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Behavioural and ecophysiological responses of marine benthos to ocean acidification and warming

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Behavioural and ecophysiological responses of marine benthos to ocean acidification and warming

Ee Zin Ong

A thesis submitted to University of Plymouth in partial fulfillment for the degree of

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Author's Declaration

At no time during the registration for the degree of *Doctor of Philosophy* has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

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Climate change is one of those things that everyone seems to have an opinion about, but few people understand.

Thom Hartmann

The Last Hours of Ancient Sunlight

Contents

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How to start the acknowledgement after a 4-years long crazy and amazing adventure?! This particular book, would not be possible without the help of many incredible people who came across my path.

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Abstract

The atmospheric carbon dioxide concentrations (CO_2) are projected to rise from 400 ppm to 1000 ppm by the end of 21st century. The increase in atmospheric CO_2 has been absorbed by the ocean resulting in a process called ocean acidification. Concurrently, the increase of atmospheric CO_2 also intensifies the greenhouse effect, resulting in an increase of ocean surface temperature. The progressive modification of ocean environment is expected to affect marine ecosystems via changes in species behaviour, physiology and survival that will determine population, interaction within the community, with implications for biodiversity-mediated ecosystem functioning. Therefore, studying the combined effects of both drivers is fundamental for our understanding of future ocean environments. This PhD thesis reveals the impacts of both acidification and warming on behavioural and physiological responses of estuarine intertidal key species (*Cerastoderma edule* and *Scrobicularia plana*) and community. The community response to stressors can be due to direct effects of climate change on individual species as well as indirect effects via alterations of trophic interactions. In summary, this PhD reveals the impacts of lowered pH and elevated temperature on estuarine intertidal sediments at different levels of biological organisation. The observed distinct sensitivity of marine intertidal species to these stressors highlights the importance of ecosystem-based approaches incorporating all interactions.

Chapter 1

General introduction

Coastal shallow habitats such as estuaries are the transition zones between the marine and freshwater environment and are greatly influenced by the changes in land and sea. Estuaries are considered geomorphologically dynamic and ephemeral systems, thus creating a unique combination of different habitat types (Meire et al., 2005). These habitats deliver considerable amounts of important ecosystem services and functions that benefit humans directly and indirectly. They are a source of raw materials and food, regulate water and cycle nutrients , serve as coastal protection and erosion control, provide habitat for animals and plants and serve as sites for recreation (Barbier et al., 2011; Costanza et al., 1997; Meire et al., 2005; O'Higgins et al., 2010). Estuaries are among the most productive ecosystems, yielding at least 4.1 trillion US$ worth of ecosystem goods and services annually (Costanza et al., 1997). However, these habitats are impacted worldwide by multiple stressors which alter the benefits and services provided by coastal ecosystems (e.g. Airoldi and Beck 2007; Barbier et al. 2011). The exponential growth of human populations is the major driver underlying the overexploitation and threats faced by coastal ecosystems (Beck et al., 2001; Halpern et al., 2008; Worm et al., 2006). Increasing anthropogenic activities such as land-use change and coastal developments result in the run-off of pollutants and nutrients, removal of sediments, and ultimately lead to alteration and destruction of natural ecosystems (Halpern et al., 2008; Seitz et

al., 2014; Syvitski et al., 2005). Global change is another stressor on top of the other local changes, which poses further threats to the functioning of coastal ecosystems (see 1.1, Feely et al., 2010; Scavia et al., 2002).

1.1 Atmospheric carbon increase

Carbon dioxide (CO_2) is a colourless gas that occurs naturally in the Earth's atmosphere. Since the pre-industrial revolution, atmospheric CO_2 concentration has risen from 280 to 406.93 ppm at present considerably exceeding the range of the past 800,000 years and at an unprecedented fast rate (Stocker et al. 2013; Dlugokencky and Tans 2017; Lüthi et al. 2008). This increase in global CO_2 emissions is mainly caused by anthropogenic activities via burning of fossil fuels, cement production, and deforestation (Sabine et al., 2004). Furthermore, IPCC has predicted an additional increase to between 421 and 936 ppm by the end of the century (Stocker et al., 2013). The increase of atmospheric CO_2 concentrations along with other greenhouse gases have led to warming of the climate on Earth by around 0.6 °C over the past century and the global mean sea surface temperature is projected to further increase by 1.0 to 4.1 °C by the end of the century (i.e. ocean warming, see 1.1.2) according to four Representative Concentration Pathway (RCP) scenarios (Collins et al., 2013). In order to prevent the worst case CO_2

emissions scenario, an agreement has been reached at the Paris Climate Summit (12 December 2015) to limit the global temperature below 2 °C above pre-industrial era at the end of 21st century. Moreover, approximately one third of the anthropogenic atmospheric CO_2 has been absorbed by the ocean and this process is altering ocean carbonate chemistry and pH, resulting in ocean acidification (see 1.1.1, Gattuso et al., 2015; Stocker et al., 2013). Recently, the United Nations have acknowledged the impacts of ocean acidification and asserted it in the Sustainable Developmental Goal 14.3. This Goal is now mandatory for most of the countries in the world to minimise and address the impacts through enhancement of scientific cooperation at all levels (https://sustainabledevelop ment.un .org/sdg14).

1.1.1 Ocean acidification

The ocean, which covers at least 70% of the Earth's surface, is buffering the atmospheric CO_2 increase and acts as a net carbon sink (8.7 ± 2.0 $GtCO_2$/yr) through sedimentation of particulate carbon and inorganic carbon (Emerson and Hedges, 2008; Le Quéré et al., 2018). Furthermore, marine organisms in the ocean play a role in channelling inorganic carbon (i.e. CO_2) from the atmosphere into surface waters, down into the deeper ocean and ultimately into rocks via a combination of processes, e.g. photosynthesis, remineralisation, calcification of marine

calcifiers, gravitational settling of marine snow etc. (i.e. the biological pump, (Passow and Carlson, 2012)). Moreover, the primary mechanism driving the excess atmospheric CO_2 emissions into the ocean is the solubility pump, which in turn leads to the change of ocean carbonate chemistry (Doney et al., 2009). When atmospheric CO_2 dissolves in the seawater, CO_2 (in aqueous phase) reacts with water molecules to form carbonic acid (H_2CO_3). The unstable H_2CO_3 will dissociate into a hydrogen ion (H^+) and bicarbonate ion (HCO_3^-). HCO_3^- will further dissociate into hydrogen (H^+) and carbonate ion (CO_3^{2-}). Therefore, when CO_2 interacts with seawater, four different inorganic carbon forms are formed by the following chemical equilibrium (Equation 1) (Dickson, 2010).

$$CO_2(g) \leftrightarrow CO_2(aq) + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+ \quad (1)$$

The formation of hydrogen ions in turn affect the pH of seawater as pH is expressed by the function $pH = -\log_{10}[H^+]$; hence, ocean carbonate chemistry is strongly associated to the seawater pH. When the partial pressure of atmospheric CO_2 increases, so will the gas exchange in the ocean seawater surface, shifting the carbon equilibrium in the equation (1) to the left, leading to an increase in hydrogen ion concentrations and a decrease in carbonate ion concentrations (i.e. a reduction in saturation states, see Figure 1.1). Consequently, seawater pH decreases which

results in ocean acidification (Caldeira and Wickett, 2003; Doney et al., 2009).

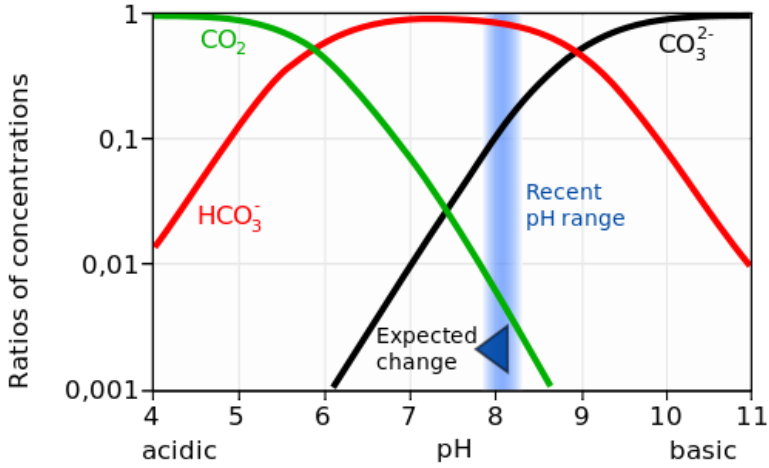

Figure 1.1 Bjerrum plot of change in seawater carbonate system from ocean acidification. Source: Karbonatsystem_Meerwasser_de.svg

Since the preindustrial period, average surface ocean pH has dropped by 0.1 units and is estimated to further decrease between 0.3 and 0.4 units by the year 2100 (Orr et al., 2005). Furthermore, studies reported that carbonate ion concentrations decreased by 16% since preindustrial times and will further decrease by about 50-55% by the end of 21st century (Brewer, 1997; Doney et al., 2009; Orr et al., 2005; UNEP, 2009).

Over the past 25 million years, the pH of the open ocean has remained between 8.0 to 8.3 due to the strong buffering capacity of the ocean (Widdicombe and Spicer, 2008). Coastal shallow habitats, however, experience a dynamic daily and seasonal

fluctuation in combination with other stressors such as pollution and eutrophication (Hofmann et al. 2011; Duarte et al. 2013) might explain the higher degree of acidification found in these habitats as compared to the open ocean (Salisbury et al., 2008). The pH variability observed in coastal habitats is different from the open ocean (Provoost et al. 2010; Wootton et al. 2008) and the pH is often lower than the predicted pH for future oceans. This natural variability of coastal habitats can therefore lead to local adaptation and an increased resilience of species to environmental changes (Vargas et al., 2017). Evidence showed that the Permo-Triassic mass Boundary extinction has concurred with a rapid injection of a large quantity of CO_2 into the atmosphere which caused the destructive loss in biodiversity (Clarkson et al., 2015); therefore, biodiversity and ecosystem functioning may be exceptionally vulnerable to the increase of atmospheric CO_2 concentration.

The reduction of calcium carbonate saturation states (e.g. aragonite and calcite) has a well-known impact on calcifying marine organisms (e.g. corals, molluscs and echinoderms), reducing their capacity to deposit calcium carbonate as their shell and outer skeleton under acidic conditions (Doney et al., 2009; Orr et al., 2005). However, recent studies have pointed out that calcification or dissolution of marine calcifying organisms do not exclusively depend on the saturation states of calcite and

aragonite (Beniash et al., 2010; Ries et al., 2009; Roleda et al., 2012). Theory suggests that elevated hydrogen ion concentrations (H^+), instead of carbonate ions (CO_3^{2-}) are presumably the main driver determining the calcification or dissolution of marine calcifiers (Cyronak et al., 2016). High H^+ concentration shifts the proton gradient between external seawater and intracellular fluid, thus inhibiting marine calcifiers from sustaining pH homeostasis (Cyronak et al., 2016), which in turn increase the energetic burden of the calcifiers (Sokolova et al., 2012). Furthermore, a recent study demonstrated that the seawater bicarbonate ion and hydrogen ion ratio [HCO^{3-} /H^+] rather than carbonate ions is essential for the blue mussel *Mytilus edulis* to regulate its calcification (Thomsen et al., 2015). In order to reveal the biomineralisation mechanisms of marine calcifiers, more research is needed to understand its physiological processes as well as their adaptability to ocean acidification (Thomsen et al., 2015).

In general, calcifying marine organisms are more negatively affected by elevated pCO_2 conditions than non-calcifying marine organisms (Kroeker et al., 2010). However, marine animals (regardless whether calcifiers or non-calcifiers) that ingest acidified water may experience physiological disruption due to the shift in acid-base balance (Pörtner et al., 2004; Seibel and Walsh, 2003). Under hypercapnia, marine animals attempt to

restore their acid-base balance back to its "optimum setting" before hypercapnia, a process which is particularly energy consuming as it requires an increase in active ion transport over the cell membranes (Pörtner et al., 2004). As a consequence, more energy is needed for basal maintenance, implying that less energy will be allocated to processes such as activity, development, growth and reproductions which results in a reduction of overall fitness and condition (Sokolova et al., 2012; Thomsen and Melzner, 2010; Wood et al., 2008). However, the sensitivity of marine organisms to hypercapnia is tightly linked to their activity, life style, stage of development (with larvae and juveniles typically more vulnerable), is species-specific and might depend on the interaction with other conditions, such as ocean warming (Byrne, 2011; Kroeker et al., 2010; Pörtner et al., 2004; Widdicombe and Spicer, 2008).

1.1.2 Ocean warming

Apart from regulating and absorbing the atmospheric CO_2, oceans also play a significant role in regulating Earth's climate change and variability by taking up a large fraction of the solar radiation (Solomon et al., 2007). An increase in greenhouse gas emissions enhances climate forcing or radiative forcing where more solar irradiance is kept in the atmosphere, rather than radiated back to the space, eventually warming up the surface of Earth (Lashof and Ahuja, 1990). The ocean absorbs and stores a

large quantity of heat (> 80 %), 1000 times more heat than the atmosphere, due to the high heat capacity (Levitus et al., 2005). This heat has been mainly stored in the upper layers of the ocean and plays a major role in climate change (Solomon et al., 2007). IPCC reported that the upper 75 m of the ocean has heated up by approximately 0.44 °C from 1971 till 2010 and this will continue to rise between 1.0 and 4.1 °C by 2100 (Collins et al., 2013; Rhein et al., 2013). Furthermore, ocean surface isotherms have migrated more rapidly in comparison than on land between 1960 and 2009 (Solomon et al., 2007). Although the average ocean temperature increases at a relatively slow rate, ocean warming becomes more extreme due to seasonal variation, especially in spring and winter, which may lead to shifts in distribution range and changes in phenology, e.g. the timing of reproduction and growth (Burrows et al., 2011).

Temperature is one of the most important factors influencing living organisms as it affects the molecules' kinetic energy and biochemical reactions, thus, altering the organisms' behaviour and physiology (Madeira et al., 2012). Years of research have shown that increases in seawater temperature trigger a series of ecosystem responses including a poleward shift in the distribution of marine fishes (Perry et al., 2005), population breakdown (Pörtner and Knust, 2007) and the alteration of food availability and food web structure (Pörtner and Farrell, 2008).

Furthermore, a long-term community-based study showed that an increase of 3.5 °C in seawater temperature significantly deteriorated temperature-sensitive algae and thus temporarily increasing the food availability for invertebrate grazers (Schiel et al., 2004). This study suggests that the responses of marine benthic communities to ocean warming are strongly linked to the direct thermal effects on key species as well as to indirect effects from (changed) ecological interactions (Schiel et al., 2004). In addition, a meta-analysis reported that above 80% of the observations on the response of marine taxa to elevated temperature (e.g. abundance, calcification, composition, demography, distribution as well as phenology) were consistent with the expected impacts of future ocean warming (Poloczanska et al., 2013).

1.2 Evil twins: Global warming and ocean acidification

Given the importance of temperature for biological patterns and processes, and the fact that ocean acidification does not occur in isolation, it is crucial to include temperature dependency in both field and experimental studies in order to obtain precise predictions of ocean acidification effects on the performance of species in future oceans (Hofmann et al., 2010). Currently, there is still a lack of knowledge about the combined effects of both

stressors on marine invertebrates (Byrne and Przeslawski, 2013). Thus it is essential to assess the possible effects on marine organisms as both stressors might act synergistically, antagonistically or additively[1] on the physiology and ecology of marine organisms (Byrne and Przeslawski, 2013; Darling and Cote, 2008). Furthermore, studies suggested that the unparalleled rates of ocean acidification and warming may limit the timeframe for marine organisms to genetically adapt to the changing conditions in their natural environment, particularly when the reproduction rates of the species are low (Kelly et al., 2012; Sunday et al., 2011). However, meta-analysis, theories and literature reviews proposed that the sensitivity of marine animals to ocean acidification and warming are correlated to life-stage development, mobility and adaptation capability, the species' thermal window, habitat and region and greatly differ among species (Byrne, 2011; Byrne and Przeslawski, 2013; Clements and Hunt, 2015; Fabry et al., 2008; Gazeau et al., 2014; Kroeker et al., 2010; Parker et al., 2013; Pörtner et al., 2004; Pörtner, 2008).

[1] Synergistic effect refers to an aggravating interaction, illustrating that the presence to one driver magnifies the response to another driver. Antagonistic effects refer to mitigating interactions, illustrating that the presence of one driver ameliorates the response to another driver. Finally, additive effects can be either aggravating or mitigating interactions, indicating that the presence of one driver does not alter the effect size of another driver (Boyd et al, 2018).

According to the theoretical framework suggested by Pörtner and Farrell (2008), the combination of both stressors can affect the functional performance of species (e.g. behaviour, competitiveness, foraging, growth and reproduction) by narrowing their thermal windows (Pörtner, 2008; Pörtner and Farrell, 2008, Figure 1.2), The theory is well supported by several works published on ocean acidification; however, there are is a growing number of studies where the model is unable to explain observed positive and neutral effects of ocean acidification (Brennand et al., 2010; Dupont et al., 2010a; Gianguzza et al., 2014). Alternatively, these contradictory findings can be solved in the light of performance curves proposed by Gianguzza et al. (2014), that incorporate an organism's fitness, growth and metabolism under different environmental conditions (Figure 1.3). This approach provides a good mechanistic understanding of the effects of ocean warming and acidification on an organism's physiology and energy budget.

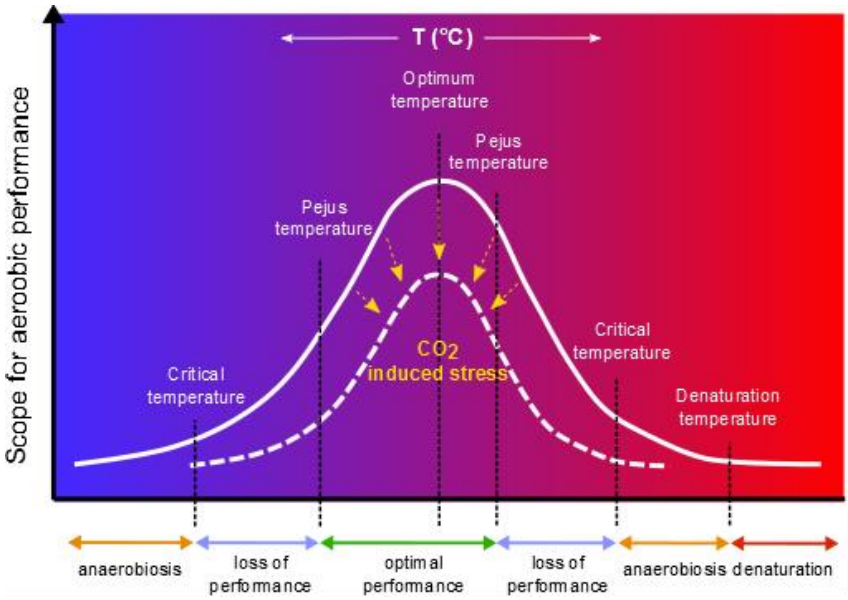

Figure 1.2 The thermal windows of aerobic performance show optima, pejus, critical and denaturation temperatures. The solid white line represents a single environmental factor situation and the dotted white line represents a combination of multi-stressors (i.e. ocean acidification) situation that can negatively affect the aerobic performance of an organism and therefore narrow the tolerance window for another environmental factor (i.e. temperature). Modified from Pörtner and Farrell (2008).

In addition, both drivers may have an impact on the ecological niche of a species. A stress situation arises when both pH and temperature change and an organism finds itself outside its ecological niche (Figure 2 in Van Straalen, 2003). The organism will not able to grow and reproduce when it is outside its own niche, however, it allows short-term survival for the organism.

Figure 1.4 shows the chemical equilibrium of CO_2 in a seawater system and the effects of ocean acidification and warming on the marine environment. The figure highlights that both drivers occur simultaneously. Therefore, there is a pressing need to

quantify the combined effect of both drivers to marine species, communities and ecosystems.

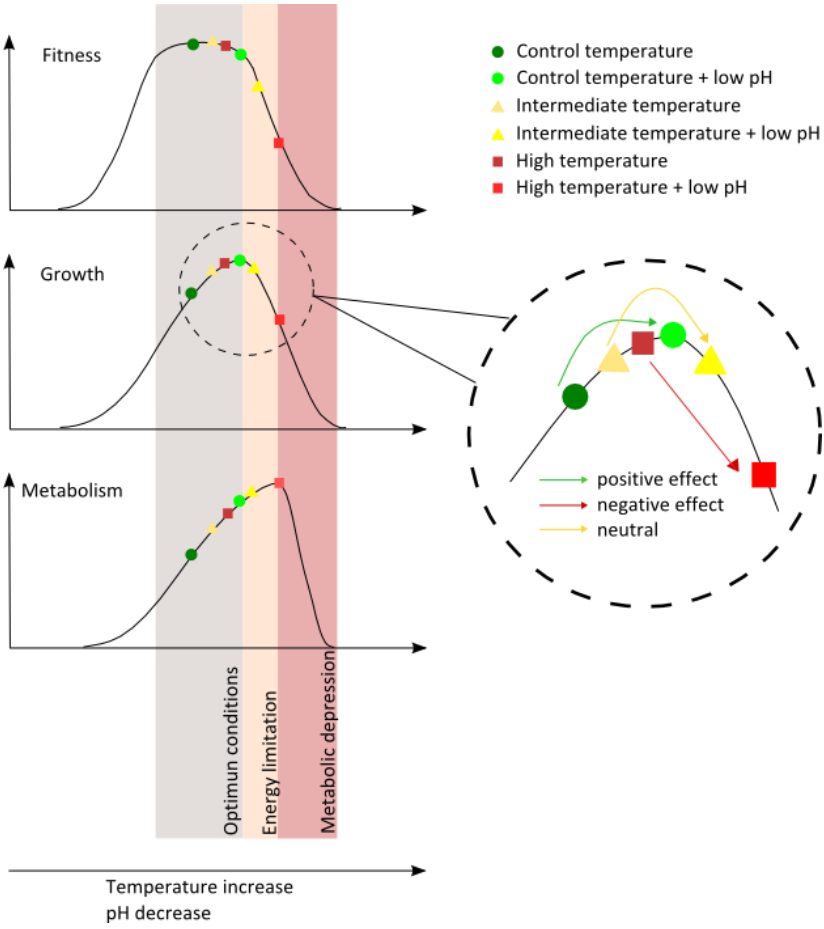

Figure 1.3 Performance curves of an organism's fitness, growth and metabolism under different temperature and pH conditions. Optimal conditions represent the natural range of growth and metabolism without any adverse consequence for fitness or survival. An increase in metabolism is often associated with an increased growth until the end point of energy limitation. Then, any further increase in metabolism will lead to a reduction of growth. Both drivers (e.g. elevated temperature and lowered pH) are causing an increase in metabolism until the point of metabolism depression and/or mortality. Modified from Gianguzza et al. (2014).

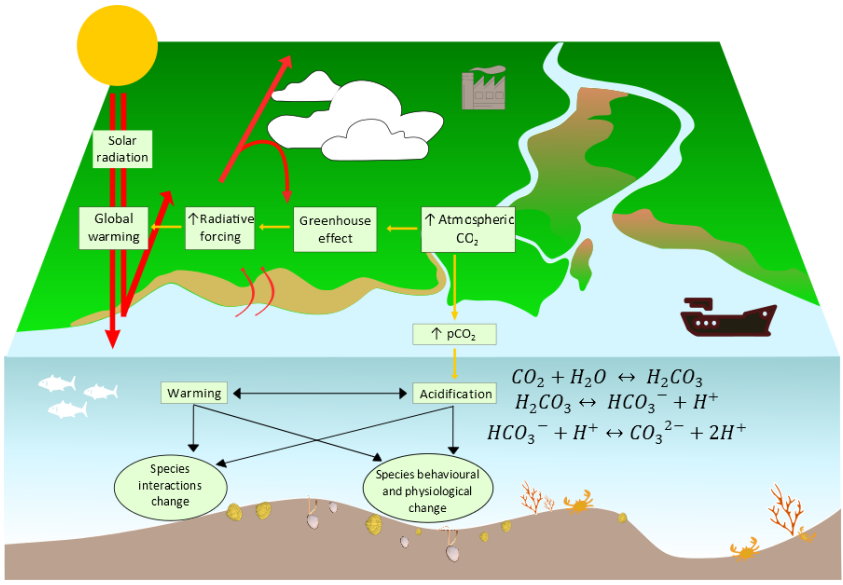

Figure 1.4 An overview of global warming and chemical dynamics of atmospheric CO_2 interacting with seawater in an estuarine ecosystem. The oval shapes represent consequences of ocean acidification and warming.

In order to facilitate comparison between studies, IPCC has proposed the application of standard exposure scenarios which refer to those environmental changes (e.g. pH and temperature) predicted to occur at the surface of open water (Riebesell et al., 2011). However, most of the studied marine species inhabit a highly variable coastal environments such as estuaries, where pH and temperature fluctuate far more drastically than in the open ocean (e.g. Vargas et al., 2017). This high variability results from processes such as riverine discharge and pollution (Salisbury et al., 2008). It has been demonstrated that coastal habitats experience intense pH fluctuations, often with higher variability than what would be expected via the sole equilibration process with the atmosphere (Hofmann et al., 2011;

Wootton et al., 2008). Thus, the higher environmental variability in coastal areas compared to the open ocean, limits the direct extrapolation of the effects of ocean acidification and warming between both environments (Duarte et al., 2013). Moreover, this environmental variability can result in associated local adaptations; e.g. physiological responses within a species can be different depending on whether the applied drivers are within or outside of the natural range of variability experienced by the organism (Calosi et al., 2017; Vargas et al., 2017). Therefore, appropriate terminology is used throughout the thesis, i.e. when the applied conditions are within the natural range of the organism or community, terms such as "lowered pH" and "elevated temperature" are used instead of "ocean acidification" and "ocean warming". In this way, we can distinguish between the different physiological and eco-evolutionary processes involved (plasticity versus selection) and still provide interesting information on the responses of organisms to pH and temperature.

1.3 Bivalves

Bivalvia is one of six classes within the phylum Mollusca and are present in both marine and freshwater systems. There are around 7500 species of bivalves and the common families include oysters, scallops, cockles, mussels and clams (Gosling, 2003). The

common features of bivalves are their two symmetrical shells called valves that are hinged dorsally covering the body. Two adductor muscles, anterior and posterior adductors, are responsible for the opening and closing of the shell valves. The mantle is responsible for the production of the calcareous shell valves; it consists of three layers (e.g. periostracum, prismatic and nacreous layers) that protect the organs of the bivalve. Furthermore, the extensions of the mantle form siphons at the posterior end, allow bivalves to feed inside the sediment; however, this is species dependent (Gosling, 2003).

The origin of bivalves carbonate shells comes from HCO_3^- present in seawater, metabolic CO_2 and tissue carbonate (Greenaway, 1971; Nicol, 1960). The calcification of bivalves occurs in the mantle and blood, and the carbonic anhydrase within the bivalve is responsible for the acceleration of $CaCO_3$ formation (Greenaway, 1971; Nicol, 1960). $CaCO_3$ is secreted in the extrapallial fluid between the mantle and shell (Greenaway, 1971) and bivalves can thus regulate the chemistry of the medium their shell is derived from (Wilbur, 1972). The secretion of their shell in fluid compartments is physically isolated from the external seawater (Wilbur, 1972). Thus, even under elevated pCO_2 conditions, bivalves are able to maintain an elevated pH at their site of calcification by converting increased HCO_3^- to CO_3^{2-}, and then depositing the CO_3^{2-} as their shells. However, this

mechanism is largely dependent on the efficiency of their proton-regulating mechanism (Ries et al., 2009), which may be affected by ocean acidification.

The shells or outer skeletons of bivalves are made from polymorphs of $CaCO_3$ such as aragonite, high- (> 4 mol % $MgCO_3$) and low-magnesium calcite (< 4 mol % $MgCO_3$) (Ries et al., 2009). Ries and colleagues (2009) suggested that organisms which calcify using the less soluble low-magnesium calcite would be more resilient to ocean acidification than those that using the more soluble aragonite and high-magnesium calcite. In addition, bivalves have the ability to produce a protective external organic layer or periostracum that isolates their shell or outer skeleton from ambient seawater (Ries et al., 2009). The protective organic layers' structure, composition and coverage differ amongst organisms (Ries et al., 2009). Organisms with extensive coverage of the external organic layer (e.g. blue mussel) generally display greater resilience to high pCO_2 conditions than those with a poor or partial coverage (e.g. scallops, oysters, clams, and cockles) (Ries et al., 2009; Schade et al., 2016).

