Research and Development*. Elsevier.

Stohs, S. J. & Bagchi, D(1995) Oxidative mechanisms in the toxicity of metal-ions. *Free Radical Biology and Medicine,* 18**,** 321-336.

Stumpp, M., Hu, M.Y, Melzner, F, Gutowska, M.A, Dorey, N, Himmerkus, N, Holtmann, W.C, Dupont, S.T, Thorndyke, M.C & Bleich, M (2012) Acidified seawater impacts sea urchin larvae pH regulatory systems relevant for calcification. *Proceedings of the National Academy of Sciences of the United States of America,* 109**,** 18192-18197.

Stumpp, M., Wren, J, Melzner, F, Thorndyke, M.C & Dupont, S.T (2011) CO_2 induced seawater acidification impacts sea urchin larval development I: Elevated metabolic rates decrease scope for growth and induce developmental delay. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology,* 160**,** 331-340.

Sunday, J. M., Calosi, P, Dupont, S, Munday, P.L, Stillman, J.H & Reusch, T.B.H (2014) Evolution in an acidifying ocean. *Trends in Ecology & Evolution,* 29**,** 117-125.

Sunday, J. M., Fabricius, K.E, Kroeker, K.J, Anderson, K.M, Brown, N.E, Barry, J.P, Connell, S.D, Dupont, S, Gaylord, B, Hall-Spencer, J.M, Klinger, T, Milazzo, M, Munday, P.L, Russell, B.D, Sanford, E, Thiyagarajan, V,Vaughan,M.L.H, Widdicombe, S & Harley, C.D.G (2017) Ocean acidification can mediate biodiversity shifts by changing biogenic habitat. *Nature Climate Change,* 7**,** 81.

Suzuki, K. T., Someya, A, Komada, Y & Ogra, Y (2002) Roles of metallothionein in copper homeostasis: responses to Cu-deficient diets in mice. *Journal of Inorganic Biochemistry,* 88**,** 173-182.

Taboada, G., Gaxiola, G, Garcia, T, Pedroza, R, Sanchez, A, Soto, L.A & Rosas, C (1998) Oxygen consumption and ammonia-N excretion related to protein requirements for growth of white shrimp, *Penaeus setiferus* (L.), juveniles. *Aquaculture Research,* 29**,** 823-833.

Taylor, P., Lichtschlag, A, Toberman, M, Sayer, M.D.G, Reynolds, A, Sato, T & Stahl, H (2015) Impact and recovery of pH in marine sediments subject to a temporary carbon dioxide leak. *International Journal of Greenhouse Gas Control,* 38**,** 93-101.

Thirumoorthy, N., Kumar, K.T.M, Sundar, A.S, Panayappan, L & Chatterjee, M (2007) Metallothionein: An overview. *World Journal of Gastroenterology,* 13**,** 993-996.

Thomsen, J., Gutowska, M.A, Saphorster, J, Heinemann, A, Trubenbach,K, Fietzke, J, Hiebenthal, C, Eisenhauer, A, Kortzinger, A, Wahl, M& Melzner, F (2010) Calcifying invertebrates succeed in a naturally CO_2-rich coastal habitat but are threatened by high levels of future acidification. *Biogeosciences,* 7**,** 3879-3891.

Thomsen, J. & Melzner, F (2010) Moderate seawater acidification does not elicit long-term metabolic depression in the blue mussel *Mytilus edulis*. *Marine Biology: Springer-Verla*, 157, 2667–2676.

Tipping, E., Smith, E.J, Lawlor, A.J, Hughes, S & Stevens, P.A (2003) Predicting the release of metals from ombrotrophic peat due to drought-induced acidification. *Environmental Pollution,* 123**,** 239-253.

Todgham, A. E. & Stillman, J.H (2013) Physiological responses to shifts in multiple environmental stressors: Relevance in a changing world. *Integrative and Comparative Biology,* 53**,** 539-544.