Some bivalves live attached to hard substrates or lie on the sea floor. However, the majority of bivalves bury themselves into the sediment (Gosling, 2003) and often comprise the majority of

macrofauna[2] community biomass. Burrowing is performed by their muscular foot and provides them the protection offered by mud, sand and gravel from predation. Meanwhile, they are capable of maintaining the contact with the surface water by extending their siphons. Bivalves feed and breathe as water is pumped in via the inhalant siphon, passing through the gills where suspended food particles are filtered and oxygen is absorbed through the exhalant siphon. When the food particles are less nutritious or abundant, above a certain threshold concentration, a proportion of the filtered material is expulsed as pseudofaeces (Bayne and Newell, 1983; Newell and Cornwell, 2004). In terms of feeding modes, bivalves can be divided into two main categories, deposit feeders and suspension feeders (Gosling, 2003, Figure 1.4). Deposit feeders obtain their nutritional requirements by uptake of the organic matter in the sediment via their long inhalant siphons (Lopez and Levinton, 1987; Reise, 1983). On the other hand, suspension feeders obtain their food by filtering suspended particles from the water column (Newell, 2004). Tellinid bivalves such as *Scrobicularia plana* and *Limecola balthica* are capable of feeding in both modes (Hughes, 1969).

[2] Macrofauna (a.k.a. macrobenthos) are invertebrates that live on the surface or in the subsurface of sediment. The most common macrofauna found in estuarine and coastal areas are gastropods, crustaceans, annelid worms, bivalves and insect larvae. They can be collected using 0.5 or 1 mm mesh screens.

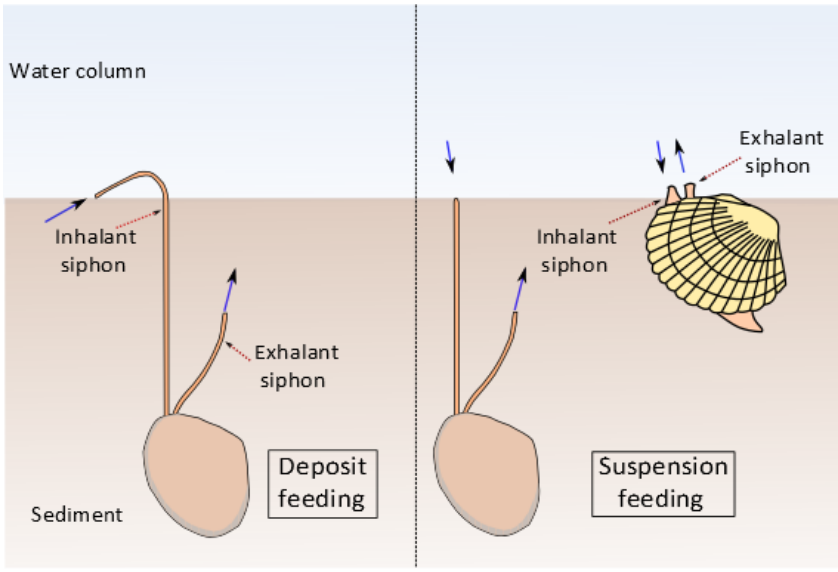

Figure 1.5 Two different feeding modes of bivalves: left shows the deposit feeding of *Scrobicularia plana* and right shows the suspension feeding of *Scrobicularia plana* and *Cerastoderma edule.* Blue arrows indicate the flow of water and food entering through the inhalant siphon, then passing through cilia on the gills and mantle surface, and exiting via the exhalant siphon.

1.3.1 Ecological and economical importance of bivalves

Bivalves contribute to various essential ecosystem services including food provisioning to higher trophic levels, biogenic habitat structure to support diverse intertidal species and biological filtration to clear or detoxify estuarine water (Barbier et al., 2011; Gutiérrez et al., 2003; Jackson et al., 2008; Parker et al., 2013). Bivalves are able to homogenise sedimentary structures, increase the volume of oxygenated sediment by tenfold and the sediment porosity by 10-20% (e.g. *Lampsilis siliquoidea*, McCall et al. 1979). Furthermore, McCall and colleagues found that *L. siliquoidea*'s behaviour (e.g. burrowing, respiration and feeding)

may stimulate the intensity and location of microbial activity in sediments. In addition, many bivalves were shown to be capable of influencing the organic degradation and nitrogen transformation processes in estuarine sediments (Pelegri and Blackburn, 1995).

Bivalves play an important ecological role as bioturbators. Bioturbation by marine benthic organisms has various kinds of important implications on the ecology and functioning of benthic ecosystems (Volkenborn et al., 2012). Bioturbation changes the distribution of porewater, the characteristics of sediment (Aller, 1981; Berg et al., 2001; Volkenborn et al., 2007) and alters the macro-, meio- and microbenthic community structure in the sediment (Marinelli et al., 2002; Meyers et al., 1987; Volkenborn and Reise, 2006; Woodin et al., 1998). Moreover, bivalves also mix the sediment layer through their sediment reworking and bio-irrigation activities (e.g. common cockle; Mermillod-Blondin et al. 2004). These activities also pressurise the porewater and increase solute exchange across the water-sediment interface, i.e. bioadvection (Wethey and Woodin, 2005; Woodin et al., 2010).

Bivalves play a crucial role in the benthic environment as they are capable of delivering oxygen to the sediment in three potential pathways (Pelegri and Blackburn, 1995). Firstly, through burrowing, bivalves expel oxygenated water from their mantle cavity. Secondly, the movement of siphons for feeding

and respiration purposes may facilitate the transportation of oxygen-rich overlying water into the sediment. Thirdly, via diffusion of oxygen through the siphon to the sediment (Pelegri and Blackburn, 1995). Another study observed an oxidised zone around the siphon of *Mya arenaria*, indicating that oxygen diffused from the siphon to the surrounding sediment (Henriksen et al., 1983).

Most bivalves can collect organic and inorganic suspended particles from the water column through suspension feeding. The undigested particulate matter is ejected by the bivalve as pseudofaeces and deposited on the sediment surface layer (Newell, 2004). The combination of these processes (i.e. filtration and biodeposition) reduces the turbidity in the water column by applying "top-down" grazer control on phytoplankton, thus, enhancing the sunlight penetration into the seafloor and fuelling the primary production of seagrass and microphytobenthos (Newell, 2004; Newell and Koch, 2004). Furthermore, bivalves are capable of recycling nutrients (i.e. nitrogen and phosphorus) back to the water column through excretion and biodeposition which is beneficial for phytoplankton production (Newell, 2004). Therefore, the overall regulation of filtering and depositing is extremely important for the functioning of the pelagic and benthic ecosystem.

According to Food and Agriculture Organization of the United Nations (FAO) (http://www.fao.org/fishery/statistics/yearbook /en), there was an increasing trend in world global bivalve production either through capture fisheries or aquaculture from 2008 to 2015 (Figure 1.6). The highest global harvest value of marine molluscs produced in 2014 (excluding cephalopods) accounted for around 20.5 billion US$ which represents 6.8% of the total world fishery production (capture and aquaculture), followed by 2013 and 2015 with 19.3 and 18.9 billion US$, respectively. Clams, cockles and arkshells contributed the highest economic value throughout these years followed by scallops, pectens and mussels. According to Narita et al. (2011), this value could rise up to 100 billion US$ depending on the consumption rates in Asia.

Given the important role of bivalves in the delivery of critical ecosystem services in estuarine and shallow coastal ecosystems, it is important to study the performance of bivalves in future seas. Any climatic and non-climatic stressors (i.e. climate change, ocean acidification, pollution, and overfishing,) that threaten the growth and health of a bivalve population, e.g. change in species physiology, distribution and trophic interaction, might ultimately affect the overall ecosystem functioning and stability.

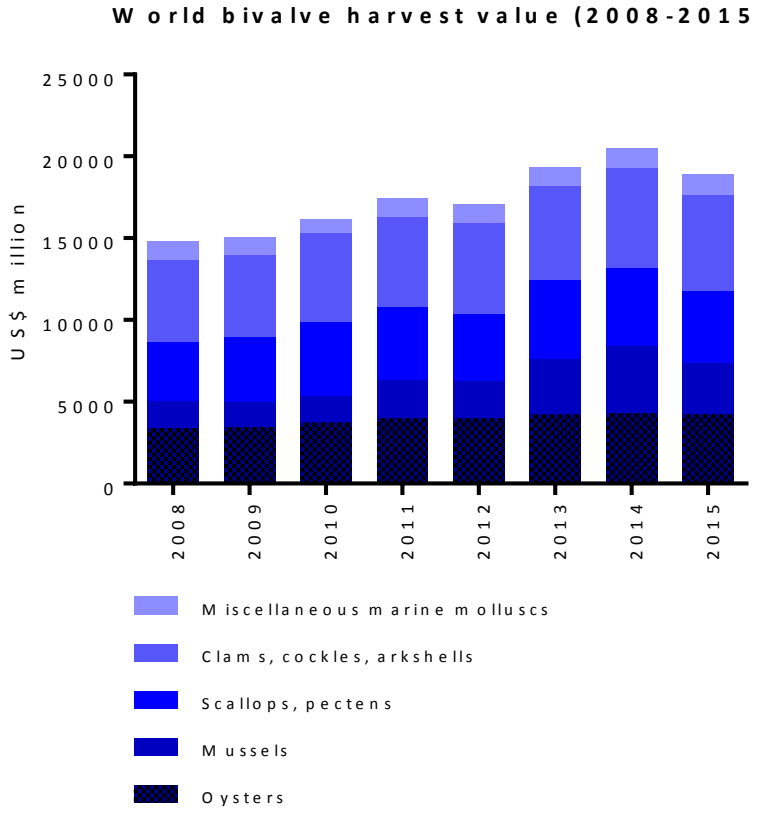

Figure 1.6 World bivalves harvest value including capture and aquaculture from 2008 - 2015 in US$

1.3.2 How do marine bivalves cope with environmental change?

Environmental change can be stressful to organisms and influence their performance and fitness. In general, there are three biological mechanisms that help organisms to mitigate these unfavourable circumstances: (1) behavioural change to avoid or reduce the stress, (2) develop stronger resistance against

the stress by reducing sensitivity, increasing tolerance or plasticity, and (3) stimulate recovery mechanisms (Huey et al., 2002). However, a trade-off between these mechanisms and energy is required (for details see Figure 1.7), i.e. additional energy is needed, causing less energy to be allocated to other activities or functions (Sokolova et al., 2012). Marine bivalves, which are particularly sensitive to ocean acidification (see section 1.1.1), can thus utilise these mechanisms to cope with stressful conditions. Especially, behavioural change are expected to cascade on to ecosystem diversity and functioning, given the importance of behavioural activities, such as filtration, bioturbation, and irrigation (see 1.3.1).

Behavioural change is often considered to be the first indicator of stress response and changing environmental conditions (Mench, 1998; Townsend et al., 2014). Behavioural change can be influenced by internal physiological processes and/or external environmental processes (e.g. Hughes, 1988). One example of behavioural changes is the migration of fish (Perry et al., 2005). Behavioural changes at a more local scale is demonstrated by increasing siphon activity and feeding of *Macomona liliana* in response to sediment deposition disturbances (McCartain et al., 2017; Townsend et al., 2014), indicating that this behavioural change may be induced by a physiological energetics shift.

Most work on acidification effects on bivalves has focussed mainly on calcification, physiology, fitness and mortality (e.g. Gazeau et al., 2010, 2007; Kurihara et al., 2007; Pörtner et al., 2004; Pörtner, 2008; Ries et al., 2009). To date, only few studies have investigated the potential impacts of ocean acidification on marine bivalve behaviour. For example, a study on the behavioural response of the king scallop *Pecten maximus* showed that the escape performance such as clapping force was adversely affected by high pCO_2 conditions (Schalkhausser et al., 2013). Another study indicated a decline in the feeding of *Perumytilus purpuratus* on plankton under low pH (Vargas et al., 2013). Furthermore, other studies showed that the settling behaviour of larvae of the hard clam *Mercenaria mercenaria* declined (Green et al., 2013), while the burrowing behaviour of juveniles of the same species similarly decreased in response to lower sedimentary aragonite saturation and higher hydrogen concentrations (Clements and Hunt, 2014). Furthermore, behavioural responses such as postponing settlement of juveniles of the soft-shell clam *Mya arenaria* was reported in acidified sediment (Green et al., 2009). Generally, the behaviour of bivalves seems to be affected by acidification; however, more research on the behavioural response under high pCO_2 condition (i.e. combined warming and acidification) is needed before a solid conclusion can be drawn.

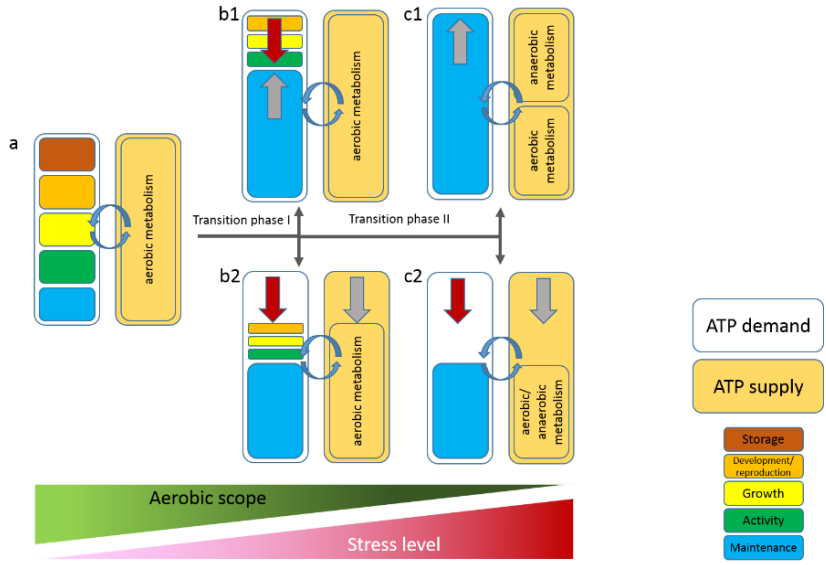

Figure 1.7 Schematic overview of the theoretical bioenergetic responses of organisms to different stress levels by including the oxygen- and capacity-limited thermal tolerance concept (OCLTT) and the dynamic energy budget (DEB). Grey arrows represent changes in ATP demand or supply when organisms are exposed to stressful conditions, red arrows represent the resulting trade-offs and blue arrows indicate the equilibrium of ATP demand and supply. A condition of low stress is represented by a whereas b and c indicate different metabolic responses under moderate and extreme stress conditions in transition phase I and II. During optimum condition (none or low stress level), the aerobic metabolism (ATP supply) of an organism is adequate to cover the energy costs for processes such as basal maintenance, activity, growth and reproduction/development (ATP demand, a). Under these conditions, the aerobic scope is high and energy allocation between processes is optimal, thus, the organism's fitness is conserved and excess energy (if present) is stored in storage tissues. Under moderate stress, costs for basal maintenance are increased to meet the ATP demand for damaged tissue reparation and stress protection (b1) or the basal metabolism and/or food assimilation is suppressed by the stressor (b2), resulting in a reduction of the aerobic scope. The onset of moderate stress causes a disequilibrium between ATP supply and demand, and subsequent acclimatisation re-establishes the energy balance by trading off energy from other processes such as growth and reproduction to cover maintenance costs. Energy storage in tissues is utilised to provide energy for these fundamental processes. During extreme stress, the gradual increase in ATP demand for basal maintenance (c1) or the gradual deterioration of aerobic metabolism overrides the supply (c2). In this stage, the aerobic scope vanishes and the metabolism shifts to partial anaerobiosis to compensate for insufficient aerobic energy supply in order to provide energy for essential maintenance costs to permit short-term survival. Ultimately, metabolic depression occurs to establish energy balance at a reduced energy stage including shutting down energy-demanding functions to ensure survival. This does not allow long-term survival of a population due to the lack of energy

investment in activity, growth and reproduction, however, it can extend the lifetime of an organism. Modified from Sokolova et al. (2012).

1.3.3 Study species

1.3.3.1 Cerastoderma edule

The common cockle *Cerastoderma edule* is a sediment-dwelling filter feeding bivalve that occurs along the east Atlantic coast from Morocco to Norway, into the Black, Mediterranean and Baltic Seas (Malham et al. 2012), particularly in sandy mud and sand (Dabouineau and Ponsero, 2009). In the intertidal area of the Eastern Scheldt estuary, cockles can occur at the highest densities of about 5000 ind.m^{-2} (Coosen et al., 1994b). The cockle represents an important diet for shorebirds (e.g. the oystercatcher *Haematopus ostralegus* and the common eider *Somateria mollissim)* and crustaceans (e.g. the brown shrimp *Crangon crangon* and shore crab *Carcinus maenas*; Beukema and Dekker 2005; Sanchez-Salazar et al. 1987; Whitton et al. 2012). Furthermore, the species' crawling and shaking behaviour can alter the upper sediment layer, which induces erosion of the sediment bed (Ciutat et al. 2006) and alteration in the benthic community structure (Flach, 1996; Van Colen et al., 2013). Common cockles are known for their commercial value and are extensively harvested by fishermen. Furthermore, common cockles are an aquaculture species in the Netherlands, Portugal and the UK, although the production in these countries has not been stable (Spencer, 2008).

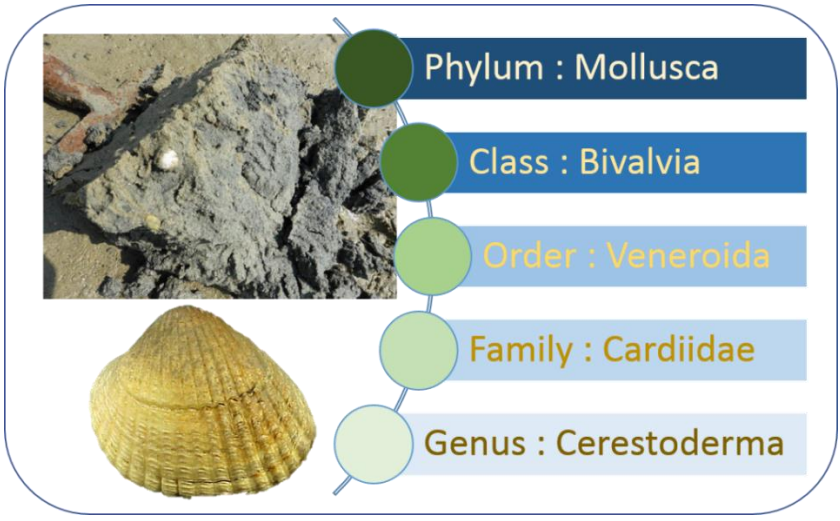

Figure 1.8 *Cerastoderma edule*

Cockles feed and respire by actively filtering seawater via their inhalant siphon and releasing it via their exhalant siphon, which is located on the surface of the sediment (Dabouineau and Ponsero 2009). Cockles are able to preselect food for ingestion through the gills, and preferentially ingest organic-rich diets (Iglesias et al., 1992). The selection efficiency was observed to be maximal when the diet's organic content was low (Urrutia et al., 2001). Under low food quality conditions (low organic content), cockles increase their pseudofaecal mucus production to reject the filtered suspension. This process limits the energy expenditure on regulating the digestion of poor quality food (Urrutia et al., 2001).

Due to the sensitivity of cockles to changing environment conditions, cockles have been used as bio-indicators for several

environmental change studies, e.g. to measure the impact of temperature change, oxidative stress, pollutants. (Bergayou et al., 2009; Freitas et al., 2007; Nilin et al., 2012). Nilin et al. (2012) used the physiological responses of cockles as indicator for mercury contamination. They observed that the survival of cockles, as well as their energy reserves and clearance rate were highly correlated with Hg content. A previous study has demonstrated that the shells of cockles are vulnerable to high seawater pCO_2 (Schade et al., 2016) suggesting that ocean acidification and/or warming in the future may place an energetic burden to cockles.

1.3.3.2 Scrobicularia plana

The peppery furrow shell *Scrobicularia plana*, is a tellinid bivalve which occurs abundantly in estuarine tidal flats and along the Atlantic coast from Norway to Senegal, and stretching out into Baltic and Mediterranean Seas (Santos et al., 2011; Tebble, 1976). The peppery furrow shell has a thin, rounded and flattened shell and is able to grow up to a length of 6.5 cm. It burrows up to 20 cm in intertidal soft bottoms with a preference for muddy sediments and can be identified on the surface of the sediment as a star-shaped marking by its inhalant siphon (Bazaïri et al., 2003; Casagranda and Boudouresque, 2005; Pizzolla, 2002). *S. plana* abundance varies geographically (Santos et al., 2011), but the species' abundance increases towards southern areas: the highest densities of 5892 ind m^{-2} were recorded in Spain (Sola,

1997), while high densities were also observed around the Atlantic coast of France with 3055 ind m^{-2}, and maximal densities of up to 1000 ind m^{-2} were recorded in the Gwendrath Estuary (South Wales) and Tamar Estuary (border between Cornwall and Devon, England (Green, 1957)). In the Scheldt estuary, the densities of *S. plana* range between 0 and 394 ind.m^{-2} (Ysebaert and Herman, 2002).

S. plana lives buried in the sediment which commonly causes it to be surrounded by adverse conditions (e.g. lack or absence of oxygen and food). Thus, this bivalve depends on its inhalant siphon to protrude through the overlying mud in order to reach the surface for respiration and filtering food (Zwarts et al., 1994). Therefore, it is critically important for *S. plana* to be able to elongate its elastic inhalant siphon allowing it to live at a safe depth far beyond the reach of surface predators (Zwarts and Wanink, 1989). In general, *S. plana* is known as a deposit-feeding bivalve that can feed directly on sediments, but it can as well feed on the suspended matter in the water column (Hughes 1969). Its siphon is regularly preyed upon by fish, birds and crabs, but the damaged tissue is able to regenerate in a few days (Hodgson, 1982). Apart from being a major diet in the food web, *S. plana* is also increasingly harvested by fishermen as human food source in France, Portugal and Spain (Langston et al., 2007).

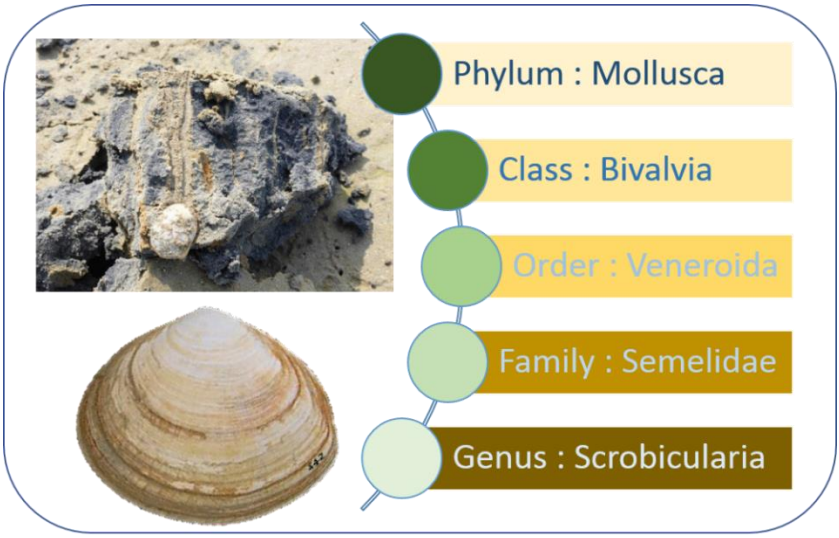

Figure 1.9 *Scobicularia plana*

Due to its sedimentary burrowing behaviour, ecological importance, wide distribution, tolerance to chemical exposure and low metabolism, *Scrobicularia* was frequently used as biomarker to monitor pollution and heavy metal contamination (e.g. Dauvin 2008; Coelho et al. 2008; Solé, Kopecka-Pilarczyk, and Blasco 2009; Bryan and Langston 1992). Interestingly, Coelho et al. (2008) reported that *Scrobicularia* plays an important role in biomagnification processes via the uptake of contaminated sediments and their subsequent transfer to economically important species at higher estuarine trophic levels.

1.4 Environments

The two model species i.e. *Cerastoderma edule* and *Scrobicularia plana* (mentioned in section 1.3.3.1and 1.3.3.2) were collected

from the estuaries of Oosterschelde and Westerschelde (Figure 1.10). In the following sections, a description of both estuaries and the sampling location is given.

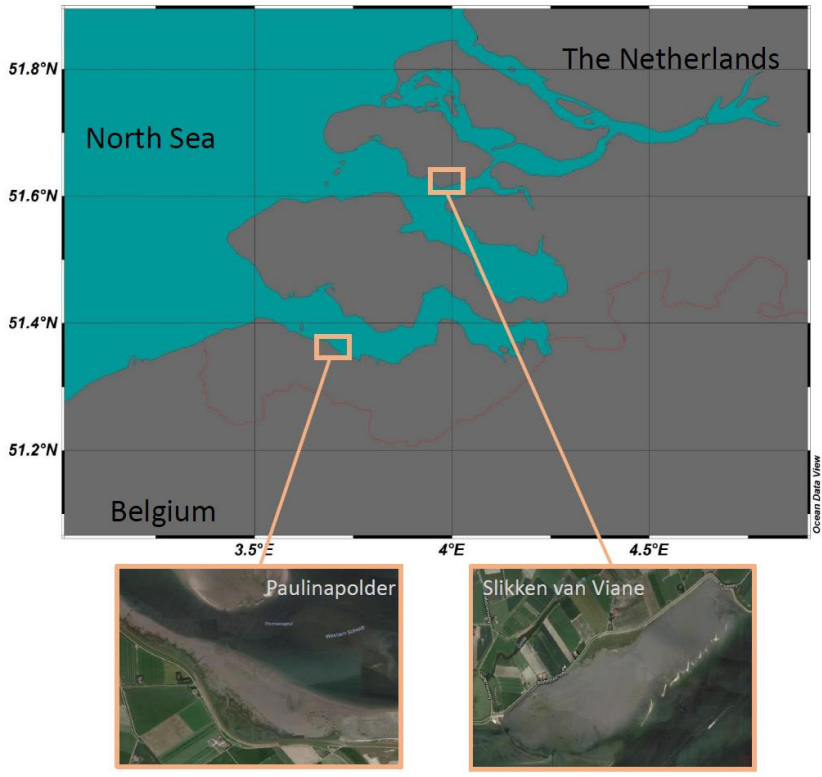

Figure 1.10 Sampling locations in the Westerschelde and Oosterschelde.

1.4.1 Oosterschelde

The Oosterschelde is an estuary located in Zeeland, the Netherlands, with an area of 351 km^2, of which 118 km^2 are tidal flats and 6.27 km^2 are salt marshes (Smaal and Nienhuis, 1992). It is the largest national park in the Netherlands (https://www.np-oosterschelde.nl/en/home.htm). The estuary is

isolated from the river input and is closed during storm surges to protect the hinterland from the North Sea by a sea-wall. This division from the sea reduced tidal exchange and created sheltered circumstances (Nienhuis and Smaal, 1994). The estuary's primary production appears to be resilient and robust and its biological communities exhibited only quantitative shifts from one dominance specific species assemblages to another (Nienhuis and Smaal, 1994). The estuary is dominated by filter feeding bivalves, mainly common cockles and blue mussels (Klepper, 1989). Both species feed on *Leptocylindrus minimus*, *Biddulphia sinensis*, *Rhizosolenia shrubsolei*, *Skeletonema costatum*, *Thalassiosira nordenskioeldii* in the Oosterschelde (Bakker et al., 1994). Moreover, it is also an important migratory route for birds (Meire et al., 1989) and a defined nursery function for some commercial fish species (Smaal and Nienhuis, 1992).

1.4.1.1 Slikken van Viane

The "Slikken van Viane" is a tidal flat situated in the northern part of the Oosterschelde, Zeeland, the Netherlands (51 ^{o} 37′ N,4 ^{o}2′ E, Figure 1.10). Viane has a semi-diurnal tidal regime with an annual average salinity of 33 (http://www.rijkswaterstaat.nl/) and the sediment type consists predominantly of fine and medium sand (Van Colen et al. 2013). The seawater surface temperature near Viane varies seasonally with a maximum yearly average of 19 °C in August and a minimum yearly average

of 5 °C in February (data retrieved in April 2018 at location Bruinisse from https://weatherspark.com/y/51298/Average-Weather-in-Brui nisse-Netherlands-Year-Round). The average annual pH near this area from 2010 and 2015 was 8.1 (retrieved on April 2018 at location Zijpe from http://live.waterbase.nl, Figure 1.11); however, a decreasing pH trend approximately of 0.2 units was observed between 1990 and 2005 (Provoost et al., 2010). According to Coosen et al. (1994a), this tidal flat (Oosterschelde) is dominated by 10 to 15 macrobenthic species, amongst the most common species Oligochaeta, Polychaeta, *Hydrobia ulvae* and *C. edule*.

Adult cockles were collected here for the experiment (chapter 2) in the low intertidal zone, where the species is highly abundant (72 ± 30 ind.m^{-2} (Van Colen et al., 2013)). This species was selected as a model species due to its vulnerability as a calcifier as well as its importance to ecosystem functioning in the estuary (see 1.3.3.1). Research has shown that climate change has shifted the distribution of intertidal marine organisms poleward by approximately 50 km per decade (Helmuth et al., 2006). Thus, this marine calcifying species may be vulnerable to future environmental changes (i.e. acidification and warming), which in the case of cockles from Slikken van Viane may ultimately lead to their extinction from this location.

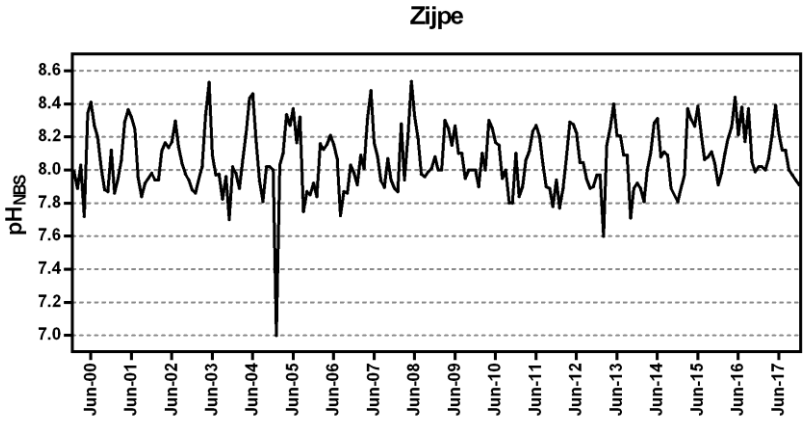

Figure 1.11 The natural pH variability (monthly, from 2000 to 2017) at Zijpe (51° 39' N, 4° 6' E) near the sampling site of our study (source: www.live.waterbase.nl).

1.4.2 Westerschelde

The Westerschelde estuary is a dynamic estuary in terms of both ecological (e.g. hydrodynamic forces) and evolutionary time scales (e.g. young and unstable habitats) that connect the North Sea and the Schelde river. Its unique climatic and hydrodynamic forces shape a highly fluctuating environment with strong spatial and temporal gradients in e.g. submersion time, temperature and salinity (Heip, 1988). The estuary hosts a low species diversity yet specialised to their environment which are resilient to a highly dynamic environment (Heip, 1988). Suspension feeders are less dominant in the turbid Westerschelde compared to the Oosterschelde (Herman et al., 1999). The dominant (in terms of carbon biomass) phytoplankton species which can be found in the Westerschelde includes

Thalassiosira sp., *Ditylum brightwellii*, *Cryptomonas* sp. (Rijstenbil et al., 1993).

1.4.2.1 Paulinapolder

Paulinapolder is a tidal flat located within the polyhaline part of Westerschelde estuary, southwest Netherlands (51° 20′ N, 3° 43′ E, Figure 1.10). This site has a semi-diurnal tidal regime with a yearly mean salinity of 24 and mean tidal range of 3.9 m (Van Colen et al., 2013). The seawater surface temperature at Paulina polder varies seasonally between a minimum of 5 °C during winter (February) to a maximum of 19 °C during summer (August) (data retrieved on April 2018 at location Terneuzen from https://weatherspark.com/y/49992/Average-Weather-in-Terneuzen-Netherlands-Year-Round). The mean annual pH between 2007 and 2017 around this area was 8.03 (retrieved on April 2018 at location Terneuzen buoy 20 from http://live.waterbase.nl, ,Figure 1.12). The pH in Westerschelde estuary is highly fluctuating but a declining trend by approximately 0.13 units was observed between 1990 and 2005 (Provoost et al., 2010), hence emphasising the importance of studying the consequences of acidification and warming in this area. Around 18 macrobenthic species are present at this location (Van Colen et al., 2012b), with the dominant macrobenthos species (in terms of biomass) being *C. edule*, *Hediste diversicolor*, *L. balthica*, and *S. plana*.

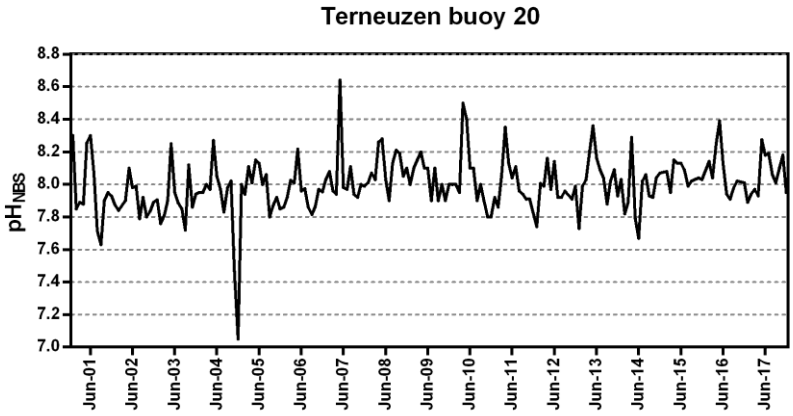

Figure 1.12 The natural pH variability (monthly, from 2000 to 2017) at Terneuzen boey 20 (51° 17' N, 3° 39' E) near to the sampling site (source: www.live.waterbase.nl).

Adult *S. plana,* macrobenthic communities and sediment were collected in the intertidal area to examine the (1) the effects of ocean acidification and warming on the behaviour of *S. plana* (chapter 3) and (2) the direct and indirect effects on the macrobenthic community associated with a potential behavioural change of *S. plana* (chapter 4).

1.5 Outline and objective of the thesis

This thesis primarily focusses on assessing the impacts of future ocean conditions on behaviour and physiology of two estuarine key species. Furthermore it assesses and discusses how such effects can change ecosystem processes and functions such as biological interactions and biogeochemistry that determine ecosystem stability and service delivery. The aim was achieved

through laboratory experiments that compared observations between current physico-chemical conditions and manipulated temperature, carbonate chemistry and pH, which mimicked the prediction for a future high pCO_2 ocean. The link between all chapters of the thesis are summarised in Figure 1.13. The selected species can be maintained in good condition for sufficient duration in laboratory setting. Furthermore, they have different biological and functional traits sediment that and differently alter functioning of shallow coastal sediments.

In chapter 2 we investigated the effects of ocean acidification and warming on the condition and ecophysiology of common cockle *C. edule*, by means of a laboratory experiment. The experiment was conducted by incubating the studied species with sediment under lowered pH and elevated temperature, in a fully crossed design. We consciously chose this shallow filter feeder species because of its necessity to deposit aragonite shells and its ecological and economic importance as a key role species in shallow coastal habitats. The experiment sheds light into how the combined effects of both stressors affect the condition and ecophysiology of the cockle, by integrating important parameters such as condition index, calcification, respiration and clearance rates, in order to underpin our understanding on the dynamic energy budget, and thermal tolerance of the species in a high pCO_2 warmer ocean (Sokolova et al., 2012).

Figure 1.13 Overview of the thesis chapters including studied responses, species and communities.

In line with chapter 2, we examined another tidal flat species, the peppery furrow shell *S. plana* by incubating it under combined stressors of ocean acidification and warming (chapter 3). In this chapter, we assessed the surface and subsurface behavioural response of the species by using non-destructive pressure sensors and time-lapse camera. First, we identified different behaviours of *S. plana* by synchronising porewater pressure signals with time-lapse images and then quantified each behaviour accordingly at during- and post-treatment. This species was chosen because of its calcifying ability, omnipresence and trophic importance as well as its capability to switch its feeding mode according to the environmental change (Hughes, 1969; Keegan, 1986; Santos et al., 2011). Our experiment

contributes to the understanding of the combined effects of acidification and warming on behavioural responses of the peppery furrow shell, whereas cascading effects of these behavioural changes on the biotic and abiotic properties of a sediment community were analysed into more detail in chapter 4.

The findings from chapter 2, 3 and 4 are integrated and discussed in the General discussion (chapter 5) that compares our results with the available literature in order to put the overall findings of the thesis into context, as well as some future research directions and challenges that are addressed.

Chapter 2

Physiological responses to lowered pH and elevated temperature synergistically reduce condition of the common cockle *Cerastoderma edule*

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2.1 Abstract

The combined effect of ocean acidification and warming on the common cockle *Cerastoderma edule* was investigated in a fully crossed laboratory experiment. Survival of the examined adult organisms remained high and was not affected by elevated temperature (+3 ˚C) or lowered pH (-0.3 units). However, the morphometric condition index of the cockles incubated under high pCO_2 conditions (i.e. combined lowered pH and elevated temperature) was significantly reduced after six weeks of incubation. Respiration rates increased significantly under low pH, with highest rates measured under combined warm and low pH conditions. Calcification decreased significantly under low pH while clearance rates increased significantly under warm conditions and were generally lower in low pH treatments. While synergistic effects were not observed in these three separate physiological responses, the interactive effect of lowered pH and elevated temperature on cockle condition has to be explained by the cumulative impacts of these different responses. The observed physiological responses suggest that the reduced food intake under hypercapnia is insufficient to support the higher energy requirements to compensate for the higher costs for basal maintenance and growth in future high pCO_2 waters.

2.2 Introduction

Estuaries are among the most productive marine ecosystems, supporting a high abundance and diversity of organisms (Beck et al., 2001). Other ecosystem services provided by these systems include disturbance regulation (e.g. flood control, storm protection, nutrient cycling, biological control, habitat creation and others (e.g. Barbier et al., 2011; Meire et al., 2005)), contributing to an estimated total annual monetary value of 4.1 trillion US$ (Costanza et al., 1997). However, these habitats are gradually degraded by increasing human activities compromising their function as feeding, nursery and breeding habitats (Seitz et al., 2014). In addition, coastal habitats like estuaries are in the frontline of current environmental change, including climate change (Scavia et al., 2002). The excess of CO_2 emissions produced by the burning of fossil fuels, cement production, and deforestation (Sabine et al., 2004) are interacting with the global climate and the ocean, causing warming and changes in ocean carbonate chemistry (Doney et al., 2009). To date, approximately 30% of the anthropogenic CO_2 in the atmosphere is being absorbed by oceans and is altering their chemistry, a process referred to as ocean acidification (OA) (Caldeira and Wickett, 2003; Sabine et al., 2004), with an estimated reduction in pH of 0.3 - 0.4 units by the end of the 21st century for the open ocean (Caldeira and Wickett, 2003; Feely et

al., 2004; Orr et al., 2005). High temporal fluctuations in pH (Wootton et al., 2008) may mask the effect of rising pCO_2 in coastal habitats in the short-term; yet recent analyses show that rates of acidification are an order of magnitude higher in coastal habitats as compared to the open ocean (Provoost et al., 2010; Wootton et al., 2008), suggesting that these shallow water marine habitats might be particularly vulnerable to rising pCO_2 concentrations.

Over the last two decades, the consequences of changes in ocean carbonate chemistry on various life stages of calcifying marine organisms have been intensively investigated, including studies of OA effects on calcification, survival, acid-base regulation, metabolism, reproduction and immunity (reviewed in Gazeau et al., 2013; Kroeker et al., 2010; Parker et al., 2013). Calcifying organisms like corals, shellfish and crustaceans have been shown to be particularly vulnerable to OA (Kroeker et al., 2010). For example, the reduction in the success of fertilisation and embryogenesis, and in the number, growth and survival of hatchlings in the Baltic tellin *Limecola balthica* at pH 7.5 (Van Colen et al., 2012a) illustrates the adverse impacts OA can have on early life history processes. Another example is an 87-day study by Range et al. (2014) demonstrating that juvenile clam *Ruditapes decussatus* decreased their rates of respiration, clearance and ingestion, whereas excretion rates were increased

under mimicked acidic conditions (e.g. pH 7.5 and 7.8). Furthermore, OA was shown to disrupt behavioural processes (e.g. finding shelter, ability to detect food, prey and predators), for example in adult hermit crabs (*Pagurus bernhardus)*, that might potentially affect the population fitness and their effects on ecosystem functioning (Briffa et al., 2012). In short, OA does impact marine calcifying organisms in terms of morphology, physiology and behaviour.

Ocean acidification does not occur in isolation (Byrne and Przeslawski, 2013). The Intergovernmental Panel on Climate Change (IPCC) has predicted an increase in global mean temperature ranging between 1.0 - 4.1 ˚C based on four CO_2 Representative Concentration Pathway (RCP) scenarios (Collins et al., 2013). While the separate effects of changes in seawater temperature and pH are well documented, comparatively few studies have hitherto focused on the combined effects of seawater temperature rise and acidification. Both stressors may act synergistically, antagonistically or additively on the condition and physiology of marine animals (Darling and Cote, 2008), and their combined effects can reduce the functional performance of species (e.g. foraging, growth, reproduction, competitiveness and behaviours) at ecosystem levels through narrowing of species' thermal windows (Pörtner, 2008; Pörtner and Farrell, 2008). Ocean acidification coupled with warming

increased the energy metabolism of the adult Pacific oyster *Crassostrea gigas* (Lannig et al., 2010). Furthermore, Talmage and Gobler (2011) showed additive negative effects of both stressors on the mortality of bay scallops *Argopecten irradians* and hard clam *Mercenaria mercenaria* larvae, and larvae reared under high temperature and low pH conditions accumulated less lipids, were smaller and had an extended time to metamorphosis. Some studies on bivalves found that elevated temperatures had more pronounced impacts than lowered seawater pH on survival, immune response, growth and development (e.g. *Mytilus galloprovincialis* and *Mytilus edulis* (Gazeau et al., 2014; Mackenzie et al., 2014; Vihtakari et al., 2013)). On the other hand, Duarte et al. (2014) concluded that calcification rates and total weight of adult Chilean mussels (*Mytilus chilensis*) decreased more in response to lowered pH than to elevated temperature. In short, the effects of OA and warming are thus highly species-specific and can depend on the life stage, biogeography and environmental context.

To properly understand the impacts of future ocean scenarios on coastal ecosystems, it is crucial to assess the combined effects of temperature change and OA on species that play a key role in the ecosystem. The common cockle *Cerastoderma edule* is a sediment-dwelling filter-feeding bivalve that represents an important dietary component for shorebirds (e.g. the oystercatcher

Haematopus ostralegus and the common eider *Somateria mollissima)* and crustaceans (e.g. the brown shrimp *Crangon crangon* and the shore crab *Carcinus maenas* (Beukema and Dekker, 2005; Sanchez-Salazar et al., 1987; Whitton et al., 2012). Furthermore, the species' subsurface crawling and shaking behaviour causes disturbance of the upper sediment layer, which can induce erosion of the sediment bed (Ciutat et al., 2006) and alterations in the benthic community (Flach, 1996; Van Colen et al., 2013). Common cockles occur along the east Atlantic coast from Morocco to Norway, and along the Black, Mediterranean and Baltic Seas (Malham et al., 2012), where they are extensively harvested by fishermen. According to the Food and Agriculture Organization of the United Nations (FAO) (http://www.fao.org/fishery/statistics/global-aquaculture-production/en), the average annual harvest of common edible cockles was 17,073 tonnes (from 2008 till 2014). The shells of cockles are solely composed of aragonite, which is a more soluble calcium carbonate polymorph than calcite (Cubillas et al., 2005). Hence, cockle shell formation is expected to be particularly vulnerable to OA and low pH conditions could therefore also reduce the species' physiological tolerances to other environmental alterations such as ocean warming (Pörtner and Farrell, 2008; Widdicombe and Spicer, 2008). Here we experimentally investigated the physiology and condition of cockles under high temperature and low pH both single and combined stressor conditions. By exposing cockles to

changes in pH and temperature, we expected to find an enhanced energy demand related to the support cost of tissue protection systems; for example, damage repair mechanisms, the regulation of acid-base balance and ion transport (Sokolova et al., 2011). These compensatory metabolic strategies can facilitate organisms to cope with stress at the short term but may disrupt the energy budget balance at the longer term, ultimately affecting fitness of the population (Sokolova et al., 2011).

2.3 Material and methods

2.3.1 Collection and incubation of cockles

In June 2015, adult cockles were collected during low tide in the lower intertidal zone at the "Slikken van Viane", Oosterschelde estuary, The Netherlands (51° 37′ N, 4° 2′ E) and were transported within two hours to the laboratory. Forty-four randomly chosen cockles (average shell length of 27.7 ± 3.2 mm (SD)) were cleaned and added to each of 12 aquaria (41 cm x 31 cm x 40 cm) that had been filled with sediment (median grain size: 224 ± 1.7 μm) to a height of 15 cm and allowed to acclimatise to preset laboratory conditions (15 °C, salinity of 33 and pH of 8.1) for 3 days, reflecting average seawater surface temperature and pH in June at the sampling location (retrieved at location Zijpe (pH) and

Bruinisse [3] (temperature) from http://live.waterbase.nl and https://weatherspark.com/y/51298/Average-Weather-in-Bruinisse-Netherlands). All cockles burrowed in the upper first centimetre of the sediment within one hour with their siphons flush or slightly extending from the sediment-water interface.

There were two factors in our fully orthogonal experiment: temperature (ambient or elevated) and pH (ambient or lowered). Seawater was stored in four barrels (250 L) and pumped from each barrel to the three aquaria and circulated back to the barrel continuously. The seawater in the aquaria and barrels was aerated and the seawater pH was manipulated in the barrels. A stirrer was installed into each barrel in order to homogenise seawater. To maintain salinity, a quarter of the seawater was renewed per week which took less than 20 min for the whole setup (i.e. 4 treatments, 12 aquaria). All aquaria were subjected to a 12:12h light:dark regime. Cockles were fed twice a week with 1 mL of commercial Shellfish Diet 1800 (Reed Mariculture Inc., composed of 40% *Isochrysis*, 15% *Pavlova*, 25% *Tetraselmis* and 20% *Thalassiosira weissflogii*) diluted with 3 L of seawater and distributed equally in the barrels. We acknowledge that the food quality and quantity used might not completely resemble those present in the field. However, we deliberately chose to use a

[3] Note that both locations (Zijpe and Bruinisse) where seawater pH and temperature data were collected, are only approximately 1 km apart.

mixture of dead microalgae to avoid bias in our experiment related to temperature and pH effects on food quantity and quality (Hinga, 2002; Thomas et al., 2012) that we were unable to account for.

After three days of acclimatisation under ambient conditions, the pH of the seawater was decreased by 0.1 pH unit and the temperature was increased by 1 ˚C per day over 3 days, until a pH value of -0.3 units and a temperature of +3 ˚C was achieved (see below). These conditions were maintained for 6 weeks (see Table 2.1 and Figure 2A.1). At the site of cockle collection field pH data for mid-June and July ranged between 8.0 and 8.2 (data retrieved at location Zijpe from 2010 to 2015 from http://live.waterbase.nl) and the daily average seawater temperature ranged from 15 to 18 ˚C (https://weather spark.com/y/51298/Average-Weather-in-Bruinisse-Netherlands). The manipulated combined pH and temperature conditions thus enable us to study realistic OA effects at two ambient temperatures, i.e. the average daily temperature and the highest temperature during the period of the experiment.

ProMinent Dulcometers coupled with Hamilton glass pH electrodes were used to control the seawater pH through the controlled bubbling of CO_2 in the lowered pH treatments. The pH values were logged every 10 min using pH electrodes and a Consort data logger (Model: C3040). All pH electrodes, including

Hamilton (S/N: 16458 and 16451) and Consort electrodes (SP10B-50), were calibrated weekly using Hanna instruments NBS buffer solutions (pH 4.01, 7.01 and 9.18). All pH measurements were reported according to the NBS scale. Meanwhile, seawater temperature was regulated by temperature heater/chiller controllers (Teco Refrigeration technologies; Model: TK200H) and HOBO Pendant data loggers (Model: UA-002-08) were used to record seawater temperature every 10 min. Vertical profiles of sediment porewater pH (for a description on the profiling set up, see Braeckman et al., 2014b) obtained during pilot tests with the used sediment demonstrated a strong gradient in pH with a steep and gradual decline from the sediment-water interface until 0.5 cm depth where the pH stabilises. Importantly, Figure 2A.2 indicates that the applied pH manipulation created similar differences (~0.3 pH units) between ambient and lowered pH treatments in both the water column and sediment.

30 mL seawater samples were collected from each tank on a weekly basis and filtered through GF/C filters for subsequent quantification of total alkalinity (TA). These samples were temporally stored at 4 °C prior to subsequent Gran titration using a Mettler Toledo G20 compact titrator and a glass electrode calibrated using Hanna instruments buffer solutions (pH 4.01 and 7.01) and a TRIS buffer solution (pH 8.1, MERCK Production Chemicals). The obtained pH, TA, temperature and salinity

values were used to determine parameters of the carbonate chemistry (e.g. partial pressure of carbon dioxide (pCO_2), saturation state of the seawater with respect to aragonite (Ωa), total inorganic carbon concentration (C_T) etc.) through CO_2SYS software (Pierrot et al., 2006) using the thermodynamic constants of Mehrbach (1973).

2.3.2 Cockle condition and physiology

Mortality of cockles was checked on a daily basis and dead cockles were removed. Cockle physiology was measured as respiration rates, clearance rates and calcification rates after 3 and 6 weeks of incubation. To this purpose, 6 - 10 cockles corresponding to a total wet weight of 61 - 70 g were removed from the sediment of each of the three aquaria per treatment, and placed inside their respective incubation chamber (Ø = 19 cm, height = 30 cm, volume = 8.2 L (Braeckman et al., 2014b)), along with the seawater from their respective barrels. Subsequently, respiration, clearance and calcification rates were determined according to the methodologies described below. Temperature was maintained throughout all incubations by working in a climate room set at the desired temperature, i.e. 14.9 ± 0.5 and 17.9 ± 0.5 °C. After the measurements, the soft tissues of individuals were separated from their shell. Individual shells and soft tissues were allowed to dry in an oven at 60 °C for 2 days

and weighed. The *condition indices* (CI) of cockles were determined using equation (1) from Lucas and Beninger (1985).

$$CI(\%) = \frac{dry\ tissue\ weight}{dry\ shell\ weight} \times 100 \quad (1)$$

Respiration rates of batches of 6 - 10 cockles with a total wet weight of 61 - 70 g were measured using Pyroscience sensor technology. Sensor spots were glued on the inner wall of incubation chambers; when the sensor spots are excited by red light, they show an oxygen-dependent infrared emission. Lens Spot Adapters were placed on the outside of incubation chambers facing the sensor spot and connected with spot fibers to a FireSting O_2 logger, which allowed continuous display and recording of the O_2 concentration (μmol L^{-1}) of the seawater. Seawater oxygen concentrations were logged continuously for 2 h and 40 min and respiration rates were calculated as in equation (2).

$$R\ (\mu mol\ g^{-1}\ DW\ h^{-1}) = \frac{V(R_0 - R_1)}{g(t_1 - t_0)} \quad (2)$$

where t_0 and t_1 represent initial and final time (h) of measurement, respectively; R_0 and R_1 represent the oxygen concentrations (μmol L^{-1}) at time t_0 and t_1; g is the dry flesh weight (g) of cockles and V is the seawater volume (L) of the incubation chamber after correction for the biovolume of the cockles. An additional incubation chamber without cockles was used as a blank to correct for bacterial respiration.

Following the respiration measurements, oxygenation of the seawater in each incubation chamber was restored and 1 mL of Shellfish Diet 1800 was injected into each incubation chamber. After an initial mixing period of 30 min, seawater samples were collected to quantify the algal cell density over time. Samples were collected by removing 10 mL of seawater from the incubation chamber using a syringe, while at the same time injecting the same volume of seawater using a second syringe in order to keep the seawater volume in the chamber constant. Samples were collected at t_0 (30 min after the injection of food into seawater in order to homogenise the mixture before samples were taken to avoid bias) and at t_1 (after 150 min of incubation) from each chamber.

In order to calculate the volume of seawater cleared by cockles, algal cell concentrations were quantified using a Coulter Multisizer equipped with a 100-µm aperture tube. Hence, *clearance rates* were calculated from the exponential decline in the cell concentration in the incubation chamber following equation (3), modified from Widdows and Navarro (2007),

$$CR(L\ h^{-1}\ dry\ weight^{-1}) = \frac{V*(log_e C_1 - log_e C_2)}{t*g} \quad (3)$$

where V represents the volume of water in the incubation chamber, t is the time interval in hours, C_1 and C_2 represent the shellfish diet cell concentration at the start and end of the

measurement, and g represents the dry flesh weight (g) of cockles present in an incubation chamber.

Calcification rates were estimated using the alkalinity anomaly-method according to the modification proposed by Gazeau et al. (2015) to correct for other processes (e.g. mineralisation and assimilation) that can affect TA independent from calcification. This method has been broadly used for short-term incubation as it is non-destructive and based on parameters that are easily collectable and accurately quantifiable. Two samples of 25 mL seawater were collected at the beginning and end of a 2-hour incubation period. These seawater samples were analysed to determine the total alkalinity (TA), concentrations of nutrients (NH_4^+, NO_3^-+NO_2^- and PO_4^{3-}), and *calcification rates* were calculated as shown in equation (4) modified from Gazeau et al. (2015).

$$G^*TA\ (\mu mol\ g^{-1}\ DW\ h^{-1}) = \frac{\Delta {NH_4}^+ - \Delta TA - \Delta NO_x - \Delta {PO_4}^{3-}}{2 * g * (t_1 - t_0)} \quad (4)$$

where t_1 and t_0 represent time (in hours) at the end and at the beginning of incubation, respectively; ΔNH_4^+, ΔTA, ΔNO_x and ΔPO_4^{3-} (in µmol kg^{-1}) are the differences in concentrations of ammonium, total alkalinity, nitrate plus nitrite and phosphate between t_0 and t_1, and g is the dry flesh weight (g) of the cockles present in the incubation chambers.

2.3.3 Statistical analysis

One-way analysis of variance (ANOVA) was used to compare seawater carbonate chemistry between treatments. For statistical data analysis, non-normal data were Log_{10} transformed to improve normality, and homogeneity of variances was checked using Levene's test. When significant differences between treatments were identified by the ANOVA, post-hoc tests (Tukey HSD pairwise tests) were performed. A 2 x 2 contingency analysis was used to examine the difference in survival proportion between pH and temperature levels at the end of the experiment (i.e. after 6 weeks of incubation). In order to test the effects of temperature (ambient, elevated), pH (ambient, lowered) and time (three weeks, six weeks) on the conditional and physiological response variables (condition index, respiration, clearance and calcification rate), we used a series of linear mixed effects models with appropriate error structures for each response variable. Since cockles were taken on two occasions (after weeks three and six) from each aquarium, we accounted for the non-independence of these data by allowing random intercepts for aquaria identity. Prior to analysis, data were Log_{10} transformed to improve normality in case of non-normal data, and the model assumptions were checked using Q-Q plots. Data were modelled using a Gaussian error distribution. For Gaussian models we calculated the *F*-statistic and associated *P*-values for

each effect, using the Kenward-Roger method to estimate degrees of freedom. Analyses were conducted using the R software version 3.3.1 (R. Core Team, 2014) with packages lme4 (Bates et al., 2015), lmerTest and pbkrTest (Kuznetsova et al., 2015).