Toulmond, A (1973) Tide-related changes of blood respiratory variables in the lugworm A*renicola marina* (L.). *Respiration Physiology: Elsevier*, 130-144.

Towle, E. K., Enochs, I.C & Langdon, C (2015) Threatened Caribbean coral Is able to mitigate the adverse effects of ocean acidification on calcification by increasing feeding rate. *Plos One,* 10(9), e0139398.

Tripati, A. K., Roberts, C.D & Eagle, R.A (2009) Coupling of CO_2 and ice sheet stability over major climate transitions of the last 20 million years. *Science,* 326**,** 1394-1397.

Truchot, J. P (1976) Carbon dioxide combining properties of the blood of the shore crab *Carcinus maenas (L)*: carbon dioxide solubility coefficient and carbonic acid dissociation constants. *Journal of Experimental Biology*, 45-57.

Tynan, E., Clarke, J.S, Humphreys, M.P, Ribas-Ribas, M, Esposito, M, Rerolle, V.M.C, Schlosser, C, Thorpe, S.E, Tyrrell, T, & Achterberg, E.P (2016) Physical and biogeochemical controls on the variability in surface pH and calcium carbonate saturation states in the Atlantic sectors of the Arctic and Southern Oceans. *Deep-Sea Research Part Ii-Topical Studies in Oceanography,* 127**,** 7-27.

Urakawa, H., Kita-Tsukamoto, K & Ohwada, K (1999) Microbial diversity in marine sediments from Sagami Bay and Tokyo Bay, Japan, as determined by 16S rRNA gene analysis. *Microbiology-Uk,* 145**,** 3305-3315.

Urbina, M. A., Walsh, P.J, Hill, J.V & Glover, C.N (2014) Physiological and biochemical strategies for withstanding emersion in two galaxiid fishes. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology,* 176**,** 49-58.

Van Hoey, G., Guilini, K, Rabaut, M, Vincx, M & Degraer, S (2008) Ecological implications of the presence of the tube-building polychaete *Lanice conchilega* on soft-bottom benthic ecosystems. *Marine Biology,* 154**,** 1009-1019.

Vannela, R (2012) Are we “digging our own grave” under the oceans? Biosphere-level effects and global policy challenge from plastic(s) in oceans. *Environmental Science and Technology*, 7932-7933.

Vargas, C. A., Lagos, N.A Lardies, M.A, Duarte, C, Manriquez, P.H, Aguilera, V.M, Broitman, B, Widdicombe, S & Dupont, S (2017) Species-specific responses to ocean acidification should account for local adaptation and adaptive plasticity. *Nature Ecology & Evolution,* 1, 0084.

Varley, D. G. & Greenaway, P(1992) The effect of emersion on haemolymph acid-base balance and oxygen levels in *Scylla-serrata* Forskal (Brachyura, Portunidae). *Journal of Experimental Marine Biology and Ecology,* 163**,** 1-12.

Venn, A. A., Tambutte, E, Holcomb, M, Laurent, J, Allemand, D & Tambutte, S (2013) Impact of seawater acidification on pH at the tissue-skeleton interface and calcification in reef corals. *Proceedings of the National Academy of Sciences of the United States of America,* 110**,** 1634-1639.

Verkaik, K., Hamel, J.F & Mercier, A (2017) Impact of ocean acidification on reproductive output in the deep-sea annelid *Ophryotrocha* sp (Polychaeta: Dorvilleidae). *Deep-Sea Research Part Ii-Topical Studies in Oceanography,* 137**,** 368-376.

Vitousek, P. M., Mooney, H.A, Lubchenco, J & Melillo, J.M (1997) Human domination of Earth's ecosystems. *Science,* 277**,** 494-499.

Wage, J., Hardege, J.D, Larsson, T.A, Simakov, O, Chapman, E.C, Arendt, D & Rotchell, J.M (2015) Effects of low seawater pH on the marine polychaete *Platynereis dumerilii*. *Marine Pollution Bulletin,* 95**,** 166-172.

Wake, B. (2019) Experimenting with multistressors. *Nature Climate Change,* 9**,** 357-357.

Wang, J., Russell, B.D, Ding, M.W & Dong, Y.W (2018) Ocean acidification increases the sensitivity of and variability in physiological responses of an intertidal limpet to thermal stress. *Biogeosciences,* 15**,** 2803-2817.

Wang, T., Knudsen, P.K Brauner,C.J, Busk, M, Vijayan, M.M & Jensen, F.B (1998) Copper exposure impairs intra- and extracellular acid-base regulation during hypercapnia in

the fresh water rainbow trout (*Oncorhynchus mykiss*). *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology,* 168**,** 591-599.

Watson, G. J., Murray, J.M, Schaefer, M & Bonner, A (2017) Bait worms: a valuable and important fishery with implications for fisheries and conservation management. *Fish and Fisheries,* 18**,** 374-388.

Watson, G. J., Pini, J.M & Richir, J (2018) Chronic exposure to copper and zinc induces DNA damage in the polychaete *Alitta viren*s and the implications for future toxicity of coastal sites. *Environmental Pollution,* 243**,** 1498-1508.

Weeks, J. M., Jensen, F.B & Depledge, M.H (1993) Acid-base status, hemolymph composition and tissue copper accumulation in the shore crab *Carcinus-maena*s exposed to combined copper and salinity stress. *Marine Ecology Progress Series,* 97**,** 91-98.

Weis, J. S (2014) *Tolerance*. Physiological, developmental and behavioral effects of marine pollution: Springer, Dordrecht.

Weiss, I. M., Tuross, N, Addadi, L & Weiner, S (2002) Mollusc larval shell formation: Amorphous calcium carbonate is a precursor phase for aragonite. *Journal of Experimental Zoology,* 293**,** 478-491.

Wernberg, T., Smale, D.A & Thomsen, M.S (2012) A decade of climate change experiments on marine organisms: procedures, patterns and problems. *Global Change Biology,* 18**,** 1491-1498.

Widdicombe, S., Beesley, A, Berge,J.A, Dashfield, S.L, McNeill, C.L, Needham, H.R & Oxnevad, S (2013) Impact of elevated levels of CO_2 on animal mediated ecosystem function: The modification of sediment nutrient fluxes by burrowing urchins. *Marine Pollution Bulletin,* 73**,** 416-427.

Widdicombe, S., Dashfield, S.L, McNeill, C.L, Needham, H.R, Beesley, A, McEvoy, A, Oxnevad, S, Clarke, K.R & Berge, J.A (2009) Effects of CO_2 induced seawater acidification on infaunal diversity and sediment nutrient fluxes. *Marine Ecology Progress Series,* 379**,** 59-75.

Widdicombe, S., McNeill, C.L, Stahl, H, Taylor, P, QueiroS, A.M, Nunes, J & Tait, K (2015) Impact of sub-seabed CO_2 leakage on macrobenthic community structure and diversity. *International Journal of Greenhouse Gas Control,* 38**,** 182-192.

Widdicombe, S. & Needham, H (2007) Impact of CO2-induced seawater acidification on the burrowing activity of *Nereis virens* and sediment nutrient flux. *Marine Ecology Progress Series,* 341**,** 111-122.

Widdicombe, S., Spicer, J & Kitidis, V (2011) *Chapter 9: Effects of ocean acidification on sediment fauna*. Ocean Acidification: OUP Oxford.

Widdicombe, S. & Spicer, J.I (2008) Predicting the impact of ocean acidification on benthic biodiversity: What can animal physiology tell us? *Journal of Experimental Marine Biology and Ecology,* 366**,** 187-197.