2.4 Results

Seawater carbonate chemistry, temperature, pH and salinity for each treatment throughout the experiment are shown in Table 2.1 and Figure 2A.1. Temperatures in the lowered pH-elevated temperature treatment and in the elevated temperature treatment were maintained at 2.57 ± 0.13 (SD) ˚C and 2.74 ± 0.15 ˚C, respectively, above the control treatment (15.4 ± 0.68 ˚C). pH treatments were kept constant throughout the experiment with average values of 8.10 ± 0.01 (SD) and 8.20 ± 0.01 for the control and elevated temperature treatment, respectively. Seawater pH was reduced by 0.37 ± 0.03 and 0.30 ± 0.02 pH units, respectively, in the lowered pH and lowered pH-elevated temperature treatments in comparison with the control (pH 8.11 ± 0.06). Throughout the experiment, total alkalinity (3568 ± 131 μmol kg^{-1}) and salinity (34.2 ± 1.0) did not differ between treatments (Table 2.1). Aragonite concentration remained favourable for calcification in all treatments, with average aragonite saturation states of 6.0, 3.7, 6.2, and 3.6 in control, lowered pH, elevated

temperature and lowered pH-elevated temperature treatments, respectively. During the experiment, survival remained high (> 93%) and did not differ significantly among treatments (Figure 2.1). Dead cockles laying on top of the sediment were removed immediately during the daily observations. There was no difference in survival between ambient and lowered pH treatments ($\chi^2_1 = 2.4$, P = 0.12) or between ambient and elevated temperature treatments ($\chi^2_1 = 2.4$, P = 0.12) at the end of the 6-week incubation (Table 2A.1). There was no three-way interaction on *condition index* (CI) of cockles between temperature, pH and time ($F_{1,8} = 0.69$, $P = 0.43$, Figure 2.1). Similarly, there was no two-way interaction between pH and time ($F_{1,9} = 3.8$, $P = 0.082$). However, there was a significant two-way interaction between temperature and pH ($F_{1,8} = 7.5$, $P = 0.025$) indicating that reduced pH led to a decrease of 16% in CI in cockles held at 18 ˚C but had no effect on CI for cockles held at 15 ˚C (Figure 2A.3a). Furthermore, there was a significant two-way interaction between temperature and time ($F_{1,9} = 8.2$, $P = 0.020$), indicating that there was no difference in CI between the two temperatures at week 3, but that by week 6 cockles held at 18 ˚C had a lower CI (4.98%± 0.29 (SE)) than those held at 15 ˚C (5.95% ± 0.13, Figure 2A.3b). There were no main effects of time ($F_{1,11} = 3.8$, $P = 0.077$) and pH ($F_{1,9} = 2.9$, $P = 0.12$), but there was a significant effect of temperature ($F_{1,9} = 7.3$, $P = 0.025$).

Table 2.1 The average (± SD) seawater carbonate chemistry parameters in four treatments during the 6-week experiment: temperature (Temp), pH, salinity, total alkalinity (TA), partial pressure of carbon dioxide (pCO_2,), total inorganic carbon concentration (C_T), concentration of bicarbonate ion and carbonate ion (HCO_3^- and CO_3^{2-}) and saturation state of the seawater with respect to aragonite (Ωa). Values between brackets represent maximum and minimum values during the experiment. Post-hoc tests indicated significant ($p < 0.05$) difference between treatments but not within the same temperature and pH treatment level.

Treatment	Control	Lowered pH	Elevated temperature	Lowered pH-elevated temperature
Temp (°C)	15.4 ± 0.7 (17.3; 13.1)	15.3 ± 0.6 (16.3; 15.6)	18.2 ± 0.2 (19.2; 15.6)	18.0 ± 0.2 (19.0; 15.5)
pH	8.11 ± 0.06 (8.20; 8.00)	7.75 ± 0.04 (7.98; 7.50)	8.2 ± 0.06 (8.38; 8.08)	7.82 ± 0.08 (8.06; 7.53)
Salinity	34.4 ± 0.9 (36; 33)	33.8 ± 0.7 (35; 33)	34.5 ± 1.1 (36; 33)	34.2 ± 1.2 (37; 33)
TA ($\mu mol.kg^{-1}$)	3568 ± 102 (3667; 3372)	3563 ± 104 (3766; 3415)	3546 ± 72 (3696; 3471)	3600 ± 77 (3768; 3525)
pCO_2 out (µatm)	325 ± 27 (367; 286)	746 ± 260 (1065; 288)	335 ± 40 (385; 280)	870 ± 264 (1055; 322)
C_T ($\mu mol.kg^{-1}$)	3007 ± 71 (3108; 2917)	3283 ± 168 (3557; 2965)	3039 ± 71 (3142; 2924)	3332 ± 67 (3406; 3211)
HCO_3^- ($\mu mol.kg^{-1}$)	2616 ± 54 (2687; 2540)	3018 ± 223 (3327; 2559)	2628 ± 73 (2707; 2474)	3071 ± 132 (3174; 2805)
CO_3^{2-} ($\mu mol.kg^{-1}$)	379 ± 32 (440; 352)	237± 75 (395; 165)	400 ± 29 (440; 357)	231 ± 81 (423; 180)
Ωa	6.0 ± 0.5 (7.2; 5.4)	3.7 ± 1.2 (6.1; 2.5)	6.2 ± 0.4 (6.8; 5.6)	3.6 ± 1.3 (6.6; 2.8)

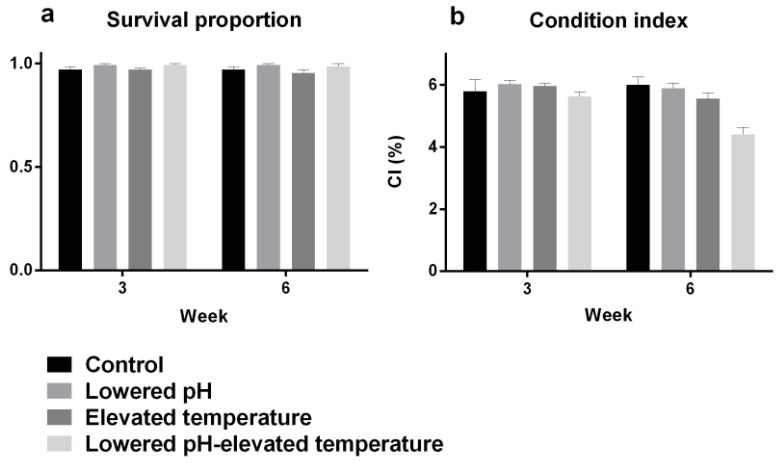

Figure 2.1 Effects of temperature and pH on survival (a) and condition index (b) after 3 and 6 weeks of incubation in the four treatments. Error bars represent standard errors (±SE).

We observed no three-way interaction between temperature, pH and time on *respiration rates* ($F_{1,8}$ = 0.096, P = 0.76, Figure 2.2) and no two-way interactions between temperature and time ($F_{1,9}$ = 0.96, P = 0.35) or temperature and pH ($F_{1,8}$ = 0.020, P = 0.89). However, a significant interaction between pH and time ($F_{1,9}$ = 6.4, P = 0.033) reflected that respiration rates decreased between weeks three and six for the ambient pH treatment but increased between weeks three and six for the lowered pH treatment (Figure 2A.4). There were no main effects of time ($F_{1,11}$ = 0.18, P = 0.68), pH ($F_{1,9}$ = 2.9, P = 0.12) or temperature ($F_{1,9}$ = 1.4, P = 0.28). In terms of *clearance rates*, there was no three-way interaction ($F_{1,8}$ = 4.9, P = 0.057) and no two-way interactions between temperature and pH ($F_{1,8}$ = 0.061, P = 0.81) or pH and time ($F_{1,9}$ = 3.1, P = 0.11). There was, however, a borderline non-significant

trend for an interaction between temperature and time ($F_{1,9}$ = 5.0, P = 0.053), illustrating that there was no difference in clearance rate between the two temperatures at week 3 but that by week 6 clearance rates were greater for cockles held at 18 ˚C compared to those held at 15 ˚C (Figure 2.2b). There were no main effects of time ($F_{1,11}$ = 3.9, P = 0.074) and pH ($F_{1,9}$ = 2.2, P = 0.17). Finally, a significant effect of temperature ($F_{1,9}$ = 6.8, P = 0.028) indicated that clearance rates increased with elevated temperature treatments (5.93 $L.h^{-1}$ ± 0.11) as compared to the ambient temperature treatments (5.39 $L.h^{-1}$ ± 0.19, Figure 2A.5). No three-way interaction between temperature, pH and time ($F_{1,8}$ = 1.09, P = 0.33), nor two-way interactions between temperature and pH ($F_{1,8}$ = 0.016, P = 0.90), temperature and time ($F_{1,9}$ = 0.0074, P = 0.93) or pH and time ($F_{1,9}$ = 0.22, P = 0.65) were found on *calcification rates* (Figure 2.2c). There was no main effect of temperature ($F_{1,9}$ = 0.97, P = 0.35) on calcification rate but a significant effect of pH ($F_{1,9}$ = 5.9, P = 0.038) demonstrated a higher calcification rate under ambient pH as compared to low pH conditions (120.4 % higher, Figure 2A.6a). Furthermore, there was a significant effect of time ($F_{1,11}$ = 5.1, P = 0.045) indicating a lower calcification rate at week 3 compared to week 6 (98.8% lower, Figure 2A.6b).

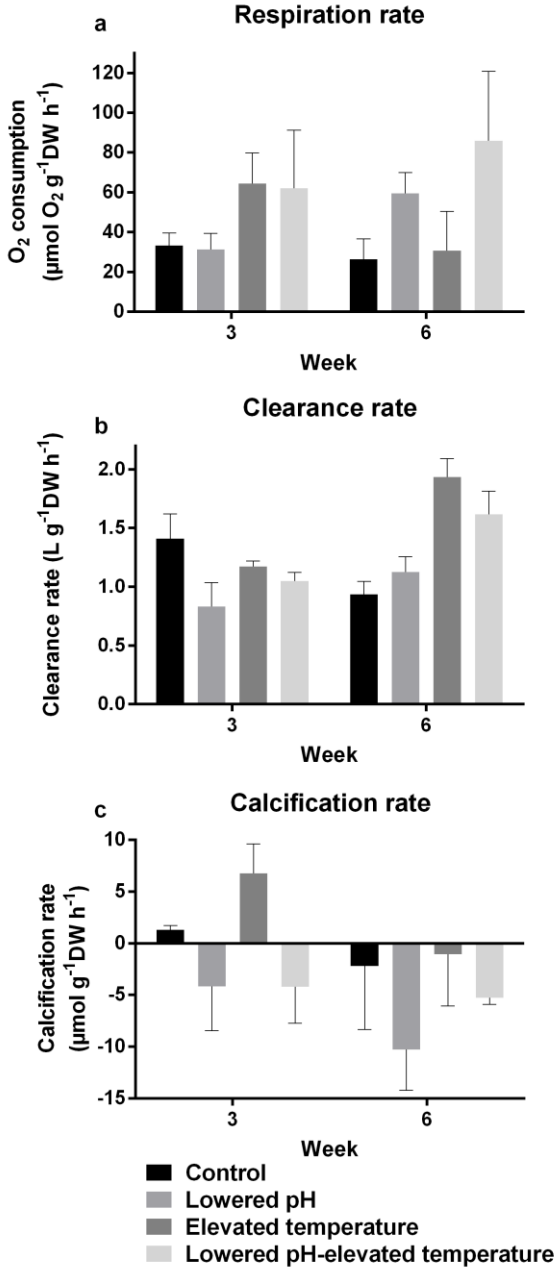

Figure 2.2 Physiological responses measurement of cockles (respiration, clearance and calcification rates) at week 3 and week 6 incubated in four treatments. (Error bars represent standard errors (±SE), n = 3 replicates per treatment).

2.5 Discussion

According to Pörtner and Farrell (2008) and Pörtner (2012), OA may narrow the thermal window of aquatic animals, probably through a reduction in functional capacity of tissue caused by the accumulation of CO_2. Our study, which mimicked the conditions projected for a future high pCO_2 ocean, i.e. lowered pH and elevated temperature, showed no effect of both applied stressors, alone or in combination, on the survival of cockles. Schade et al. (2016) demonstrated equally low mortality rates of the same species collected from the Baltic Sea in seawater with a pH of 7.4 to 7.8 (the latter being the ambient pH for the studied population). The lack of a mortality effect under increased temperature and lowered pH might be due to temperatures used which are well within the thermal window of the studied species (4 – 38 °C, (Compton et al., 2007; Rygg, 1970)). Furthermore, the 6 week duration of our experiment might have been too short to demonstrate mortality and could therefore rather represent a moderate level of environmental stress (Sokolova et al., 2012).

Bivalve condition indices represent a good reflection of the energy available for growth of bivalves (Lucas and Beninger, 1985; Nilin et al., 2012). Consequently, such indices give a realistic indication of bivalve fitness at longer time scales than can usually be studied under laboratory conditions and are therefore used to assess the condition of bivalves under variable

environmental conditions (e.g. Dove and Sammut, 2007; Nilin et al., 2012; Norkko et al., 2005). We found a temperature and time effect on the CI of cockles with lower CI at the high temperature treatments after six weeks of incubation. The temperature effect corroborates Hiebenthal et al. (2013) who reported that CI of *M. edulis* and *Arctica islandica* decreased with increasing temperature. Furthermore, we also observed a synergistic effect of lowered pH and elevated temperature on cockle condition with a pronounced additive negative effect on CI related to low pH in the combined treatment. This finding illustrates that physiological responses to low pH (see below) aggravate the negative effect of ocean warming on cockle condition.

This study shows that the respiration rates of cockles were significantly higher under acidified conditions after 6 weeks of incubation, while such effect was absent after three weeks. We hypothesise that different compensatory mechanisms for energy homeostasis under low pH can explain this time-dependent effect. At the short term cockles may have compensated the enhanced costs for basal maintenance through the allocation of energy from storage tissue (lipid and glycogen), whereas at the longer term (e.g. after 6 weeks), when the storage material has been depleted, increased respiration was required to maintain homeostasis (Sokolova et al., 2012) and support the need for a higher expression in biomineralisation-related enzymes and

acid-base regulation (Beniash et al., 2010). Similar upregulation of the aerobic respiration after two months of incubation at a pH 7.70 was reported by Thomsen and Melzner (2010) for blue mussels (*M. edulis*) and after an 11-weeks of incubation at pH 7.5 by Beniash et al., (2010) for *C. virginica*. Furthermore, increased oxygen uptake with decreasing pH is found in other marine taxa as well, e.g. ophiuroid brittlestar (*Amphiura filiformis*) and arctic pteropods (*Limacina helicina* (Comeau et al., 2010; Wood et al., 2008)). Opposite effects have also been demonstrated, e.g. for *M. chilensis* (Navarro et al., 2013) and juvenile and adult Mediterranean mussels (*M. galloprovincialis*) (Michaelidis et al., 2005), but Gazeau et al. (2014) and Fernández-Reiriz et al. (2012) found no effect of pH on respiration in the latter species. Furthermore, the respiration rates of cockles at the control pH at week 3 were significantly higher than at week 6 (see Figure 2A.4), which might be due to a slight increase in pH over time. Alternatively, this decrease in respiratory activity may have resulted from ageing of, or more generally from bottling effects on, the experimental animals. However, this explanation is contradicted by the condition index, which remained constant throughout the experiment.

Since no pseudofaeces production was observed during the measurements, the rate of food ingestion equals the rate of clearance multiplied by the cell concentration of the diet (Iglesias

et al., 1992; Lu and Blake, 1997). In this study, clearance rates were predominantly affected by temperature, with higher clearance rates in the elevated temperature treatments. Similarly, Smaal et al. (1997) found a positive relation between cockle clearance rates and elevated temperature in a field setting, which indicates that the cockles incubated under laboratory conditions in this study reacted similarly to those from the field. Walne (1972) observed that clearance rates of *Ostrea edulis* scaled down by 45%, and of *C. gigas* and *M. edulis* by 25% when temperature was lowered from 20 to 10 ˚C. In general, clearance rates in our study were lower under low pH conditions but this effect was not statistically significant ($p = 0.17$). However, both short and long-term observations on adult mussels (*M. chilensis*), juvenile clams (*R. decussatus*), adult noble scallops (*Chlamys nobilis*) and adult green-lipped mussels (*Perna viridis*) have demonstrated negative clearance rate responses to low pH (Fernández-Reiriz et al., 2011; Liu and He, 2012; Navarro et al., 2013). In general, marine bivalves thus seem to reduce costs related to filtration in low-pH waters rather than to ingest more food to cope with hypercapnia.

Shell dissolution rates of bivalves are often associated with the properties of a protective external organic layer or periostracum that separates the shell from the ambient seawater (Ries et al., 2009). As cockles have a thin periostracum of around 2 μm (Hall-

Spencer et al., 2008) and their shell is solely composed of aragonite - the most soluble polymorph of carbonate (Cubillas et al., 2005; Taylor, 1973), cockles may be particularly vulnerable to OA. We observed a reduction in calcification rates at lowered pH despite the fact that the seawater remained oversaturated with respect to aragonite ($\Omega_{aragonite}$ = 3.7 and 3.6 in lowered pH and lowered pH-elevated temperature treatments, respectively). Similarly, e.g. Beniash et al. (2010) and Ries et al. (2009) found a reduction of calcification of juvenile and adult oysters (*C. virginica*) in low-pH seawater that remained oversaturated with respect to calcite and aragonite. Clearly, the reduction of calcification or dissolution of bivalves does not solely depend on the calcite and aragonite saturation state. According to Cyronak et al. (2016), elevated proton concentrations (H^+), rather than the concentration of carbonate ions (CO_3^{2-}), is likely to be responsible for the reduction of calcification rates of marine calcifying organisms. Elevated [H^+] in acidic seawater alters the proton gradient between intracellular fluid and external seawater, thus hampering calcifying organisms from maintaining pH homeostasis (Cyronak et al., 2016). A recent study on the blue mussel *M. edulis* indicated that seawater [HCO_3^-]/[H^+] ratio is crucial in regulating calcification rates of the mussel (Thomsen et al., 2015). Our result shows that calcification rates were higher at week 3 (1st July 2015) than that of week 6 (28th July 2015 (Figure 2A.6b)). We hypothesise that this reduction in calcification over

the course of the experiment results from a shift in energy allocation. Cockles start to spawn at the site of collection around July (Rueda et al., 2005) and spawning cockles were observed in the last week of the experiment. It therefore seems likely that cockles have allocated relatively more energy towards gonad production than to calcification by the end of the 6-week incubation.

In summary, our results indicate synergistic effects of lowered pH and elevated temperature on the condition index of the common cockle *Cerastoderma edule*. Since synergistic effects were not found for the separate physiological responses addressed in this study, the interactive effect of lowered pH and elevated temperature on cockle condition must be explained by the cumulative impact of different responses. We hypothesise in *C. edule* that the reduced food intake under low pH conditions is insufficient to support the higher energy requirements in future high pCO_2 oceans to compensate for (1) higher basal maintenance in warmer and more acidic waters, and (2) growth in low-pH waters. Furthermore, cockles may allocate additional energy from energy storage pools to cover the increasing maintenance demand (e.g. tissue repair and maintenance) but this mechanism will leave less energy available for reproduction and growth, which will in turn likely have repercussions on populations of cockles and their roles in ecosystem functioning,

e.g. their influence on recruitment of other benthic species (Flach, 1996; Van Colen et al., 2013), the mediation of benthic primary production (Swanberg, 1991), and as a food source for higher trophic levels (Beukema and Cadée, 1996). In order to disentangle the different mechanisms that will determine future population stability of bivalves, and of benthic invertebrates in general, future research needs to address the different components that govern energy allocation under multiple, combined stressor scenarios.

2.6 Acknowledgments

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2.7 Appendices

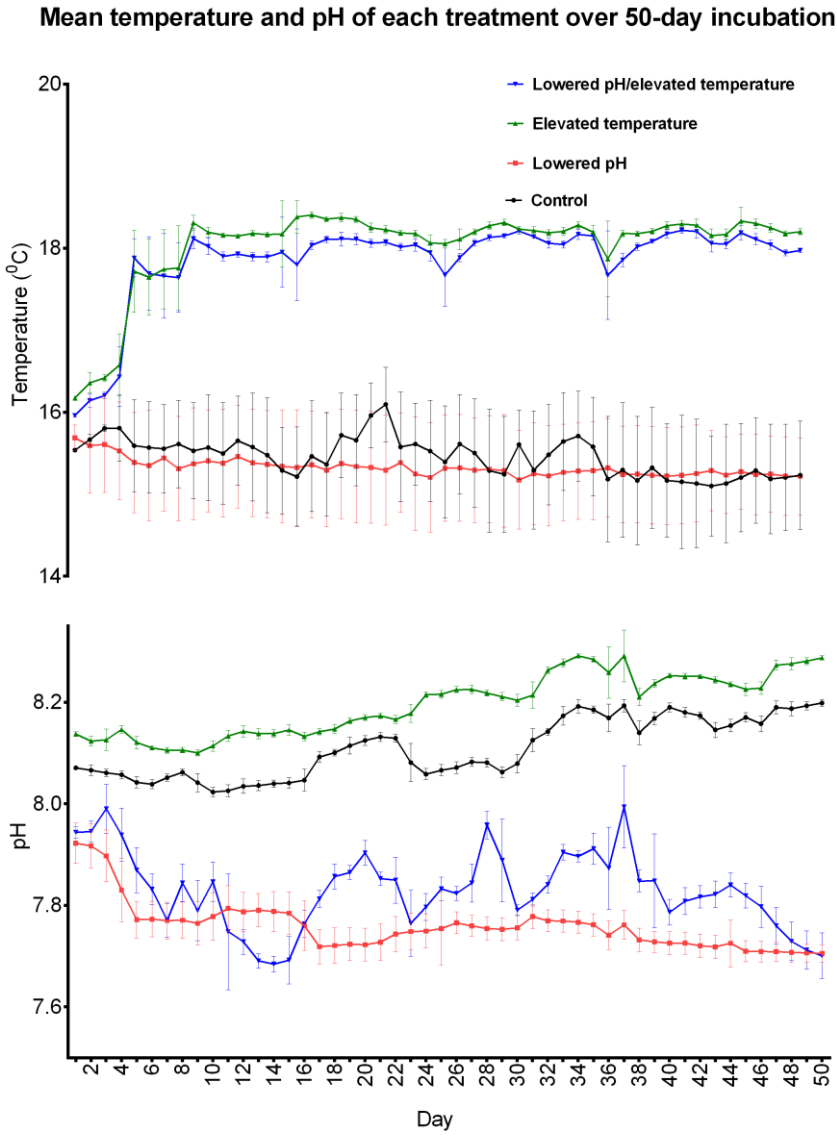

Figure 2A.1 Variations of daily temperature and pH in four treatments for 50-day incubation. The experiment started at 6th day of the incubation. Two conditional and physiological responses measurements were conducted at day-26 and day-47. Error bars represent standard deviation (±SD).

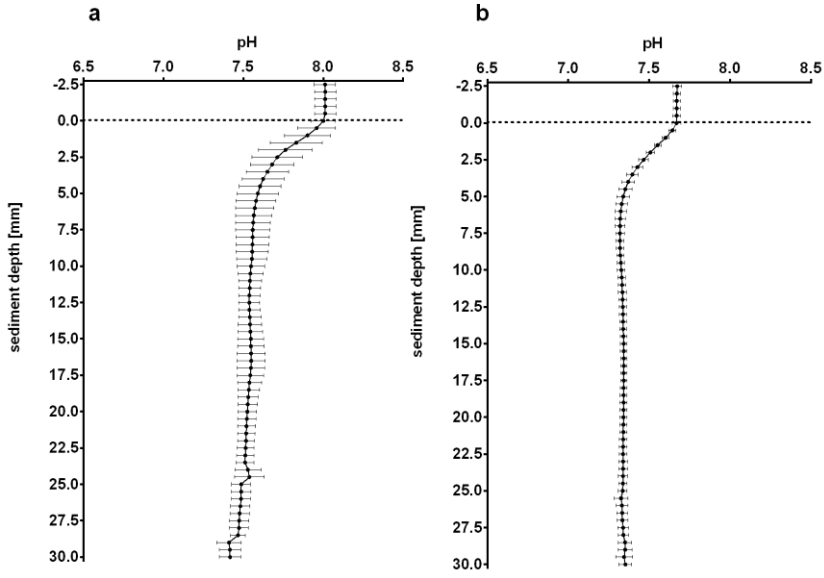

Figure 2A.2 pH sediment profile of muddy sand sediment at ambient pH (a) and lowered pH (b). Error bars represent standard deviation (±SD).

Table 2A.1 Numbers of cockle surviving in each aquarium for the four treatments. After week 3 and 6, a number of cockles (column 'measurement') was taken out from each aquarium for conditional and physiological measurements. Remaining cockles represent the number of cockles left in aquaria after those measurements.

		Week									
Treatment	Replicate	1	2	3	3 measurement	remaining cockles	4	5	6	6 measurement	remaining cockles
Control	1	43	43	43	14	29	29	29	29	18	11
Control	2	44	42	42	13	29	29	29	29	14	15
Control	3	44	44	44	13	31	31	31	31	17	14
Lowered pH	1	44	44	44	12	32	31	31	31	15	16
Lowered pH	2	44	44	44	13	31	31	31	31	17	14
Lowered pH	3	44	44	44	13	31	31	31	31	13	18
Elevated temperature	1	44	44	43	13	30	30	30	30	16	14
Elevated temperature	2	44	44	43	13	30	29	28	28	16	12
Elevated temperature	3	44	43	43	14	29	28	28	28	18	10
Combined	1	44	44	43	14	29	29	28	28	23	5
Combined	2	44	44	44	14	30	30	30	30	18	12
Combined	3	44	44	44	13	31	31	31	31	16	15

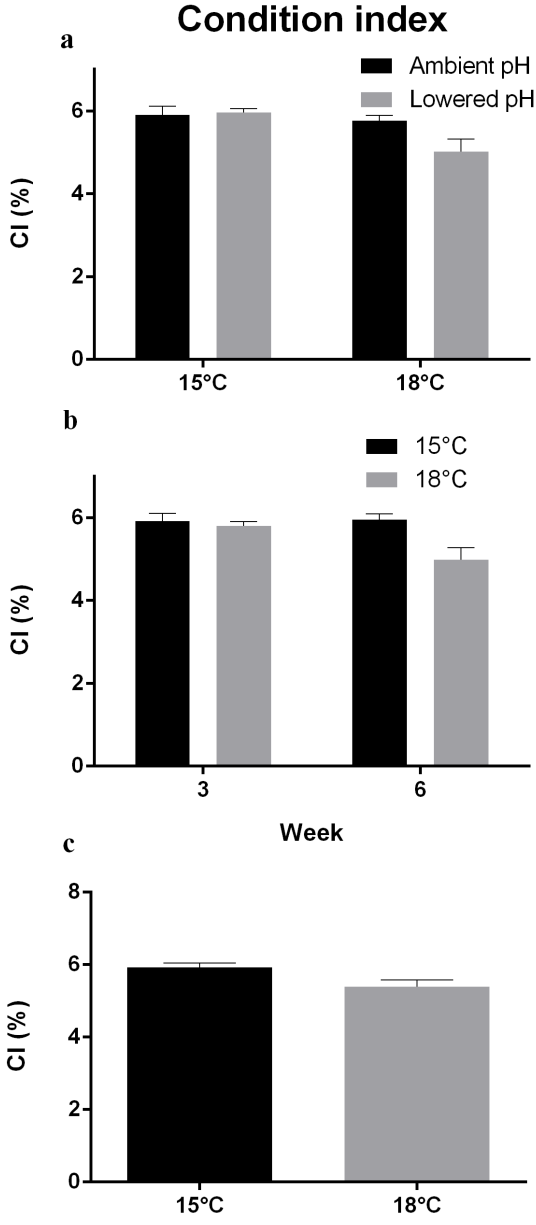

Figure 2A.3 Condition indices of cockles. a) The interaction effect between temperature and pH ($F_{1,8}$ = 7.5, P = 0.025), b) the interaction effect between temperature and time ($F_{1,9}$ = 8.2, P = 0.020) and c) the main effect of temperature for the pooled pH and time values show that CI was higher at 18 °C than 15 °C ($F_{1,9}$ = 7.3, P = 0.025). Error bars show standard errors (±SE).