Williams, R., Wright, A.J, Ashe, E, Blight, L.K, Bruintjes, R, Canessa, R, Clark, C.W, Cullis-Suzuki, S, Dakin, D.T, Erbe, C, Hammond, P.S, Merchant, N,D, O'Hara, P.D, Purser, J, A,Radford, N, Simpson, S.D, Thomas, L & Wale, M.A (2015) Impacts of anthropogenic noise on marine life: Publication patterns, new discoveries, and future directions in research and management. *Ocean & Coastal Management,* 115**,** 17-24.

Wittmann, A. C. & Portner, H. O (2013) Sensitivities of extant animal taxa to ocean acidification. *Nature Climate Change,* 3**,** 995-1001.

Wolf-Gladrow, D. A. & Rost, B.R (2014) Ocean acidification and oceanic carbon cycling. *Global Environmental Change: Springer Science+Business Media Dordrecht.*

Wolf-Gladrow, Z. A (2001) *CO_2 in seawater: Equilibrium, kinetics, isotopes*. Elsevier.

Wood, H. L., Spicer, J.I & Widdicombe, S (2008) Ocean acidification may increase calcification rates, but at a cost. *Proceedings of the Royal Society B-Biological Sciences,* 275**,** 1767-1773.

Xie, L. T. & Klerks, P.L (2004) Fitness cost of resistance to cadmium in the least killifish (*Heterandria formosa*). *Environmental Toxicology and Chemistry,* 23**,** 1499-1503.

Xu, K. D., Tang, Z.R, Liu, S.B, Liao, Z, Xia, H, Liu, L.W, Wang, Z.M & Qi, P.Z (2018) Effects of low concentrations copper on antioxidant responses, DNA damage and genotoxicity in thick shell mussel *Mytilus coruscus*. *Fish & Shellfish Immunology,* 82**,** 77-83.

Yamamoto-Kawai, M., McLaughlin, F.A, Carmack, E.K, Nishino, S & Shimada, K (2009) Aragonite undersaturation in the Arctic Ocean: Effects of ocean acidification and sea ice melt. *Science,* 326**,** 1098-1100.

Yu, P. C., Matson, P.G, Martz, T.R & Hofmann, G.E (2011) The ocean acidification seascape and its relationship to the performance of calcifying marine invertebrates: Laboratory experiments on the development of urchin larvae framed by

environmentally-relevant *p*CO($_2$)/pH. *Journal of Experimental Marine Biology and Ecology,* 400**,** 288-295.

Yuan, X. T., Chen, A.H, Zhou, Y.B, Liu, H.Y & Yang, D.Z (2010) The influence of cadmium on the antioxidant enzyme activities in polychaete *Perinereis aibuhitensis grube* (Annelida: Polychaeta). *Chinese Journal of Oceanology and Limnology,* 28**,** 849-855.

Zange, J., Grieshaber, M.K & Jans, A.W.H (1990) The regulation of intracellular ph estimated by p-31-nmr spectroscopy in the anterior byssus retractor muscle of *Mytilus edulis* L. *Journal of Experimental Biology,* 150**,** 95-109.

Zeng, X. F., Chen, X.J & Zhuang, J(2015) The positive relationship between ocean acidification and pollution. *Marine Pollution Bulletin,* 91**,** 14-21.

Zhang, C., Yu, Z.G, Zeng, G.M Jiang, M, Yang, Z.Z, Cui, F,. Zhu,M.Y, Shen, L.Q & Hu, L (2014) Effects of sediment geochemical properties on heavy metal bioavailability. *Environment International,* 73**,** 270-281.

Zhu, Q. Z., Aller, R.C & Fan, Y.Z (2006) Two-dimensional pH distributions and dynamics in bioturbated marine sediments. *Geochimica Et Cosmochimica Acta,* 70**,** 4933-4949.

Zlatkin, R. & Heuer, R (2019) Ocean acidification affects acid–base physiology and behaviour in a model invertebrate, the California sea hare (*Aplysia californica*). *Royal Society Open Science*, 6: 191041