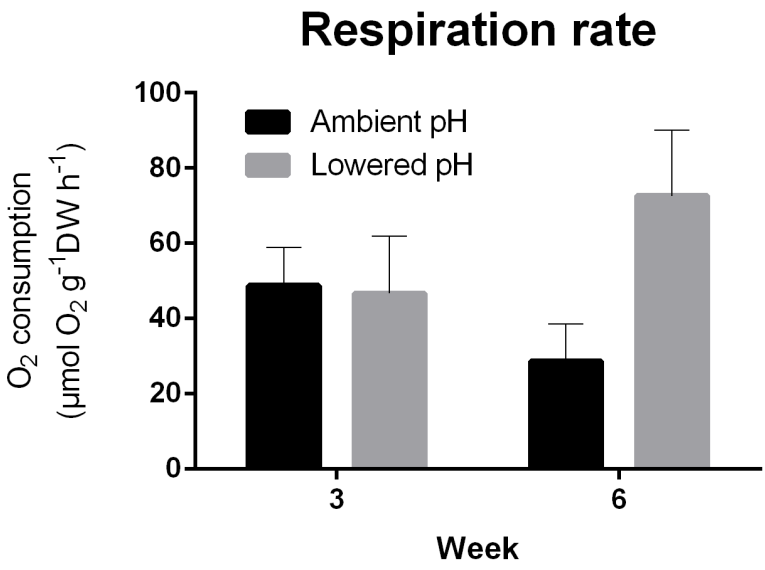

Figure 2A.4 The interaction effect between time and pH ($F_{1,9}$ = 6.4, P = 0.033). Analysis was conducted on Log_{10} transformed data but raw data are plotted here. Error bars show standard errors (±SE).

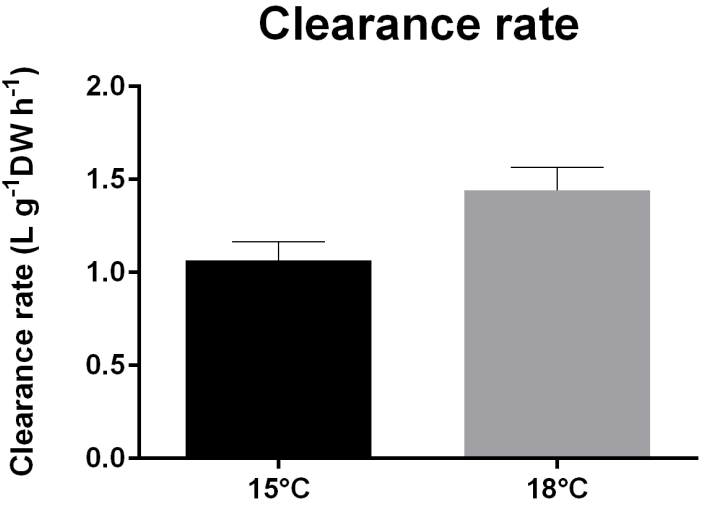

Figure 2A.5 The main effect of temperature for the pooled pH and time values shows that clearance rate was higher at 18 °C than 15 °C ($F_{1,9}$ = 6.8, P = 0.028). Analysis was conducted on Log_{10} transformed data but raw data are plotted here. Error bars show standard errors (±SE).

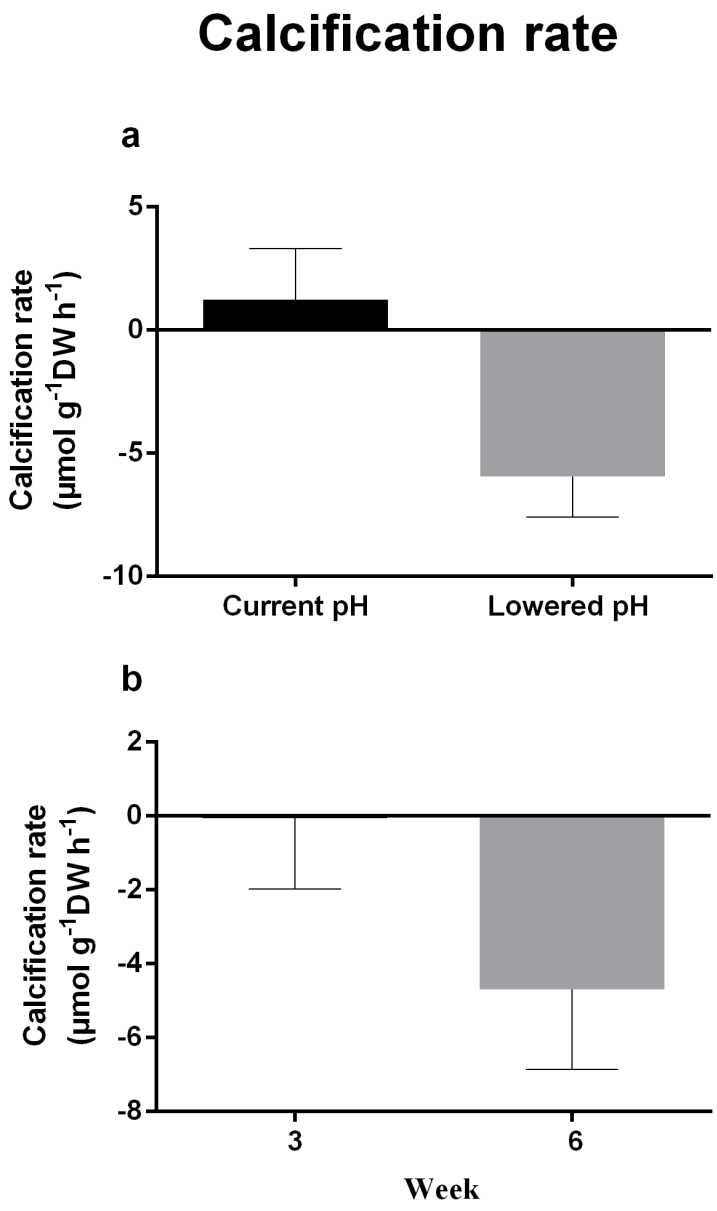

Figure 2A.6 Calcification of cockles. a) The main effect between pH for pooled temperature and time values shows that calcification rate was higher at ambient pH than at lowered pH ($F_{1,9}$ = 5.9, P = 0.038) and b) the main effect of time for pooled pH and temperature values shows the calcification rate was higher at ambient pH than at lowered pH ($F_{1,11}$ = 5.1, P = 0.045). Error bars show standard errors (±SE).

Chapter 3

Behavioural responses of *Scrobicularia plana* to ocean acidification and warming

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3.1 Abstract

Presently, behavioural responses of marine calcifying organisms to combined effects of ocean acidification and warming receive very little attention from the scientific community. Here, we investigate the combined effects of lowered pH (-0.6 units) and elevated temperature (+3 °C) on the behaviour of the infaunal bivalve *Scrobicularia plana* in a fully crossed design. Seven behaviours were identified (namely burrowing, deposit- and suspension-feeding, short positive pressurisation, valve claps, defaecation and siphon relocation) using porewater pressure sensor technology and time-lapse footage. Survival and condition were not affected by lowered pH and elevated temperature after 4 weeks of incubation; however, the feeding behaviour was altered. Suspension feeding was the dominant feeding mode before the manipulation of seawater pH and temperature. After 3 weeks of seawater manipulation, the total time spent on suspension feeding was significantly reduced under low pH treatments (pH_T 7.4), while that of deposit feeding was significantly increased under warm conditions (13 °C). In the combined low pH/high temperature treatment, *S. plana* fed almost exclusively via deposit feeding. We suggest that the switch in feeding mode is a mechanism to maintain total fresh food intake while limiting the intake of acidified water to prevent

lethal and conditional effects under energy demanding warm hypercapnic conditions.

3.2 Introduction

Excessive carbon dioxide emissions generated by human activities are altering ocean temperature, pH and carbonate chemistry at a global scale (Collins et al., 2013; Doney et al., 2012; Orr et al., 2005). The average global ocean surface temperature is estimated to increase by up to 3.2 °C by the end of the 21st century (Collins et al., 2013). Furthermore, IPCC has predicted a reduction in seawater surface pH for the open ocean of 0.4 units by the end of the century, leading to a process called ocean acidification (Caldeira and Wickett, 2003; Gattuso et al., 2015). Currently, decreases in seawater pH are occurring at an unprecedented rate, particularly in shallow coastal seas (Provoost et al., 2010; Wootton et al., 2008). The gradual alteration of seawater pH, temperature and carbonate chemistry may challenge marine benthic individuals, populations and communities, as demonstrated by changes in behaviour, physiology, body condition, trophic interactions, shifts in species composition, ecosystem functioning and service delivery (Byrne and Przeslawski, 2013; Dupont and Pörtner, 2013; Fabry et al., 2008; Harley et al., 2006; Nagelkerken and Munday, 2016; Orr et al., 2005; Pörtner et al., 2004; Wittmann and Pörtner, 2013).

Studies estimate that organisms allocate less energy to activity and processes such as growth, reproduction and development when exposed to stressors because more energy is allocated to

basal maintenance, i.e. acid-base regulation, ion transport and damage repair mechanisms (Sokolova et al., 2012; Thomsen and Melzner, 2010; Wood et al., 2008). Furthermore, theory also suggests that acidification narrows the thermal windows of organisms (Pörtner and Farrell, 2008; Widdicombe and Spicer, 2008), particularly in calcifying marine organisms that deposit calcium carbonate ($CaCO_3$) to form their shells and outer skeletons (e.g. Gazeau et al., 2014; Ries et al., 2009). The short-term exposure to reduced levels of pH and elevated temperature rarely results in mortality of adult marine benthic organisms. The reason might be that there are different biological mechanisms such as behavioural change that help organisms to mitigate the stressful environmental conditions (Huey et al., 2002).

Behavioural change is typically the first indication of disruption of organisms under stress (Tuomainen and Candolin, 2011). Such behavioural alterations may impact the way macrofauna affects ecosystem functioning in marine soft-sediment habitats (McCartain et al., 2017; Townsend et al., 2014; Woodin et al., 2012). For example, the burrowing and feeding activities of benthic macrofauna can increase solute fluxes across the water-sediment interface via a process called bioadvection, resulting from pressurisation of the porewater (Wethey and Woodin, 2005; Woodin et al., 2010). This process may facilitate

microphytobenthos productivity (e.g. Chennu et al., 2015) and thus food supply to marine benthos, by means of a gardening effect (Hylleberg, 1975; Reise, 1983). Another example is the effect of sediment surface disturbance by deposit feeding macrofauna on mortality and/or post-settlement of juveniles (Dunn et al., 1999; Thrush et al., 1992, 1996). Hence, small disruptions of individual behaviour may subsequently impact marine benthic ecosystem biodiversity and functioning. To date, there are relatively few studies that have investigated ocean acidification effects on the behaviour of marine organisms (see e.g. Briffa et al., 2012; Clements and Hunt, 2014; Green et al., 2009; Schalkhausser et al., 2013). Moreover, there are only a limited number of studies that have investigated behavioural responses to the combined effects of ocean acidification and warming (Nagelkerken and Munday, 2016). One example is the change in escape performance of temperate king scallop *Pecten maximus* to their starfish predator under single warm and hypercapnic conditions, but the absence of such response under the combination of both stressors (Schalkhausser et al., 2014).

The peppery furrow shell *Scrobicularia plana* is a tellinid bivalve that is abundant in intertidal habitats along the Eastern Atlantic coast from Senegal to Norway and in the Mediterranean and the Baltic Sea (Santos et al., 2011; Tebble, 1976). This species occurs across a wide range in temperatures associated with seasonal

fluctuations in its distribution range, e.g. 6 to 15.5 °C in North Wales (Hughes, 1971), 10 to 27 °C in Mediterranean (Northern Tunisia, Casagranda and Boudouresque, 2005) and 13 to 28 °C in Northwest Morocco coast (Bazaïri et al., 2003). Apart from being an important dietary component in the food web, the species is increasingly harvested by fishermen in France and Portugal (Langston et al., 2007). *S. plana* has an infaunal life habit and has a fairly broad tolerance to chemical exposure, and is therefore frequently used as a bio-indicator to monitor environmental pollution such as heavy metal contamination (e.g. Bryan and Langston, 1992; Coelho et al., 2008; Dauvin, 2008; Solé et al., 2009). *S. plana* lives up to 20 cm deep in cohesive fine sediments, and it depends on its inhalant siphon for access to the sediment surface and water column for feeding and respiration (Zwarts et al., 1994). It feeds on phytoplankton and benthic diatoms through suspension feeding and deposit feeding, respectively (Hughes, 1969). This flexible feeding mode is an advantageous trait to survive in dynamic environments such as tidal flats. For example, the Baltic tellin *Limecola balthica* switches from deposit feeding at low tide emersion to suspension feeding at high tide submersion (Brafield and Newell, 1961), which may be a strategy to escape from siphon nipping by demersal fish and epibenthic crustaceans during submersion (e.g. Sandberg et al., 1996).

In this study, the results of a microcosm experiment are presented where we incubated individuals of *S. plana* under two levels of seawater pH (7.4 or 8.0) and temperature (10 or 13 °C) in a fully crossed design. We hypothesised that under future high pCO_2 ocean conditions (i.e. lowered pH and elevated temperature), *S. plana* would alter its behaviour which may have consequences for ecosystem functioning.

3.3 Material and methods

3.3.1 Collection and incubation of Scrobicularia plana

Adult individuals and fine sand sediments were collected on March 5th 2017 during low tide at the Paulina tidal flat, Schelde estuary, The Netherlands (51° 20′ 55.4′′ N, 3° 43′ 20.4′′ E) and transported within 2 h to the research facility. *S. plana* (average shell length of 29.6 ± 0.45 mm (SE)) were allocated into four incubation tank systems (95 cm × 65 cm × 40 cm). In each tank, there were one rectangular box (40 cm × 30 cm × 18 cm) in which 5 "reserved" *S. plana* were incubated prior to behavioural measurements and 10 microcosms/cylindrical jars (Ø = 12 cm and height = 20 cm, 3 for sediment porewater pH profiling and 7 for *S. plana* behaviour recordings; see Figure 3A.1; for sample size per treatment see 3.3.4). Both were filled with pre-sieved (1 mm

mesh) sediments[4], to a height of 15 cm (median grain size: 208.8 ± 2.5 μm (SE, n = 24)). The set-up was allowed to acclimatise in aerated running seawater at 10 °C, pH_T 8.0 and salinity of 30 for one week. All pH values measured on the NBS scale (pH_{NBS}) were converted to total scale (pH_T) via conversion described in Zeebe and Wolf-Gladrow (2001), using the Matlab program CO2SYS (Van Heuven et al., 2011) taking into account the seawater temperature and salinity.

This experiment was set up according to a two-factor fully crossed design; temperature (ambient or elevated: 10 or 13 °C) and pH (ambient or lowered: 8.0 or 7.4). Each incubation tank was supplied with flowing aerated seawater (650 $L.h^{-1}$) that was pumped from a holding tank to the incubation tank and circulated back by gravity to the holding tank. In order to maintain the salinity and remove the build-up of nutrients, 20% of the total volume was removed and refilled with fresh seawater on a weekly basis. All incubation tanks were subjected to a 12:12 h light: dark regime. *S. plana* were fed twice a week with 0.5 mL of commercial Shellfish Diet 1800 (Reed Mariculture Inc., consisted of 40% *Isochrysis*, 15% *Pavlova*, 25% *Tetraselmis* and 20% *Thalassiosira weissflogii*) diluted in 4 L of fresh seawater and distributed evenly to each holding tank. We recognize that the

[4] Sediment was pre-sieved in order to avoid interference of other macrobenthos with the hydraulic pressure signal.

quality and quantity of food might be quite different from those in the field. Yet, we consciously chose Shellfish diet mixture (dead microalgae) to avoid bias in our experiment due to temperature and pH effects on food quality and quantity (Hinga, 2002; Thomas et al., 2012) which we would be unable to control for.

After one week of acclimatisation, the temperature of the seawater was elevated by 1 °C and the pH_T of the seawater was lowered by 0.1 units per day over 6 days, until a temperature of 13 °C and a pH_T value of 7.4 was achieved after which these conditions were maintained for 4 more weeks (see Table 3.1 and Figure 3A.2). The seawater pH of the lowered pH treatments was manipulated and controlled via the bubbling of CO_2 using an IKS Aquastar Industrial control panel coupled with pH glass electrodes (compression-proof). All pH glass electrodes were 2-point calibrated weekly with IKS GmbH NBS buffer solutions (pH 4.01 and 7.01). The seawater temperature was regulated (Teco Refrigeration technologies; Model: TK200H) and recorded together with the pH_{NBS} every 10 min by the IKS Aquastar Industrial control panel.

On a weekly basis, temperature and pH were measured using a pH meter (HANNA Instruments, model: HI991001). Prior to measurement, the pH meter was calibrated with HANNA Instruments NBS buffer solutions (pH 4.01 and 7.01).

Furthermore, salinity was measured using a WTW portable conductivity meter (model: LF320). In addition, 200 mL of seawater was collected from the incubation tanks and filtered through GF/C filter papers for the quantification of total alkalinity (TA). These samples were stored at 4 °C prior to subsequent titration using a HydroFIA titrator (TA CONTROS Systems & Solutions GmbH), calibrated with CO_2 in Seawater Reference Material (CRM, batch 144). The TA measurements have accuracy (deviation from CRM) and precision (the standard deviation within measurements of the same batch) of 2.0 and 1.6 $\mu mol.kg^{-1}$ (n=8), respectively. The obtained values for temperature, pH, salinity and TA were used to determine other carbonate chemistry parameters (partial pressure of carbon dioxide (pCO_2), concentration of carbonate and bicarbonate ions (CO_3^{2-} and HCO_3^-) and saturation state with respect to aragonite (Ωa) and calcite (Ωc); Table 3.1) using CO2SYS software (Pierrot et al., 2006) where the thermodynamic constants of Mehrbach et al. (1973) were applied.

Table 3.1 The average carbonate chemistry of seawater in four treatments during the 4-week manipulated incubation: temperature, pH_T, salinity, total alkalinity (TA), partial pressure of carbon dioxide (pCO_2), concentration of bicarbonate and carbonate ions (HCO_3^- and CO_3^{2-}) and saturation state with respect to aragonite and calcite (Ωa and Ωc). The ± values represent standard deviation of 4 replicates. The seawater carbonate chemistry parameters were significantly differences across treatments and post-hoc tests indicate that there were significant differences between treatments, however, not within similar pH and temperature levels (see 3.3.4).

Treatment	Control	Lowered pH	Elevated temperature	Lowered pH-elevated temperature
Temperature (°C)	10.0 ± 0.30	10.3 ± 0.17	13.0 ± 0.32	13.0 ± 0.12
pH_T	8.03 ± 0.04	7.43 ± 0.01	7.98 ± 0.04	7.46 ± 0.04
Salinity	30.2 ± 0.03	30.3 ± 0.08	30.5 ± 0.15	30.6 ± 0.23
TA (µmol kg^{-1})	2587 ± 3.0	2590 ± 3.5	2590 ± 4.1	2591 ± 3.8
pCO_2 (µatm)	501 ± 52	2141 ± 73	574 ± 63	2020 ± 171
HCO_3^- (µmol kg^{-1})	2268 ± 22	2500 ± 4.8	2265 ± 30	2482 ± 12
CO_3^{2-} (µmol kg^{-1})	133 ± 10	37 ± 1.1	136 ± 13	45 ± 3.7
Ωa	2.0 ± 0.16	0.6 ± 0.02	2.1 ± 0.20	0.7 ± 0.06
Ωc	3.3 ± 0.25	0.9 ± 0.03	3.3 ± 0.32	1.1 ± 0.09

3.3.2 Hydraulic activity measurements and behavioural identification

Porewater pressure sensors were used to measure the hydraulic activity of *S. plana*. The pressure sensor (Honeywell type 26PC) was built into a plastic pipe (Ø = 1.2 cm, height = 11.5 cm) with an open top and a sealed bottom. The pressure port was positioned close to the outer wall of the plastic pipe and covered with 64 µm nylon mesh, whereas the reference port was positioned in a seawater plenum on the inside of the pipe. (Wethey et al., 2008; Wethey and Woodin, 2005). Prior to insertion of porewater pressure sensors in the sieved sediment,

sensors were calibrated in a fixed rack inside a 30-cm high water tank, which was gradually filled with different water levels from the tip until above the diaphragm of the sensors with ambient seawater. Calibration was conducted to determine the relationship between sensor output (mV) and hydraulic head.

In order to identify the sub-surface behaviours, a pilot experiment was conducted to document *S. plana* activity at the sediment surface using a GoPro (HERO 3+) to document time-lapsed images with intervals of 10 seconds. By synchronising both surface and hydraulic behaviours, we distinguished 7 *S. plana* behaviours: burrowing, deposit- and suspension-feeding, short strong positive pressurisation (most likely related to 'boost' respiration and/or feeding, e.g. *Macomona liliana* (Woodin et al., 2012)), valve claps (pseudofaeces production and coughing), defaecation and siphon relocation (see Table 3.2, Figure 3.1 and Figure 3.2).

At the start of week 1, one *S. plana* was placed on the sediment surface of each of 7 microcosms per treatment. Porewater pressure signal data generated from *S. plana* hydraulic activities were recorded using a data logger (National Instruments) with SignalExpress 2014 software at a rate of 200Hz. The hydraulic activities were recorded during light conditions for 27 h in week 1 (i.e. prior to pH and temperature manipulation) and for 26.5 h in week 5 (i.e. third week of stable pH and temperature settings).

The pressure data was smoothed by using a running median per hour in order to reduce noise (McCartain et al., 2017; Wethey and Woodin, 2005).

Table 3.2 Seven behaviours of *Scrobicularia plana* observed details from time-lapsed images and/or porewater pressure signals.

	Hydraulic activity	Surface behaviour
Burrowing	Concentrated up and down signals. Only occurs once after addition to the sediment surface	Shell crawling and moving into the subsurface with help of its foot
Deposit feeding	A period (~ 3 min) of positive pressurisation with small positive and negative peaks	Inhalant siphon stretching out and dragging along the top 3 - 5 mm sediment surface forming star-shaped marking and often followed by pseudofaeces production
Suspension feeding	A period (~ 7 min) of positive pressurisation without small positive and negative peaks. It happens more frequently during the feeding day	Inhalant siphon located near the opening hole with a little up/down movement or siphon stretching out into water column without contact with the sediment
Valve claps	High to medium magnitude negative spikes	Small sediment deposition on sediment surface during pseudo-faeces production or sediment plume formation in the water column (i.e. couching)
Positive pressurisation	Strong positive peak	Siphon stretching out into water column followed by contraction of the distal tip during a short period
Defaecation	A period of concentrated positive and negative signals (relatively low magnitude in comparison to burrowing)	Exhalant siphon repulses faeces on the surface layer of the sediment. Although exhalant siphon is not visible, a hole opening can be observed
Siphon relocation	Not analysed in pressure records	Siphon opens up a new hole

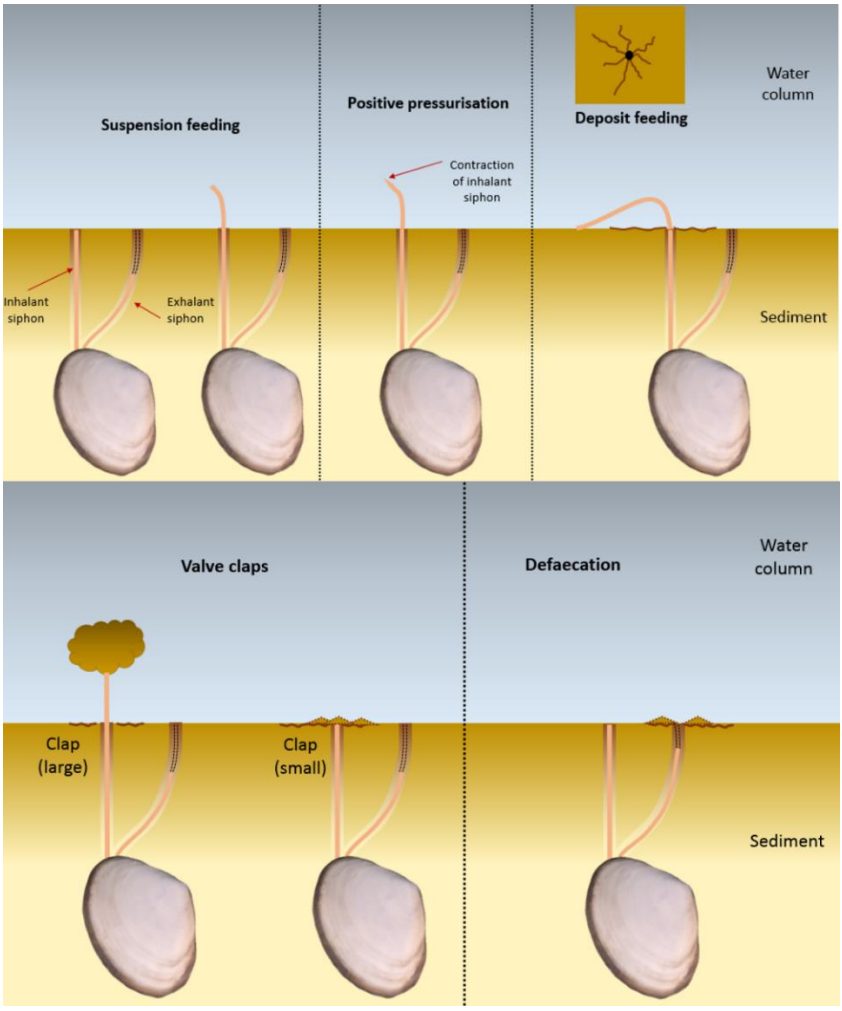

Figure 3.1 Illustrations of five behaviours of *Scrobicularia plana* documented in the experiment.

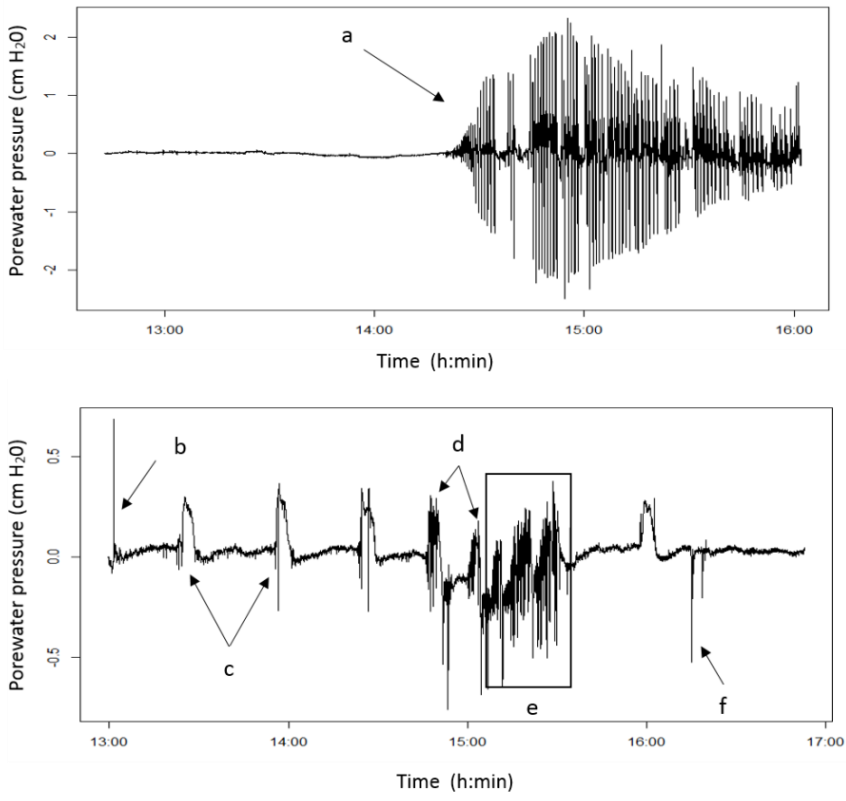

Figure 3.2 Porewater records show different hydraulic characteristics of *Scrobicularia plana* that represent specific behaviours: (a) burrowing, (b) positive pressurisation, (c) suspension feeding, (d) deposit feeding, (e) defaecation and (f) valve claps.

3.3.3 Scrobicularia plana condition index and abiotic sediment properties

At the end of the experiment (i.e. after 4 weeks of incubation), the survival and condition index of *S. plana* were determined, vertical sediment porewater pH profiles were measured, and samples for total organic matter (TOM) and pigments were collected. Survival was derived from the number of alive *S. plana*

in the rectangular box and 7 microcosms of each treatment (see section 3.3.1). The condition index (CI) was calculated by dividing the dry tissue weight (60 °C for 2 days) by dry shell weight and multiplying by 100 (Lucas and Beninger 1985). Vertical profiles of porewater pH were determined in 3 microcosms of each treatment at 3 mm sampling intervals using an Unisense pH microsensor (tip size 500 μm) with the set-up described in (Braeckman et al., 2014b). Plexiglas cores (Ø = 3.6 cm) were used to sample the top centimetre of sediment from each microcosm (with (n=7) and without (n=3) the presence of *S. plana*) to determine total organic matter (TOM) and pigments (phaeophorbide a, phaeophytin and chlorophyll *a*). TOM was calculated as the mass loss observed on the dried sample (48 h at 60 °C) after combusting at 450 °C for 2 h. Pigments were analysed using HPLC (Agilent) analysis according to Wright and Jeffrey (1997) after extraction in methanol at 4 °C overnight. Phaeopigments were derived from the sum of phaeophorbide a and phaeophytin. The microalgal freshness was calculated as the ratio of chlorophyll *a* to its degradation products (phaeopigments), while total pigments was the sum of chlorophyll *a* and its degradation product (Thiel, 1978).

3.3.4 Statistical analysis

A 2 × 2 contingency analysis was used to determine the difference in survival between the 4 treatments. One-way

analysis of variance (ANOVA) or Kruskal-Wallis tests (for non-parametric data) were used to compare seawater carbonate chemistry between treatments and behavioural differences between groups prior to manipulation, while two-way ANOVA was applied to test the effects of pH (ambient, lowered), temperature (ambient, elevated) and their interaction on behaviour (n = 4) in week 5, condition index (n = 12) and sediment food content (TOM, pigments; n = 7) in week 6. There were only 4 replicates for behavioural measurement because only 4 microcosms from each treatment had good pressure signals. We removed microcosms (replicates) from statistical analysis on food content when the *S. plana* did not survive the incubation.

The pH profiles of each treatment were compared based on a data matrix where each depth was treated as a "variable" and each pH value measured as a measure of abundance (Widdicombe et al., 2013). A two-way test for temperature, pH and their interactions was performed using the PERMANOVA + add-on package for the Primer 6 software (Anderson et al., 2008; Clarke and Gorley, 2006), based on the Euclidean distance similarity matrix which was constructed on untransformed data. Subsequently, the SIMPER analysis was conducted to determine in which depth layers any identified differences occurred.

The considered hydraulic behaviours were deposit, suspension and total feeding frequency (%) and duration (min) per event. Burrowing was not analysed because it occurred only once when individuals were placed on the sediments and thus not anymore after manipulation of seawater pH and temperature. Siphon relocation was only observed via time-lapse images and not analysed in the pressure recordings. Pseudofaeces production and deposit feeding were often tightly associated (see results) but the former was also very hard to distinguish from defaecation when both activities occurred close in time. Pseudofaeces production and defaecation were therefore not included in the analysis to avoid any misinterpretation. From the data collected, we calculated two feeding preference indices (%) by standardising number of deposit feeding events per total number of feeding events and by standardising total time of deposit feeding over total time of feeding (i.e. sum of deposit and suspension feeding). For statistical data analysis, non-normal data were Log_{10} transformed to improve normality, and homogeneity of variances was checked using Levene's test. When significant differences between factors were identified by the ANOVA, post-hoc tests (Tukey HSD pairwise tests) were performed. All analyses were determined using R-software version 3.4.1 (R Core Team, 2014).

3.4 Results

3.4.1 Behavioural observations

All *S. plana* burrowed into the sediment within the first 7 hours after their addition to the microcosm. Afterwards there was no additional burrowing. During deposit feeding, the inhalant siphon of *S. plana* extended onto the sediment surface in random directions, taking up deposits till a depth of ~ 3 mm. During suspension feeding, the inhalant siphon was mostly located near the burrow opening but sometimes extended into the water column. Prior to manipulation of seawater temperature and pH, deposit feeding events lasted approximately 3 min per event whereas suspension feeding events lasted longer (ca 7 min per event). Also prior to manipulation, suspension feeding was the dominant feeding mode both in number of feeding events (73.8 ± 4.8 (SE) %) and in time spent feeding (85.2 ± 3.0 %). Occasionally, the inhalant siphon was stretched out into the water column and contracted during a short period, presumably for respiration purposes as described in Woodin et al. (2012) for *Macomona liliana*. Pseudofaeces production was observed when the inhalant siphon produced small deposits at the sediment surface or sediment plumes in the water column, which could be recognised by their darker colour compared with the surrounding sediment. During defaecation, the exhalant siphon

ejected faeces onto the sediment surface. The production of pseudofaeces, plumes and faeces often co-occurred with deposit feeding. The linkage with hydraulic signatures is given in Table 3.1.

3.4.2 pH sediment profile

There was no interaction between temperature and pH on the sediment pH vertical profiles (Pseudo-F = 0.50, $p_{(perm)}$ = 0.69), however, there was a pH effect (Pseudo-F = 3.82, $p_{(perm)}$ = 0.03), indicating that lowered pH affected the shape of the sediment pH profiles (Figure 3.3). SIMPER showed that the observed differences between treatments of control (pH_T 8.0) and lowered pH (pH_T 7.4) mainly occurred in the upper 0.6 cm layers (25.8%).

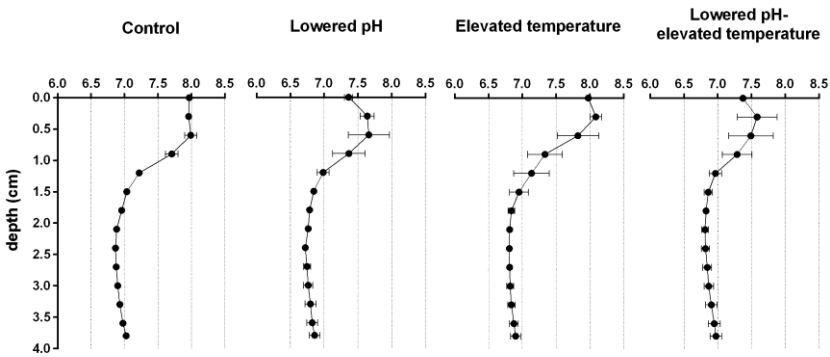

Figure 3.3 Sediment vertical pH_T profiles for 4 treatments: Control, Lowered pH, Elevated temperature, and Lowered pH-elevated temperature. X-axis is sediment depth in cm. Error bars represent standard errors (± SE) of 3 replicates.

3.4.3 Sediment organic matter and pigment concentration

There were no significant single or combined effects of pH and temperature on total organic matter (TOM), and pigment concentrations (Table 3.3, Table 3A.1). There was no two-way interaction and pH effects on microalgal freshness; however, there was a temperature effect reflecting that the freshness increased under elevated temperature conditions ($F_{1,19} = 5.49$, $P = 0.03$, Figure 3.4b).

Table 3.3 Average value (± SE) of sediment characteristics measured in four treatments at the end of experiment [total organic matter (TOM), pigments (chlorophyll *a*, phaeophorbide a, phaeophytin, phaeopigments and total pigments) and microalgal freshness].

Treatment	Control	Lowered pH	Elevated temperature	Lowered pH-elevated temperature
TOM (%)	1.14 ± 0.05	1.13 ± 0.04	1.20 ± 0.06	1.20 ± 0.14
Pigments (μg/g)				
Chlorophyll *a*	3.38 ± 0.40	2.69 ± 0.18	3.08 ± 0.85	2.74 ± 0.69
Phaeophorbide a	0.17 ± 0.03	0.15 ± 0.02	0.14 ± 0.03	0.15 ± 0.07
Phaeophytin	0.18 ± 0.02	0.15 ± 0.01	0.11 ± 0.01	0.12 ± 0.03
Phaeopigments	0.35 ± 0.05	0.30 ± 0.03	0.25 ± 0.04	0.27 ± 0.09
Total pigments	3.73 ± 0.45	2.99 ± 0.20	3.33 ± 0.89	3.01 ± 0.78
Freshness	9.84 ± 0.27	9.23 ± 0.65	11.49 ± 1.1	11.19 ± 0.99

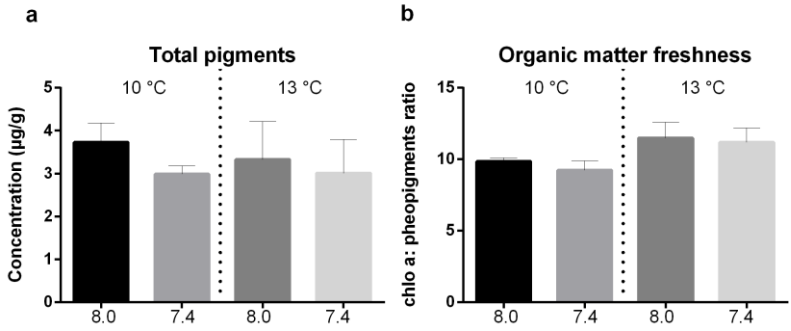

Figure 3.4 Effects of temperature and pH on total pigments and microalgal freshness in four treatments. Error bars represent standard errors (± SE).

3.4.4 Conditional and behavioural responses

There was no significant difference in survival between 10 and 13 ° C ($\chi^2_1 = 2.97$, P = 0.23) nor between pH_T 7.4 and 8.0 ($\chi^2_1 = 0.82$, P = 0.66). There were also no significant effects of temperature, pH or their interaction on the condition index (Table 3.4 and Figure 3.5e).

We found no significant differences for any of the documented behaviours between the four groups before manipulation of seawater conditions (Figure 3A.3, Figure 3A.4). However, there were significant effects of temperature or pH on some behaviours after manipulation. Prior to the manipulation of seawater temperature and pH, suspension feeding was the dominant feeding mode. No effects of temperature and pH were found on the total feeding event (sum of suspension and deposit feeding) and the total time spent feeding after 3 weeks of seawater manipulation (Figure 3.5a,b and Table 3.3). However,

elevated temperature significantly increased the frequency of deposit feeding (0.79 ± 0.19 (SE) events.h^{-1}) in comparison to the control temperature (0.24 ± 0.05 events.h^{-1}) ($F_{1,12}$ = 9.34, P = 0.01)(Figure 3.5b) but the duration per event did not change significantly between treatments (Figure 3.5c). There were no pH or temperature x pH interaction effects on frequency or duration per deposit feeding event. Similarly, there was no pH effect ($F_{1,12}$ = 0.36, P = 0.56) or temperature x pH interaction effect on the total time spent on deposit feeding ($F_{1,12}$ = 0.24, P = 0.64), but there was a significant temperature effect on total time spent on deposit feeding ($F_{1,12}$ = 5.31, P = 0.04)(Table 3.4 and Figure 3.5a), illustrating that *Scrobicularia* increased the time spent on deposit feeding at 13 °C. Low pH significantly reduced the frequency of suspension feeding (-0.27 - 0.006 events.h^{-1} as compared to control pH)($F_{1,12}$ = 6.27, P = 0.03, Figure 3.5b). Similarly, low pH (pH_T 7.4) significantly lowered total time spent on suspension feeding compared to ambient pH (pH_T 8.0)(60% lower, $F_{1,12}$ = 10.63, P = 0.01)(Figure 3.5a). There were no interactive ($F_{1,12}$ = 1.24, P = 0.29) and temperature ($F_{1,12}$ = 2.0, P = 0.18) effects on the duration of a suspension feeding event, but low pH did decrease the duration from 6.4 to 4.4 min per suspension feeding event at 10°C and from 5.7 to 1.4 min at 13 °C (52% lower, $F_{1,12}$ = 8.37, P = 0.01)(Figure 3.5d). The effects of elevated temperature and lowered pH on deposit and suspension feeding, respectively, resulted in a change in feeding preference. A significantly higher %

of deposit feeding events and total time spent on deposit feeding occurred at 13 °C ($F_{1,12}$ = 5.8, P = 0.03 and $F_{1,12}$ = 9.17, P = 0.01, respectively). Interestingly, *S. plana* fed almost exclusively via deposit feeding under the lowered pH and elevated temperature treatment.

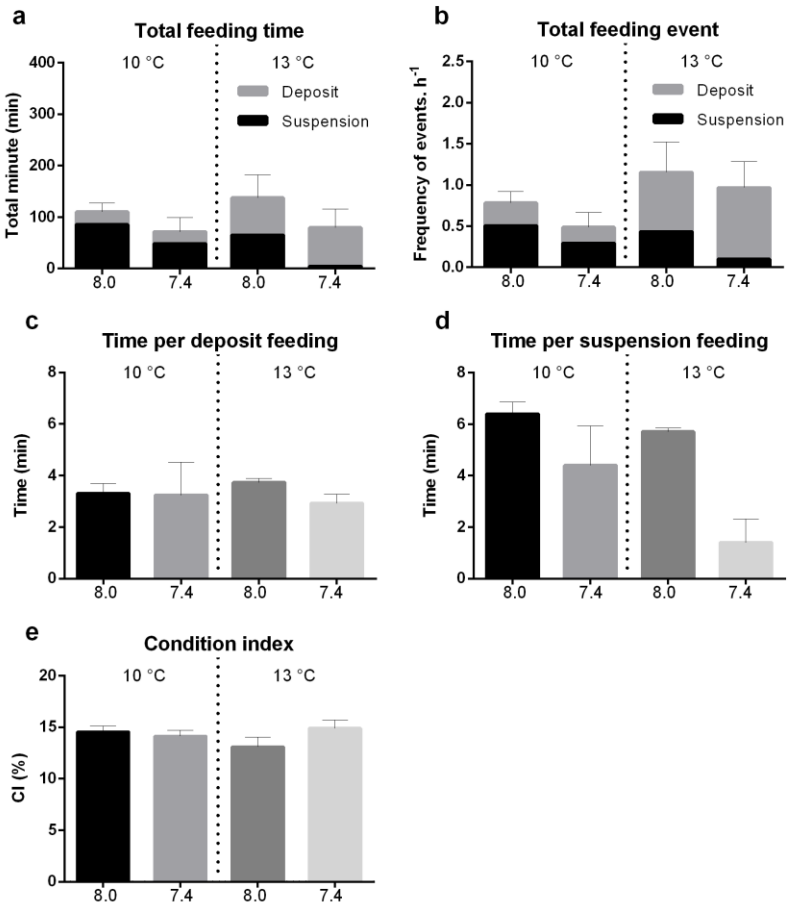

Figure 3.5 Effects of temperature and pH on behaviour events [e.g. (a) total feeding time includes deposit and suspension feeding, (b) total feeding events includes deposit and suspension feeding, (c) time per deposit event, (d) and suspension feeding event and (e) condition index] during treatment. Error bars represent standard errors (± SE).

Table 3.4 Results of two-way ANOVA testing the condition and behaviour parameters in *Scrobicularia plana* exposed to the four treatments. Significant results are in bold.

	Df	Mean squares	F value	Pr (>F)
Condition index				
Temperature	1	0.32	0.07	0.80
pH	1	2.71	0.56	0.46
Interaction	1	11.68	2.43	0.13
Residuals	35	4.81		
Deposit feeding				
Temperature	1	0.47	9.34	**0.01**
pH	1	0.0001	0.002	0.96
Interaction	1	0.02	0.43	0.52
Residuals	12	0.05		
Suspension feeding				
Temperature	1	0.05	1.64	0.22
pH	1	0.18	6.27	**0.03**
Interaction	1	0.008	0.29	0.60
Residuals	12	0.03		
Total time spent on deposit feeding				
Temperature	1	6.28	5.31	**0.04**
pH	1	0.44	0.37	0.55
Interaction	1	0.28	0.24	0.64
Residuals	12	1.18		
Total time spent on suspension feeding				
Temperature	1	5.63	3.22	0.10
pH	1	18.57	10.63	**0.01**
Interaction	1	2.15	1.23	0.29
Residuals	12	1.75		
Percentage of deposit feeding events				
Temperature	1	5.41	5.8	**0.03**
pH	1	0.14	0.15	0.70
Interaction	1	1.26	1.36	0.27
Residuals	12	0.93		
Percentage of deposit feeding time				
Temperature	1	8.34	9.17	**0.01**
pH	1	0.02	0.02	0.90
Interaction	1	1.13	1.24	0.29
Residuals	12	0.91		
Time per deposit feeding				
Temperature	1	0.01	0.006	0.94
pH	1	0.77	0.40	0.54
Interaction	1	0.54	0.28	0.61
Residuals	12	1.92		
Time per suspension feeding				
Temperature	1	0.78	2.00	0.18
pH	1	3.28	8.37	**0.01**
Interaction	1	0.49	1.24	0.29
Residuals	12	0.39		

	Df	Mean squares	F value	Pr (>F)
Temperature	1	0.72	2.56	0.14
pH	1	0.23	0.82	0.38
Interaction	1	0.01	0.04	0.84
Residuals	12	0.28		
Total feeding time				
Temperature	1	1234	0.29	0.60
pH	1	9365	2.19	0.17
Interaction	1	362	0.08	0.78
Residuals	12	4285.3		

3.5 Discussion

The hydraulic behaviour observations show that *Scrobicularia plana* did not manoeuvre in the sediment after burrowing. This is in accordance with the original observations by Hughes (1969) describing that horizontal migrations of this species are uncommon. This observation supports that *S. plana* may have a more flexible feeding strategy in comparison to other bivalves such as *Limecola balthica*, *Tellina tenuis*, and *Macomona liliana*, all of which rely more on surface deposit feeding and often migrate horizontally to new areas in search of fresh food resources (Brafield and Newell, 1961; McCartain et al., 2017; Townsend et al., 2014; Yonge, 1949). Moreover, *S. plana* fed predominantly via suspension feeding prior to manipulation, which is in accordance with stable isotope data from the population at the site of collection with showed that *S. plana* consumed more phytoplankton than microphytobenthos (Van Colen et al., 2010a), although the permanently submersed conditions in the

laboratory may have enhanced the contribution of suspension feeding to the species' diet in comparison with the natural semi-diurnal tidal cycle in the field.

In this study, the survival and condition of *S. plana* were not affected by future high pCO_2 conditions (i.e. acidification and warming). The lack of lethal effects in this experiment can probably be explained by (1) the fact that the high temperature condition (13°C) is still far below the average yearly highest temperature at the site of collection (19°C, https://weatherspark.com/y/49992/Average-Weather-in-Terneuzen-Netherlands-Year-Round, assessed July 2018), (2) the relatively short incubation period that might have been insufficient to manifest lethal effects (Sokolova et al., 2012), and (3) behavioural plasticity that reduced the intake of acid water while at the same time ensuring food intake through switching between feeding modes to cover higher metabolic demands. Sublethal effects on behaviour might, however, equally affect the ecosystem interaction network, especially for species that play key roles in community interactions and functioning, such as *S. plana*.

However, under low pH *S. plana* significantly decreased (but not completely ceased) suspension feeding, corroborating other studies on ocean acidification effects on suspension feeding bivalves (Fernández-Reiriz et al., 2011: *Ruditapes decussatus*; Liu and He, 2012: *Chlamys nobilis* and *Perna viridis*; Navarro et al.,

2013: *Mytilus chilensis*; chapter 2: *Cerastoderma edule*). Excessive intake of low-pH seawater could lead to physiological disruption due to the shift in acid-base balance (Pörtner et al., 2004; Seibel and Walsh, 2003). For respiration purposes, *S. plana* might have had to keep on suspension feeding in order to uptake oxygen from the aerated water column. However, both the frequency and the time per event of this feeding mode were significantly lower at pH_T 7.4, particularly under the low pH-elevated temperature treatment. Elevated temperature significantly increased the dietary contribution of deposit feeding probably to support higher energy requirements associated with a higher aerobic metabolism related to an increase in temperature (Hughes, 1970). Under the future scenario of combined acidification and warming, *S. plana* switched its feeding mode from predominant suspension feeding under control conditions (66.1 % and 74.5 % of events and total time, respectively) to almost exclusive deposit feeding (90.1 % and 94.8 % of events and total time, respectively). We hypothesise that this behavioural switch is a strategy to reduce the intake of low-pH water, and hence avoid hypercapnia that would disrupt normal physiology. The strong negative pressurisations at the end of a deposit feeding event do not only remove pseudofaeces, but also purge the intake water back to the water column before it enters the body. Furthermore, the elevated sediment pH conditions in the upper first few millimetres in comparison to the water

column in the low-pH treatments could have contributed to a reduced low-pH water intake during deposit feeding. Unfortunately, we lack pH profiles from the microcosms with *S. plana*, but similar subsurface peaks in porewater pH that overshoot water column pH have been documented in the presence of bioturbators in sediments with a high carbonate content (and hence a high buffering capacity to pH change) (Dashfield et al., 2008). In addition, the presence of infaunal species can modify pH sediment profile and create their own niche (e.g. Hu et al., 2014). Finally, the increased deposit feeding activity did not deplete microphytobenthos standing stock as sediment chlorophyll *a* content did not vary among treatments. Rather the combination of siphon relocations (that open up new burrows) and increased positive pressurisation associated with the increase in deposit feeding might have fuelled microphytobenthos productivity by promoting upwelling of nutrient-rich porewater, as has been demonstrated in *Arenicola marina* and *M. liliana* (Chennu et al., 2015; Woodin et al., 2010). The higher freshness of microalgae in the elevated temperature treatments where *S. plana* deposit-feed longer in comparison to the control temperature treatment supports this gardening effect and might explain why *S. plana* - in the presence of highly nutritious and fresh food could almost completely cease suspension feeding under future ocean conditions (i.e. lowered pH, elevated temperature).

To conclude, our results indicate behavioural change of *Scrobicularia plana* under ocean acidification and warming which might have prevented lethal and conditional effects. Nevertheless sublethal effects such as the observed change in feeding behaviour could affect ecosystem properties like water quality and benthic diversity, especially for species such as *S. plana* that play key roles in community interactions and ecosystem functioning (e.g. Baker et al., 2015; Lohrer et al., 2004; Mermillod-Blondin et al., 2005, 2004; Newell and Koch, 2004; Thrush et al., 1992). Thus, future research is required in order to determine the ecosystem response to behavioural changes of 'key species' such as the peppery furrow shell under future ocean conditions.

3.6 Acknowledgements

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3.7 Appendices

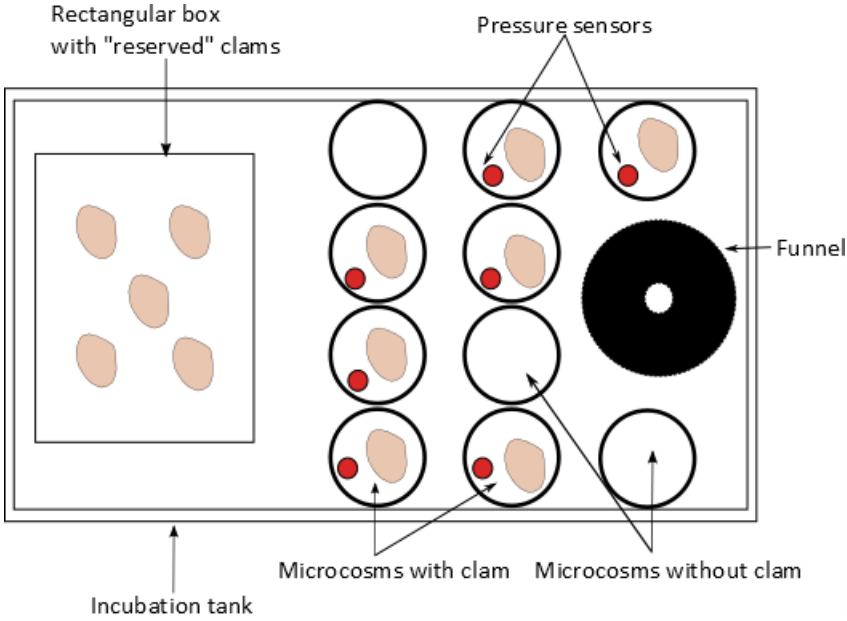

Figure 3A.1 Schematic drawing of an experimental incubation tank that contains one rectangular box and 10 cylindrical jars (microcosms) with or without *Scrobicularia plana*. Pressure sensors (red circles) were inserted in those microcosms that contains *Scrobicularia plana*.

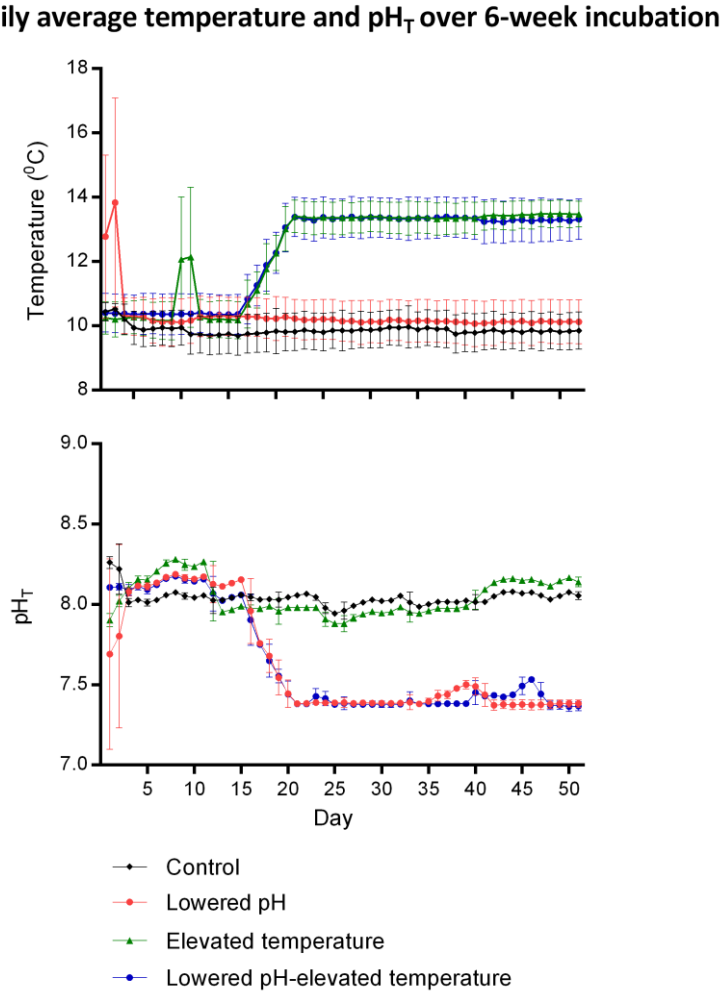

Figure 3A.2 Variations of daily temperature and pH in four treatments for 1-week acclimation and 6-week incubation. The manipulation of seawater temperature and pH_T started at day 15 until they achieved an offset of 13 °C and 7.4 and they were maintained until the end of the experiment. Error bars represent standard deviation (± SD).

Table 3A.1 Results of two-way ANOVA testing the total organic matter (TOM), and pigment concentrations in four treatments. Significant results are in bold.

	Df	Mean square	F value	Pr (>F)
TOM				
Temperature	1	0.015	0.87	0.36
pH	1	0.001	0.05	0.82
Interaction	1	<0.001	0.002	0.96
Residuals	19	0.018		
Chlorophyll *a*				
Temperature	1	0.093	0.59	0.45
pH	1	0.097	0.61	0.44
Interaction	1	0.033	0.20	0.66
Residuals	19	0.160		
Phaeophorbide a				
Temperature	1	0.256	0.95	0.34
pH	1	0.045	0.17	0.69
Interaction	1	0.003	0.01	0.91
Residuals	19	0.270		
Phaeophytin				
Temperature	1	0.137	7.87	**0.01**
pH	1	0.001	0.64	0.43
Interaction	1	0.002	1.02	0.33
Residuals	19	0.002		
Phaeopigments				
Temperature	1	0.482	3.09	0.09
pH	1	0.038	0.24	0.63
Interaction	1	0.013	0.08	0.77
Residuals	19	0.156		
Total pigments				
Temperature	1	0.143	0.88	0.36
pH	1	0.067	0.41	0.53
Interaction	1	0.030	0.18	0.67
Residuals	19	0.163		
Freshness				
Temperature	1	0.150	5.49	**0.03**
pH	1	0.014	0.50	0.49
Interaction	1	0.004	0.16	0.70
Residuals	19	0.027		

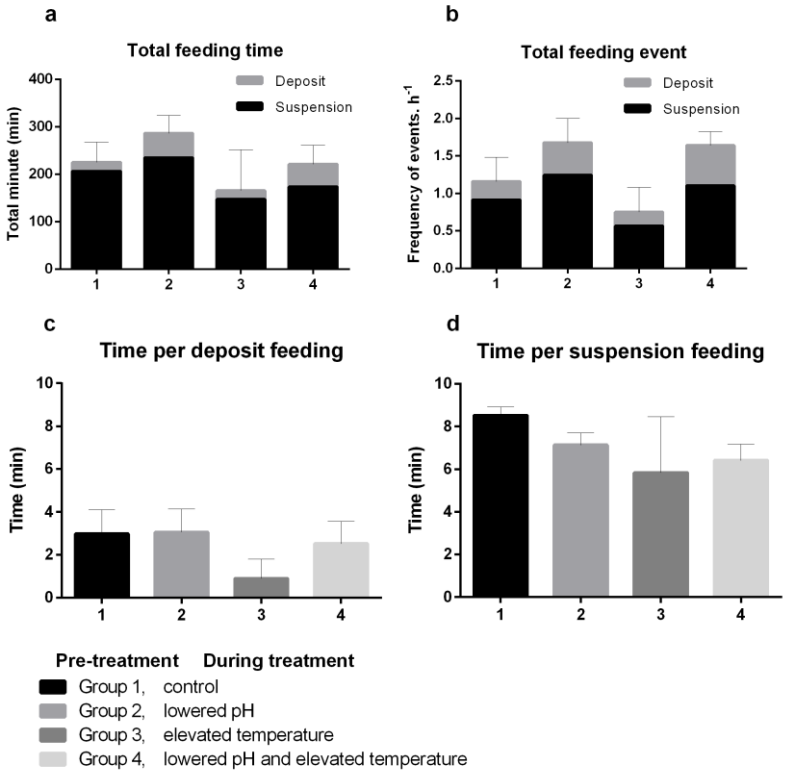

Figure 3A.3 *Scrobicularia plana* behaviour prior manipulation of seawater pH and temperature [e.g. (a) total feeding time includes deposit and suspension feeding, (b) total feeding event includes deposit and suspension feeding, (c) time per deposit event, (d) and per suspension feeding event. Legend labels represent each groups at during-treatment. Group 1, 2, 3, and 4 at pre-treatment represent control, lowered pH, elevated temperature and lowered pH-elevated temperature treatments at during-treatment. Error bars represent standard errors (± SE).

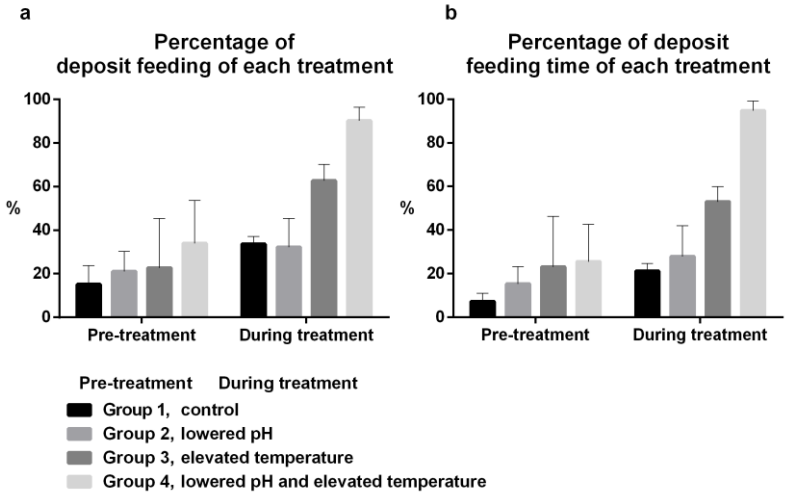

Figure 3A.4 The percentage of all feeding events that were deposit feeding events (a) and deposit feeding time (b) of *Scrobicularia plana* at pre- and during-treatment in four groups. Group 1, 2, 3, and 4 at pre-treatment represent control, lowered pH, elevated temperature and lowered pH-elevated temperature treatments at during-treatment. Error bars represent standard errors (± SE).

Chapter 4

Ocean acidification and warming change clam mediated effects on benthic macrofauna and ecosystem functioning

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4.1 Abstract

Ocean acidification and warming are two occurring phenomena that are predicted to intensify in the near future. While direct effects of both stressors on marine benthic communities are reasonably well studied, their indirect effects mediated by benthic key species and their interactions remain mostly unknown. This study examined effects of two different ocean climate conditions [ambient, lowered pH-elevated temperature (+ 3°C, - 0.4 pH units)] in the presence and absence of *Scrobicularia plana* on an intertidal soft-bottom community. *S. plana* is an infaunal tellinid bivalve with a flexible feeding strategy, which is highly beneficial to survive in dynamic environments such as tidal flats. After a 4-week incubation, the total abundance of macrofauna was significantly affected by the interactive effects of lowered pH-elevated temperature and the presence of *S. plana*. Calcifying surficial living organisms such as the gastropod *Hydrobia ulvae* and juvenile bivalves were significantly less abundant under lowered pH-elevated temperature treatments, particularly in the absence of *S. plana*. The presence of *S. plana* resulted in higher abundances of *Heteromastus filiformis*, whilst it decreased the population of *Hediste diversicolor* under both ambient and lowered pH-elevated temperature conditions. The overall adverse effects of high pCO_2 conditions on macrofauna were buffered by the increase of *S.*

plana deposit feeding via the inhibition of predator species such as *H. diversicolor*. The increase of deposit feeding caused disturbance to the burrows of *H. diversicolor*, thus inhibiting the abundance of *H. diversicolor.* In addition, the presence of *S. plana* significantly reduced porewater nitrite and nitrate concentration under the ambient condition, yet this effect disappeared under lowered pH-elevated temperature. This can be explained by the decrease of suspension feeding under low pH conditions which in turn reduced the stimulation of anaerobic nitrogen cycling. These results demonstrate *S. plana* effects on macrofauna populations, and ecosystem functioning varied between pCO_2 ocean conditions. In summary, the study illustrates that in addition to direct effects of climate change on species, populations and communities, indirect effects via alteration in the trophic interactions can significantly induce changes in soft-bottom sediment habitats.

4.2 Introduction

Burning of fossil fuels, deforestation and cement production are among the anthropogenic impacts that have dramatically increased the atmospheric carbon dioxide (CO_2) levels from 280 ppm in the pre-industrial era to 407 ppm at present (accessed on May 2018, Dlugokencky and Tans, 2017); a further increase of up to 940 ppm by the end of the 21st century is predicted, if CO_2 remains unmitigated (Collins et al., 2013) (Collins et al., 2013). About 30% of the anthropogenic atmospheric CO_2 is absorbed by the oceans which is altering the ocean carbonate chemistry and causing a reduction in ocean pH (Caldeira and Wickett, 2003; Doney et al., 2009; Sabine et al., 2004), a process referred to as ocean acidification (OA, Doney et al., 2009). The pH of the open ocean surface is projected to decrease by 0.14 and 0.4 units by 2100 under two contrasting Representative Concentration Pathways (RCP): the low CO_2 emissions scenario (RCP 2.6) and the business-as-usual (RCP 8.5) scenario, respectively (Collins et al., 2013; Gattuso et al., 2015). Presently, shallow coastal habitats are actually experiencing a greater degree of acidification compared to open oceans (Hofmann et al., 2011; Provoost et al., 2010; Wootton et al., 2008). Furthermore, increases in atmospheric CO_2 and methane concentrations will intensify the greenhouse effect, resulting in an increase of ocean surface temperature of between 1.0 and 3.2 °C by the end of this century

(Collins et al., 2013). The progressive modification of the ocean surface temperature and pH is expected to affect marine ecosystems, e.g. via changes in species behaviour, physiology and survival that will determine population fitness and interaction types and strengths within the community, with implications for biodiversity-mediated ecosystem functioning (Byrne and Przeslawski, 2013; Fabry et al., 2008; Nagelkerken and Munday, 2016; Orr et al., 2005; Pörtner et al., 2004). Moreover, as acidification and warming do not act in isolation, species and ecosystem responses may be additive, synergistic or antagonistic (Darling and Cote, 2008). Therefore, studying the combined effects of both acidification and warming is fundamental for our understanding of future ocean environments.

Over the last two decades, the effects of ocean acidification on marine calcifying organisms such as bivalves have been intensively studied. It has been suggested that the sensitivity of marine vertebrates and invertebrates (e.g. behaviour and physiology) to high pCO_2 water are often associated with specific developmental stages, thermal windows, mobility and adaptive capacity, and vary profoundly among species (Byrne, 2011; Byrne and Przeslawski, 2013; Clements and Hunt, 2015; Fabry et al., 2008; Gazeau et al., 2014; Kroeker et al., 2010; Parker et al., 2013; Pörtner et al., 2004; Pörtner, 2008). In general, calcifiers are negatively affected by ocean acidification causing the dissolution

of their $CaCO_3$ skeletons and shells (e.g. Beniash et al., 2010; Ries et al., 2009; Thomsen et al., 2015). Furthermore, the intake of acidified water during respiration and feeding disturbs the acid-base regulations involved in the production, consumption and active transportation of protons between intracellular and extracellular compartments (Pörtner et al., 2004; Seibel and Walsh, 2003). Consequently, a higher energy demand for basal maintenance is needed in warmer and hypercapnic waters, which may decrease energy allocation to processes such as activity, development, reproduction and somatic growth (Sokolova et al., 2012; Thomsen and Melzner, 2010; Wood et al., 2008). The upregulation of metabolic activities may potentially maintain organismal health, but it may result in biological deficiencies and behavioural changes in the long term (Sokolova et al., 2012).

Behavioural change is usually the first indication that organisms experience stress (Tuomainen and Candolin, 2011). Such behavioural changes may have consequences for ecosystem functioning in marine soft-sediment habitats (McCartain et al., 2017; Townsend et al., 2014; Woodin et al., 2012). Indeed, activities of macrobenthic organisms such as burrowing, feeding and sediment reworking, can enhance solute fluxes across the water-sediment interface (Wethey and Woodin, 2005; Woodin et al., 2010) that may enhance microphytobenthos productivity (e.g.

Chennu et al., 2015), i.e. gardening (Hylleberg, 1975; Reise, 1983). Moreover, sediment surface disturbance by deposit feeding of macrofauna influences the mortality and/or post-settlement survival of juveniles (Dunn et al., 1999; Thrush et al., 1992, 1996). Thus, disruptions of organismal behaviour may subsequently influence marine benthic ecosystem functioning. Relatively few studies have examined effects of ocean acidification on the behaviour of marine organisms (see e.g. Briffa et al., 2012; Clements and Hunt, 2014; Green et al., 2009; Schalkhausser et al., 2013). Moreover, there is still a general lack of studies on cumulative effects of ocean acidification and warming on behavioural responses of marine organisms (Nagelkerken and Munday, 2016).

A few soft-sediment community experiments have either focused on structural effects at the community level or functional implications on either single effects of lowered pH or elevated temperature, or in combination. For example, ocean acidification and warming modified the impacts of sea urchins and polychaetes on biogeochemical cycling and meiofauna community composition (Dashfield et al., 2008; Godbold and Solan, 2013; Widdicombe et al., 2013). However, the mechanistic linkage between structural effects and changes in functioning (e.g. biogeochemical cycling) remain poorly understood. Hale et al. (2011) observed that the abundance and diversity of molluscs

and arthropods were reduced while nematode abundance was increased under high pCO_2 conditions, suggesting that there may be both direct (i.e. physiological effect on macrofauna) and indirect (i.e. release from interference competition) impacts on the community. Indirect effects related to (changes in) species interactions have long been recognised in ecology (Darwin, 1859), but are understudied due to the complexity of ecosystems (Kordas et al., 2011; Wootton, 2002). A recent study demonstrated that grazing by amphipods and gastropods mediated the indirect effect of acidification and warming on primary producers in a seagrass community (Alsterberg et al., 2013). Indirect effects by macrofauna also play a role in benthic ecosystem functioning in tidal flats, where stressful conditions can affect the interaction network (e.g. Thrush et al., 2014). Therefore, to better understand the consequences of ocean warming and acidification on soft-sediment benthic community structure and ecosystem functioning, indirect effects, such as through ecosystem engineering and trophic interactions, should be taken into consideration.

The peppery furrow shell *Scrobicularia plana* is a widely distributed tellinid bivalve along the coasts of the European Atlantic, the Baltic and Mediterranean seas. It is considered a species of temperate to warm climates in soft-bottom communities (Santos et al., 2011; Tebble, 1976), where it is preyed

upon by fish and birds and is harvested by fishermen, especially in France, Portugal and Spain (Langston et al., 2007). This species feeds on settled or suspended fresh organic matter and detritus through deposit- and suspension-feeding, respectively (Riera et al., 1999; Van Colen et al., 2012b), irrigates its burrow, and produces (pseudo)faeces that can impact community interactions and ecosystem functioning (Norkko and Shumway, 2011). In chapter 3 of this PhD, we have demonstrated that *S. plana* switches from suspension feeding to deposit feeding when temperature increased by 3 °C, and reduced its suspension feeding when pH was lowered by 0.6 units relative to ambient conditions.

Here we present the results of a mesocosm experiment where we incubated an intertidal sediment community under ambient and lowered pH-elevated temperature conditions (+3 °C, -0.4 pH units) in the presence and absence of *S. plana*. At the end of a 4-week incubation, we determined abiotic sediment properties, sediment community aerobic respiration, the sediment-water nutrient exchange and the macrobenthos community to investigate how *S. plana* affects the community and ecosystem response to high pCO_2 conditions. We hypothesise that behavioural changes under high pCO_2 conditions may instigate changes in community interactions and functioning in sediments via direct (e.g. interference competition from bioturbation) and

indirect (e.g. alteration of food supply to benthos and secondary effects resulting from change in interference competition) mechanisms.

4.3 Material and methods

4.3.1 Collection of sediment and specimens

Adult *Scrobicularia plana* (length 31.16 ± 0.35 mm (SE)) and sediment were collected on September 2017 at a polyhaline sandflat in the Westerschelde estuary, The Netherlands (Paulina polder; 51° 20′ 55.4′′ N, 3° 43′ 20.4′′ E). Twenty rectangular boxes (length : 30 cm × width: 20 cm × depth: 17 cm) were filled to a depth of 12 cm with sediment (median grain size: 232.03 ± 0.88 μm) that was collected from a homogenous patch of equal size as the box, and where star-shaped feeding tracks of *S. plana* were absent. Disturbance was minimised by collecting sediment using Plexiglas cores (Ø = 10 cm, n= 6; Ø = 3.6 cm, n= 10) and transferring the sediment carefully into each box. Specimens and sediment were transported to the laboratory within 2 hours after collection in the field. Prior to manipulation of *S. plana* presence/absence (see further) and manipulation of seawater temperature and pH, *S. plana* and boxes filled with sediment were maintained separately for 7 days in ten incubation tanks (2 boxes per tank) filled with aerated seawater at 17 °C, a pH_T of 8.03 and a salinity of 33. pH_T of 8.03 equals the average of

continuous recordings from 2007 till 2017 measured at Terneuzen boei (Figure 1.12, http://live.waterbase.nl). A temperature of 17 °C was chosen as it was the field temperature measured at the moment of specimen collection.

4.3.2 Experimental set-up

The experiment manipulated two factors (seawater pH plus temperature and presence/absence of *S. plana*) with two levels each in a crossed design: ambient temperature and pH versus elevated temperature and lowered pH; and *S. plana* presence versus absence. There were five replicates for each combination of *S. plana* treatment x climate condition. Ten holding tanks (100 L) were used for seawater storage and channelled to the incubation tank (66 cm × 45 cm × 22 cm) using a pump; water was circulated back to the holding tanks by gravity. The seawater was oxygenated and manipulated in each incubation tank. The two boxes per tank (Figure 4A.2) were separated by a net (h = 35 cm, mesh size = 1 mm) to prevent migration of macrofauna from one box to another. Then, five *S. plana* were randomly selected and added to one of the two boxes per incubation tank. Since chapter 3 demonstrated that horizontal mobility of *S. plana* is uncommon after burrowing, we added *S. plana* into the box in a crossed pattern design (Figure 4A.2), in order to spread "the *Scrobicularia* effect" over the box. After two weeks of acclimatisation, temperature in the lowered pH-elevated temperature treatments

was increased (1 °C per two days over a period of 6 days) until 3 °C above the ambient treatments, and pH in the lowered pH-elevated temperature treatments (- 0.4 units) was decreased by 0.1 unit per day over 4 days and maintained constant for 4 more weeks. The seawater carbonate chemistry parameters during the 4-week incubation are shown in Table 4.1 and Figure 4A.1. The seawater temperature of the ambient treatments (~17 °C) was maintained by the air conditioning in the climate room, whereas the temperature for the lowered pH-elevated temperature conditions (~20°C) was regulated using temperature controllers (Refrigeration technologies; Model: TK200H). Meanwhile, the seawater pH of the lowered pH-elevated temperature treatments was manipulated and controlled by an IKS Aquastar Industrial control panel coupled with pH glass electrodes (compression-proof) via the bubbling of pure CO_2. All pH glass electrodes (pH in NBS scale) in each incubation tank were 2-point calibrated weekly with IKS GmbH NBS buffer solutions (pH 4.01 and 7.01 at 25 °C). In addition, all pH glass electrodes were inter-calibrated using a portable pH/conductivity meter (Metrohm; Model: 914), allowing conversion from the pH in NBS scale to the pH in total scale using TRIS buffer solution with a salinity of 35 (Dickson, 2010). Seawater salinity was measured weekly with a WTW portable conductivity meter (model: LF320). On a weekly basis, 150 mL of seawater was sampled from each incubation tank and filtered through GF/C filter papers for the measurement

of total alkalinity (TA). These seawater samples were 'poisoned' with $HgCl_2$ to inhibit biological activity from altering the carbon distribution in the samples (Dickson et al., 2007) and were kept at 4 °C until TA analysis via titration using a HydroFIA TA (CONTROS Systems & Solutions GmbH) at the Flanders Marine Institute (VLIZ). Calibration of HydroFIA TA (CONTROS Systems & Solutions GmbH) was accomplished using CO_2 in Seawater Reference Material (CRM, batch 166). Accuracy (deviation from CRM) and precision (the standard deviation within measurements of the same batch) were 2 and 1.9 $\mu mol.kg^{-1}$ (n = 16), respectively. All temperature, pH, salinity and TA values were inserted into CO2SYS software (Pierrot et al., 2006) to obtain seawater carbonate chemistry parameters (i.e. pH_T, pCO_2, HCO_3^-, CO_3^{2-}, Ωc and Ωa) using the thermodynamic constants of Mehrbach et al. (1973).

Every week, 40 L of seawater from each holding tank was replaced in order to maintain a constant salinity of 33 and to limit any build-up of nutrients. The seawater refreshment procedure took around 30 min for the whole set-up. All open top incubation tanks were subjected to a 12:12 hour light:dark regime. The sediment communities were fed twice a week by mixing 4 mL of concentrated commercial Shellfish Diet 1800 (Reed Mariculture Inc., consisted of 40% *Isochrysis*, 15% *Pavlova*, 25% *Tetraselmis* and 20% *Thalassiosira weissflogii*) with 10 L of fresh seawater, and

allocated evenly to each holding tank. We acknowledge that the Shellfish diet may not resemble the food supply in the field, but by adding this controlled food supply, we could largely avoid unforeseen effects of temperature and pH on food quantity and quality (Hinga, 2002; Thomas et al., 2012).

After 4 weeks of incubation under manipulated conditions, samples for sediment abiotic properties (4.3.3), sediment-water exchange of solutes (4.3.4) and the macrofauna community (4.3.5) were collected from each box. The survival of *S. plana* in every box was checked and all were alive at the end of the experiment.

Table 4.1 The mean carbonate chemistry of seawater in the ambient and lowered pH-elevated temperature treatments during a 4-week incubation: Temperature, total pH, salinity, total alkalinity (TA), partial pressure of carbon dioxide (pCO_2), concentration of bicarbonate and carbonate ions (HCO_3^- and CO_3^{2-}), and saturation state with respect to aragonite and calcite (Ωa and Ωc). The ± values represent standard errors (SE). The seawater carbonate chemistry parameters were significantly different across both climate conditions (See 4.3.6).

Treatment	Control	Future
Temperature (° C)	16.6 ± 0.12	19.6 ± 0.14
pH_T	8.09 ± 0.01	7.66 ± 0.02
Salinity	32.9 ± 0.23	33.4 ± 0.10
pCO_2 (µatm)	371 ± 5.2	1167 ± 45.6
TA (µmol kg^{-1})	2351 ± 1.8	2379 ± 1.9
HCO_3^- (µmol kg^{-1})	1916 ± 5.0	2170 ± 8.8
CO_3^{2-} (µmol kg^{-1})	177.8 ± 1.8	85.8 ± 3.2
Ωa	2.8 ± 0.03	1.3 ± 0.05
Ωc	4.3 ± 0.04	2.1 ± 0.08

4.3.3 Sediment abiotic properties

After careful removal of overlying seawater, five cores (Ø = 10 cm) were first inserted into the sediment following a crossed pattern design (Figure 4A.2, see 4.3.2). Then, a rhizon soil moisture sampler (mean pore size 0.15 μm) coupled with a 10 mL syringe was used to vertically extract the porewater in the sediment till a depth of 5 cm (n = 2 per box). The calculated radii of an influenced cylindrical area around a 5 cm rhizon for a sample of 7 mL is 1.04 cm (Seeberg-Elverfeldt et al., 2005). The collected porewater samples were stored at -20 °C awaiting subsequent dissolved inorganic nutrient analysis using automated colorimetric analysis on a San^{++} Continuous Flow Analyzer (CFA, SKALAR). The top centimetre of each core was collected and subsampled three times using 3.6 cm Ø (10 cm^2) cores: one sample for total organic matter and carbon to nitrogen ratio, one sample for granulometry and porosity, and one sample for pigment concentrations. All subsamples were stored at -80 °C prior to further processing. Total organic carbon and nitrogen content (TOC/TN) samples were lyophilised, homogenised and acidified using 1% hydrochloric acid (HCl) prior to analysis with a Flash 2000 Elemental Analyser (Thermo Fisher Scientific). Sediment granulometry was determined using a Malvern Mastersizer 2000 (Malvern Instruments, UK) via laser diffraction. Total organic matter (TOM) was calculated as the mass loss

observed on the dried sample (48 h at 60 °C) after muffling at 450 °C for 2 h. Porosity was calculated as the relative volume of porewater to the total volume of dry sediment and porewater (weight loss by drying for 48 h at 60 °C). Pigments were analysed using HPLC (Agilent) analysis according to Wright and Jeffrey (1997) after an over-night extraction with acetone at 4 °C in the dark. Sedimentary organic matter (OM) derived from primary production was calculated as the sum of Chloroplastic Pigment Equivalents (CPE: sum of chlorophyll *a* and phaeopigments (its degradation products)) (Thiel, 1978). Microalgal freshness was calculated as the ratio of chlorophyll *a*: phaeopigments (Ingels et al., 2011).

4.3.4 Oxygen and nutrient fluxes

The oxygen and nutrient fluxes across the water-sediment interface were measured from the centre core per box (Figure 4A.2). These cores were extracted from their respective boxes, closed at the bottom and filled carefully with the conditioned seawater using bubble wrap to minimize disturbance of the sediment surface. The cores were equipped with magnetic stirring rings and covered with airtight lids, fitted with two stopcocks to enable the sampling of overlying water for nutrient flux measurements. Then, cores were incubated in the dark for 4 hours following Mestdagh et al. (2018). At the beginning of the incubation, samples of 40 mL seawater were collected from the

incubation tank with a glass syringe (t_0). After 4 hours of incubation, 40 mL seawater was collected from each core with a glass syringe (t_1). Oxygen concentration of the water column in each core was measured continuously using Pyroscience rigid probes and ensured to remain above 50% saturation during the incubation. Sediment community oxygen consumption rates were determined from the linear decrease of oxygen during the incubation and nutrient exchange rates were determined from the change in nutrient concentrations between t_0 and t_1 according to the following equation:

$$Fluxes = -\frac{dC}{dt} \times \frac{V}{A}$$

where $\frac{dC}{dt}$ represents the change of oxygen or nutrient concentration in the water column (mmol L^{-1} d^{-1}), V represents the volume of overlying water (L), and A represents the sediment surface area (m^2). Note that the background nutrient concentrations of the "laboratory seawater" used in the core incubation may not resemble those in the field, therefore the reported nutrient fluxes should be considered as "potential fluxes". In addition to temperature that was logged continuously (Pyroscience dipping-probe temperature sensor), the pH of the seawater in the cores was measured at the start and end of the incubation with a portable pH/conductometer. During the closed incubations, pH_{NBS} declined by 0.02 and 0.13 units in both

ambient and lowered pH-elevated temperature treatments, respectively. Finally, the top centimetre was sliced and subsampled for sediment abiotic properties, as described in section 4.3.3.

4.3.5 Macrofauna

The remaining sediment from the rectangular box and the sediment below the top centimetre from each of the five 10-cm Ø cores was sieved through a 500-µm sieve and fixed in 8% buffered formaldehyde. All organisms were identified to species level, except for Oligochaeta and juvenile bivalves. Species richness, total abundance, Pielou's evenness and the Shannon-Wiener diversity index were determined using the Primer 6 software (Anderson et al., 2008).

4.3.6 Statistical data analysis

To compare the seawater carbonate chemistry parameters between two climate conditions, one-way analysis of variance (ANOVA) was performed. Non-normal data were Log_{10} transformed to improve normality, and homogeneity of variances was checked using Levene's test. To test the effects of condition (ambient, lowered pH and elevated temperature), *S. plana* (absence and presence) and their interaction on ecosystem properties, a series of linear mixed effects models (lmer) for each response variable were used. We took into account the non-

independence of the two boxes per tank by allowing random intercepts for tank identity. We used a Gaussian error distribution to model all other response variables. For these models, the F-statistic and associated P-values for each effect were calculated using the Kenward-Roger method to estimate degrees of freedom. For non-normally distributed data, Log_{10} transformation was used prior to analysis in order to improve normality, and Q-Q plots were used to check the model assumptions. Response variables were the averaged results when multiple samples per box were collected (n = 2 for porewater nutrients and n = 5 for all other sediment variables, see Figure 4A.2), as well as the maximum value of pigment data (maximum value of 5 samples in each box) to study possible treatment effects on microalgal patchiness. These analyses were performed using packages lme4 (Bates et al., 2015), lmerTest and pbkrTest (Kuznetsova et al., 2015) in the R software version 3.5.0 (R Core Team, 2013). Furthermore, the effects on the species richness, total macrofaunal abundance and on the abundance of the dominant species and taxa (i.e. *Hediste diversicolor*, *Heteromastus filiformis, Hydrobia ulvae, Pygospio elegans* and juvenile bivalves) were analysed with a series of generalised linear mixed models (glmer) using Poisson distribution of data and log likelihood ratio tests to calculate the Chi-Square and associated P-values for each effect. One adult *S. plana* was found in one of the replicates from the lowered pH-elevated

temperature treatment without *S. plana* (N = 4) and this particular replicate was therefore removed from the analyses mentioned above.

4.4 Results

4.4.1 Manipulation of temperature and pH_T

Temperature and pH_T of both ambient and lowered pH-elevated temperature treatments remained relatively stable throughout the experiment (Figure 4A.1). After manipulation, temperature in the ambient and lowered pH-elevated temperature treatments was maintained at 16.3 ± 0.03 °C and 19.5 ± 0.11 °C (mean ± SE), respectively. pH_T in the ambient and lowered pH-elevated temperature treatments was maintained stable at 8.08 ± 0.03 and 7.68 ± 0.04, respectively.

4.4.2 Effects on sediment abiotic properties

There were no significant differences between treatments in terms of median grain size, porosity, TOC/TN and TOM, porewater silicate, phosphate, ammonium concentrations. There was, however, a significant interaction effect of climate condition and *S. plana* on porewater nitrite and nitrate concentrations ($F_{1, 7.70} = 7.66$, P = 0.03)(Table 4A.2, Figure 4A.3), indicating that nitrite and nitrate concentrations were lower under ambient conditions in the presence of *S. plana*; yet this effect disappeared under

lowered pH/elevated temperature. Concentrations were 1.62 ± 0.50 μmol.L^{-1} when *S. plana* was present, 7.68 ± 1.50 μmol.L^{-1} when *S. plana* was absent at pH 8.1 and 17 °C, 6.25 ± 3.38 μmol.L^{-1} when *S. plan*a was present and 5.25 ± 1.19 μmol.L^{-1} when *S. plana* was absent at pH 7.7 and 20 °C. Average microalgal biomass and freshness did not differ between treatments. However, maximum microalgal biomass was significantly lower at pH 7.7 and 20 °C ($F_{1, 7.73}$ = 7.58, P = 0.03) (Table 4A.2, Figure 4A.4). Although there were no significant interactions between climate condition and *S. plana* on maximum microalgal biomass and freshness, maximum microalgal biomass was lowest while freshness was highest in the presence of *S. plana* when pH was lowered and temperature was elevated (Table 4A.2, Figure 4A.4).

4.4.3 Effects on oxygen and nutrient fluxes

There was a significant climate effect on phosphate fluxes ($F_{1, 7.77}$ = 13.98, P = 0.0006), reflecting the switch of the sediment community from a sink of phosphate (influx, -1.29 ± 0.21 mmol.m^{-2}.d^{-1}) at pH 8.1 and 17 °C towards a source (efflux, 0.82 ± 0.55 mmol.m^{-2}.d^{-1}) of phosphate at pH 7.7 and 20 °C (Table 4A.3, Figure 4A.5). Sediment-water column exchange of nitrite, nitrate, ammonium and silicate did not differ between treatments. In general, the sediment was a sink for dissolved inorganic nitrogen, independent of treatments. Sediment community oxygen consumption rates did not differ between treatments and were,

on average, higher at pH 7.7 and 20 °C in the absence of *S. plana* (Table 4A.3, Figure 4A.5).

4.4.4 Effects on macrofauna

The total macrofauna abundance was significantly affected by the interaction between climate condition and *S. plana* ($\chi^2_1 = 14.2$, $P = 0.0002$) (Table 4.2, Figure 4.1). At pH 7.7 and 20 °C, abundance was the lowest, particularly in the absence of *S. plana*. The abundances were 187.4 ± 21.0 ind.0.06 m^{-2} when *S. plana* was absent and 206 ± 33.6 ind.0.06 m^{-2} when *S. plana* was present at pH 8.1 and 17 °C; at pH 7.7 and 20 °C, the respective results were 74.5 ± 19.5 ind.0.06 m^{-2} when *S. plana* was absent and 126 ± 30.3 ind.0.06 m^{-2} when *S. plana* was present. The species richness, species evenness and Shannon diversity did not differ significantly between treatments.

There was a significant climate condition x *S. plana* interaction effect on the abundances of *Hydrobia ulvae* ($\chi^2_1 = 94.8$, $P < 0.0001$), juvenile bivalves ($\chi^2_1 = 9.39$, $P = 0.003$) and *Pygospio elegans* ($\chi^2_1 = 13.04$, $P < 0.0001$) (Table 4.3, Figure 4.2); the abundances of these species were lower at pH 7.7 and 20 °C, particularly when *S. plana* was absent. On the other hand, *S. plana* facilitated the abundance of *Heteromastus filiformis* irrespective of the climate condition ($\chi^2_1 = 17.7$, $P < 0.0001$), whereas the opposite pattern

was found for *Hediste diversicolor* ($\chi^2_1 = 8.19$, P = 0.004) (Table 4.3, Figure 4.2).

Table 4.2 Generalised linear mixed effects models comparing macrofaunal species richness and total abundance and linear mixed effects models comparing species evenness and Shannon-Wiener in the four treatments. Significant results are in bold.

	Chi df	***Chisq***	***Pr(>Chisq)***
Total species			
Interaction	1	0.16	0.69
Condition	1	1.88	0.17
Scrobicularia	1	0.22	0.64
Total abundance			
Interaction	1	14.20	**0.00**
Condition	1	7.38	**0.01**
Scrobicularia	1	22.92	**0.00**
	ndf	***ddf***	***F-ratio***	***p value***
Evenness				
Interaction	1	7.29	0.27	0.62
Condition	1	7.96	4.30	0.07
Scrobicularia	1	8.26	0.04	0.84
Shannon				
Interaction	1	7.46	0.49	0.50
Condition	1	7.90	1.04	0.34
Scrobicularia	1	8.43	0.12	0.74

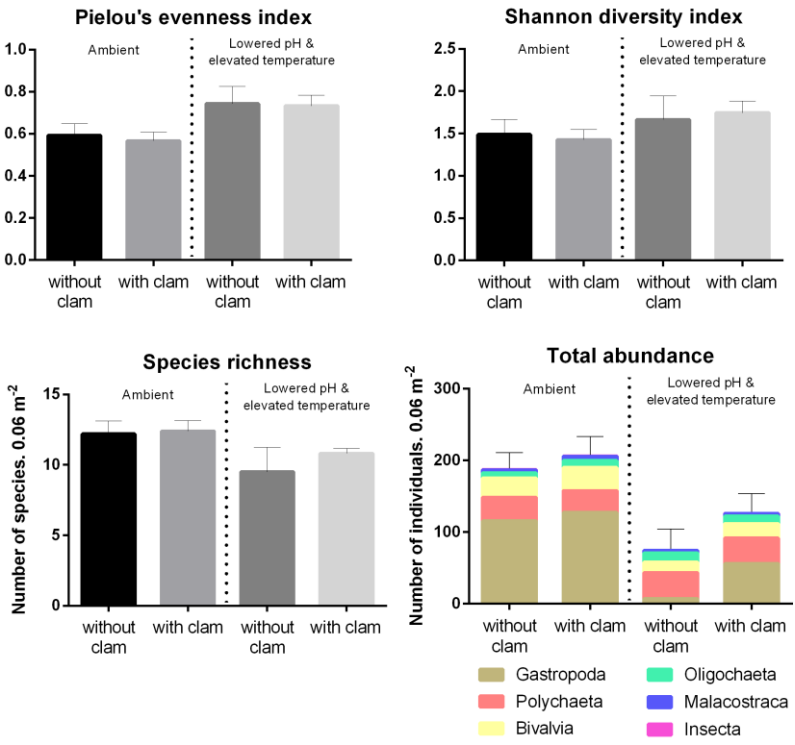

Figure 4.1 Average macrofauna diversity indices (± SE) (evenness, Shannon-Wiener index and species richness) and total macrofauna abundance in the four treatments. Clam = *Scrobicularia plana*.

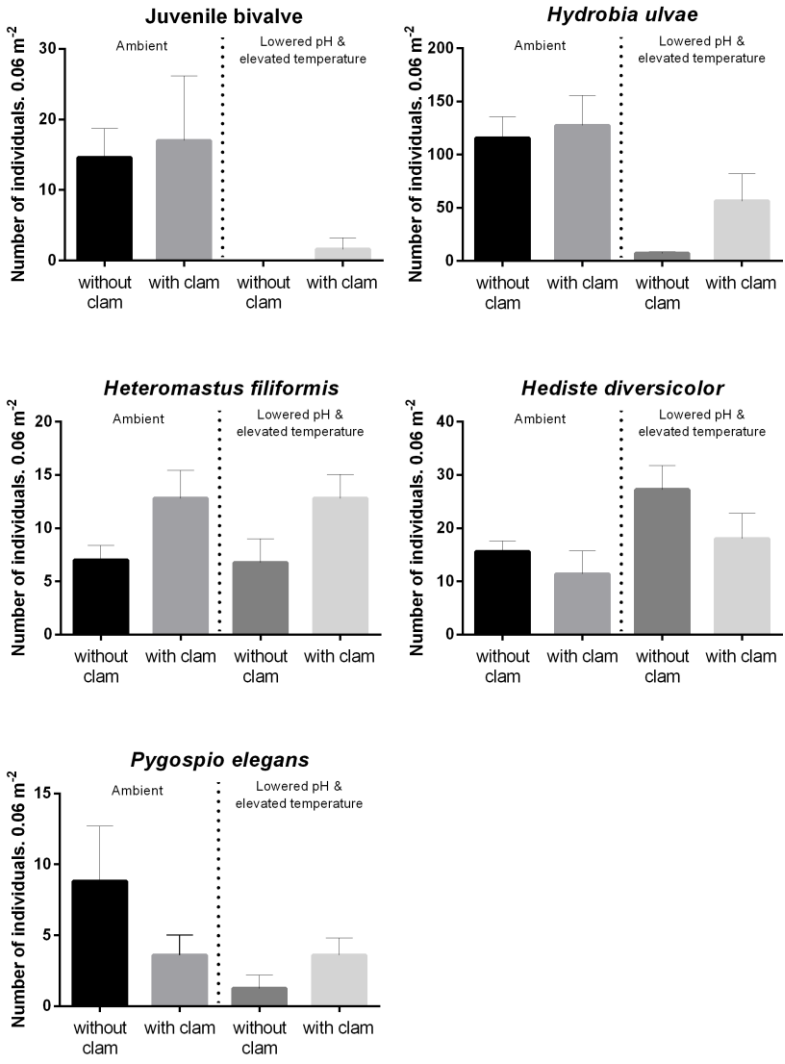

Figure 4.2 Average densities (± SE) of different macrofauna taxa in the four treatments. Clam = *Scrobicularia plana.*

Table 4.3 Generalised linear mixed effects models comparing the abundance of dominant species in the four treatments. Significant results are in bold.

	Chisq	Chi df	Pr(>Chisq)
Bivalve spat			
Interaction	9.39	1	**0.002**
Condition	6.09	1	**0.01**
Scrobicularia	2.38	1	0.12
Hydrobia ulvae			
Interaction	94.84	1	**0.000**
Condition	7.12	1	**0.008**
Scrobicularia	31.22	1	**0.000**
Heteromastus filiformis			
Interaction	0.06	1	0.81
Condition	0.03	1	0.86
Scrobicularia	17.68	1	**0.000**
Hediste diversicolor			
Interaction	0.97	1	0.97
Condition	2.82	1	0.09
Scrobicularia	8.19	1	**0.004**
Pygospio elegans			
Interaction	13.04	1	**0.000**
Condition	3.42	1	0.06
Scrobicularia	3.11	1	0.08

4.5 Discussion

The ambient pH value used in this experiment was similar to the mode of the pH recorded monthly over the last ten years at the sampling site, whereas the lowered pH value was 0.02 units below the lowest pH and which was only exceeded once (7.67, June 2014) during the same time series (Figure 1.12) Ocean acidification and warming have profound effects on marine biodiversity. Direct effects on biodiversity related to mortality of

species was frequently observed but little is known about indirect effects, and how such effects interact with the presence of key species. Density changes in key species can quickly result in alterations of biodiversity (Thrush et al., 2006) and functioning (e.g. Braeckman et al., 2010a), but sublethal behavioural effects at the individual level can have effects as well. In marine soft-sediment habitats, large organisms such as clams play pivotal functional roles (e.g. Thrush et al., 2006), mainly through sediment reworking and burrow ventilation that affect biodiversity via interference competition and nutrient regeneration, that directly and indirectly affect biological interactions between ecosystem components (Thrush et al., 2014; Van Colen et al., 2015). *Scrobicularia plana* represents such an ecosystem engineering species (sensu Jones et al., 2012) through its deposit feeding effects on sediment erodibility (Orvain, 2005) and interference competition that can have secondary effects on sediment biogeochemistry (Clare et al., 2016). Previous research (chapter 3) demonstrated that this species survives short-term (4 weeks) high pCO_2 conditions (low pH and high temperature); however, these conditions significantly altered the species' feeding behaviour. An increase in deposit feeding under warm conditions and a decrease in suspension feeding under low pH is expected to affect the benthic community directly and indirectly via change in biotic interactions and indirect effects on

biogeochemistry through changes in sediment aeration and stimulation of benthic nutrient cycling.

The presence of *S. plana* affected all abundant species populations, but these effects varied from positive to negative. Facilitation of other populations was mainly present under lowered pH and elevated temperature, except for *Heteromastus filiformis* that was more abundant in both conditions when *S. plana* was present. *H. filiformis* lives at a similar depth as *S. plana* and deposit feeds head-down in the sediment (Cadée, 1979; Mulsow et al., 2002). *H. filiformis* might thus benefit from bio-irrigation by *S. plana* that supplies oxygen for respiration at depth (Kristensen and Kostka, 2013; Norkko and Shumway, 2011) and stimulates nutrient cycling that increases microbial growth around the sediment grains (e.g. Mermillod-Blondin et al., 2004). In contrast, the presence of *S. plana* inhibited *H. diversicolor* particularly under lowered pH and elevated temperature. Disturbance of *H. diversicolor* burrows associated with deposit feeding by *S.plana* seems the most plausible explanation for the inhibition effect of *S. plana* on *H. diversicolor* (see e.g. Clare et al., 2016 for similar effect on *Corophium volutator*). Moreover, the increase in deposit feeding events under warm and low pH conditions (chapter 3) may explain why interference effects are strongest in that treatment.

Lowered pH and elevated temperature conditions strongly decreased the density of juvenile bivalves and of the gastropod *H. ulvae*. The reduction of both populations cannot be ascribed to lower recruitment during our experiment because recruitment occurs earlier (May-June) at the site of collection (Van Colen et al., 2008). Mortality thus explains the reduced densities of these calcifiers that are known to be vulnerable to ocean acidification, particularly their juvenile life stages and in combination with warming (reviewed in Kroeker et al., 2010). Remarkably, *Hydrobia ulvae*, juvenile bivalves and *P. elegans* benefitted from the presence of *S. plana*, but only under lowered pH and elevated temperature. We suggest that the release from predation by *H. diversicolor* under warm and low pH conditions in the presence of *S. plana* mainly explains this lower high pCO_2-induced mortality. Furthermore, the lowest porewater nitrate, silicate and highest maximal microalgal freshness were found in this treatment, suggesting a gardening effect related to more deposit feeding by *S. plana* in this treatment. Such higher microalgal production might additionally have contributed to the higher survival of juvenile bivalves, *H. ulvae* and *P. elegans* that all rely strongly on fresh microalgal food for their diet (López-Figueroa and Niell, 1988; Van Colen et al., 2010).

In general, the influxes of dissolved inorganic nitrogen in each treatment demonstrate a high DIN demand by the sediment

microbial community (bacteria, archaea, microphytobenthos) that exceeded the level of DIN production in the sediment through excretion and nitrification. Widdicombe et al. (2013) reported a similar high DIN demand for a sandy sediment at a pH of 8 and suggested that the high assimilation by microphytobenthos and high rates of anammox determined the DIN influx. We observed significantly lower porewater nitrite and nitrate concentrations under the ambient condition in the presence of *S. plana*. As no differences in aerobic mineralisation were found between treatments, nitrate and nitrite removal must have been driven by assimilation into microphytobenthos biomass and/or anaerobic respiration by benthic denitrification, dissimilatory nitrate reduction to ammonium (DNRA), and/or anammox. The first two processes were shown to dominate anaerobic nitrate consumption in a similar sandflat sediment in the Wadden Sea, whereas anammox was absent (Marchant et al., 2014). We hypothesize that behavioural plasticity of *S. plana* might govern this climate condition x *Scrobicularia* interaction effect by stimulating anaerobic nitrogen cycling. Chapter 3 demonstrates that the same pressurisation is created during deposit and suspension feeding, but that under ambient pCO_2, conditions this pressurisation is maintained approximately 3 times longer during suspension feeding than during deposit feeding. Consequently, predominant suspension feeding by *S. plana* under ambient conditions will irrigate a higher volume of

DIN-rich water from the water column deep into the sediment where it can be reduced via denitrification and DNRA. The low $[NO_3^-]/[NH_4^+]$ ratio in this treatment supports the hypothesis that DNRA contributes to the removal of nitrate under ambient conditions in the presence of *S. plana*. Finally, the sediment changed from a sink for PO_4^{3-} to a source of PO_4^{3-} in response to lowered pH and elevated temperature. A higher aerobic metabolism at higher temperature may result in less oxygenated sediment (Conley et al., 2009), promoting iron reduction from Fe (III) to Fe (II) and the concomitant release of PO_4^{3-} into the water column (Belias et al., 2007; Rozan et al., 2002). *Hediste diversicolor* was observed to crawl at the sediment surface in the lowered pH and elevated temperature treatment, possibly to escape from depleted oxygen conditions in the sediment (Riedel et al., 2014). Such migration to shallower depths might further have intensified the consumption by *H. diversicolor* of epifaunal and shallow living infaunal species such as *H. ulvae*, *P. elegans* and juvenile bivalves.

In conclusion, *Scrobicularia plana* was found to survive short-term combined acidification and warming, but its effects on macrofauna populations and ecosystem functioning varied between pCO_2 ocean conditions. These results demonstrate that in addition to direct effects of climate change on populations, indirect effects via change in the ecosystem interaction network

can also rapidly induce strong changes in seafloor sediment habitats.

4.6 Acknowledgements

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4.7 Appendices

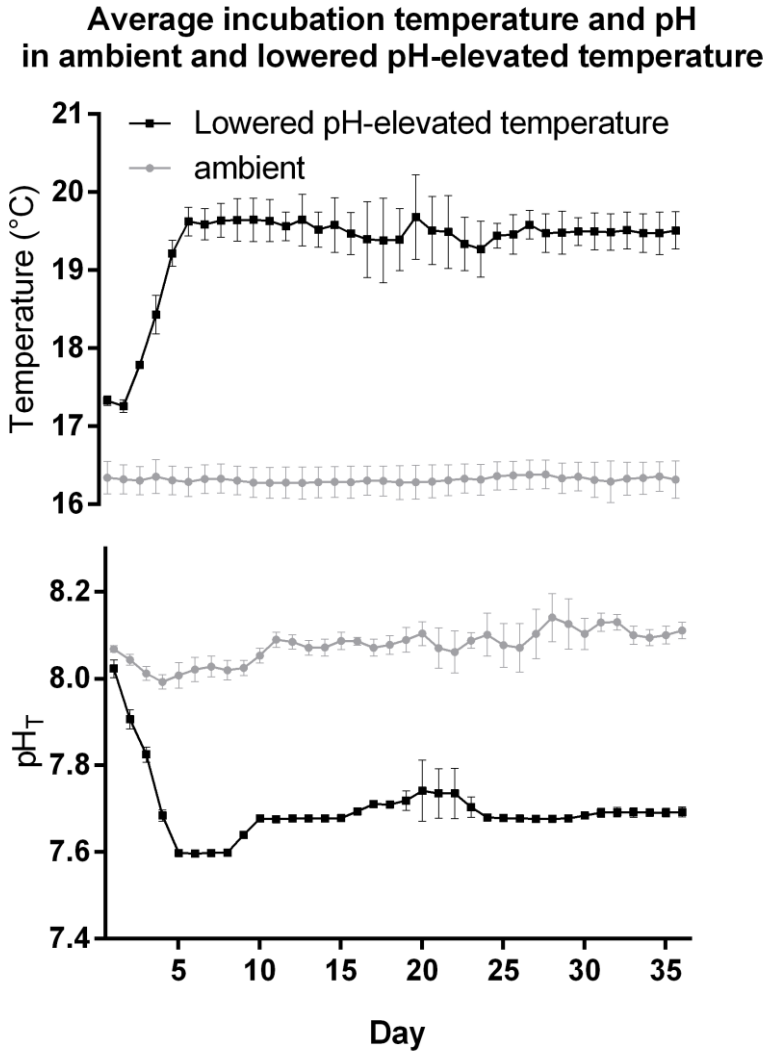

Figure 4A.1 Daily temperature and pH variation in the ambient and lowered pH-elevated temperature conditions during 4-weeks of incubation started from day 6. The manipulation of seawater temperature and pH_T started at day 1 until they reached 19 °C and 7.6 in pH_T. Error bars represent standard deviation (± SD).

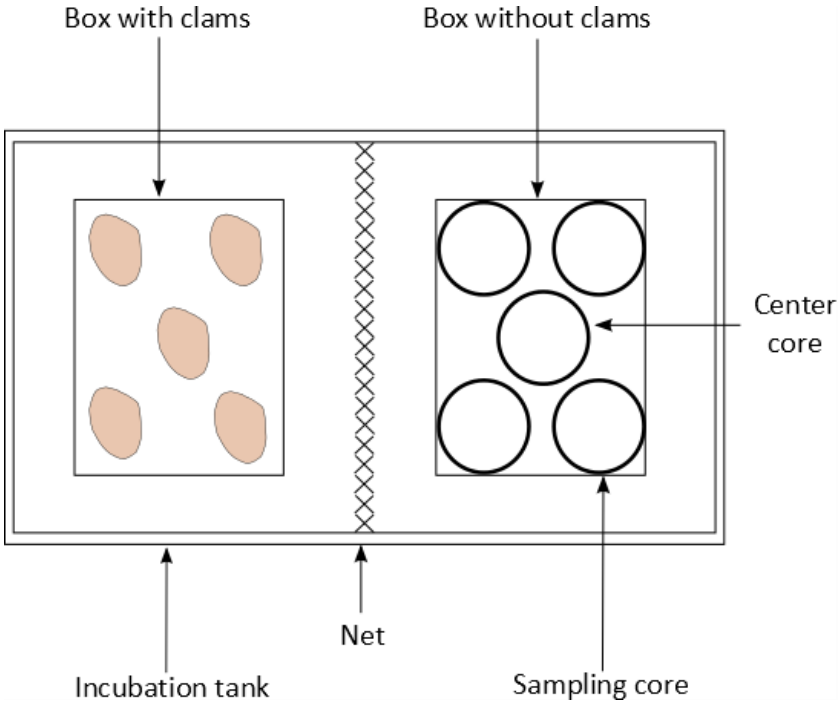

Figure 4A.2 Schematic drawing of incubation tank that contains two boxes, one with and one without *Scrobicularia plana*, separated by a net. Five cores (represent by circles) were inserted into the sediment of each box following a crossed pattern design. The centre core was used for flux measurement.

Table 4A.1 Linear mixed effects models comparing sediment and porewater characteristics in the four treatments. Significant results are in bold.

	ndf	***ddf***	***F-ratio***	***p value***
Grainsize				
Interaction	1	7.65	1.30	0.29
Condition	1	7.77	0.41	0.54
Scrobicularia	1	8.63	0.47	0.51
Porosity				
Interaction	1	7.70	0.28	0.61
Condition	1	7.73	0.81	0.40
Scrobicularia	1	8.67	1.02	0.34
C:N ratio				
Interaction	1	7.46	0.14	0.72
Condition	1	7.91	0.41	0.54
Scrobicularia	1	8.40	0.04	0.84
TOM				
Interaction	1	7.41	2.10	0.19
Condition	1	7.90	0.52	0.49
Scrobicularia	1	8.41	0.04	0.84
Porewater NO_2^- + NO_3^-				
Interaction	1	7.70	7.66	**0.03**
Condition	1	7.73	1.28	0.29
Scrobicularia	1	8.67	4.01	0.08
Porewater Si				
Interaction	1	7.70	0.73	0.42
Condition	1	7.73	3.23	0.11
Scrobicularia	1	8.67	0.89	0.37
Porewater NH_4^+				
Interaction	1	7.70	0.59	0.46
Condition	1	7.73	0.55	0.48
Scrobicularia	1	8.67	0.03	0.86
Porewater PO_4^{3-}				
Interaction	1	7.34	1.59	0.25
Condition	1	7.93	1.48	0.26
Scrobicularia	1	8.35	0.41	0.54

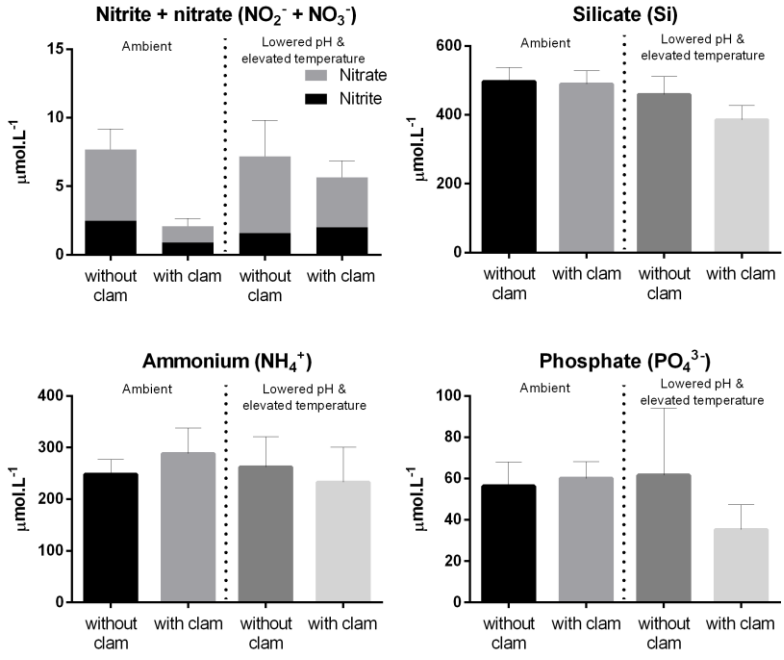

Figure 4A.3 Average concentration (± SE) of porewater nitrite and nitrate, silicate, ammonium and phosphate in the four treatments. Clam = *Scrobicularia plana.*

Table 4A.2 Linear mixed effects models comparing pigment concentrations and pigment-derived parameters in the four treatments. Significant results are in bold.

	ndf	***ddf***	***F-ratio***	***p value***
Phaeopigments				
Interaction	1	7.59	0.00	0.95
Condition	1	7.84	2.39	0.16
Scrobicularia	1	8.53	0.00	0.97
CPE (average)				
Interaction	1	7.70	0.14	0.72
Condition	1	7.73	2.63	0.15
Scrobicularia	1	8.67	0.01	0.93
CPE (maximum)				
Interaction	1	7.70	0.92	0.37
Condition	1	7.73	7.58	**0.03**
Scrobicularia	1	8.67	0.04	0.84
Freshness (average)				
Interaction	1	7.50	0.13	0.73
Condition	1	7.88	1.02	0.34
Scrobicularia	1	8.45	0.01	0.93
Freshness (maximum)				
Interaction	1	7.38	0.44	0.53
Condition	1	7.94	1.42	0.27
Scrobicularia	1	8.32	0.01	0.92

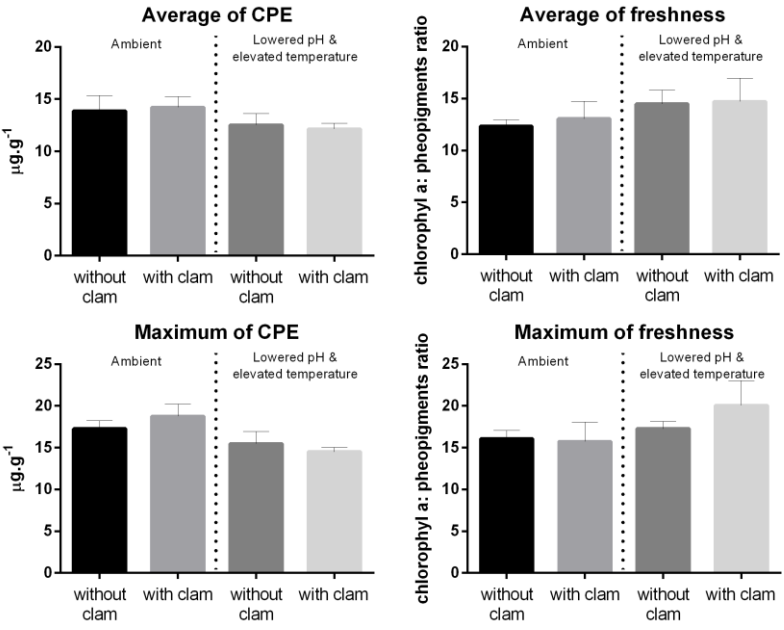

Figure 4A.4 Average and maximum of CPE concentration and freshness in the four treatments. Maximum CPE or freshness represents the maximum value of technical replicates in the four treatments. Error bars represent standard errors (± SE). Clam = *Scrobicularia plana*.

Table 4A.3 Linear mixed effects models comparing flux measurements in the four treatments. Significant results are in bold.

	ndf	***ddf***	***F-ratio***	***p value***
Oxygen consumption				
Interaction	1	7.69	0.54	0.48
Condition	1	7.75	0.93	0.36
Scrobicularia	1	8.64	0.88	0.37
NO_2^-				
Interaction	1	7.52	1.72	0.23
Condition	1	7.86	0.34	0.57
Scrobicularia	1	8.50	0.87	0.38
NO_3^-				
Interaction	1	7.29	0.84	0.39
Condition	1	7.96	0.64	0.45
Scrobicularia	1	8.27	1.23	0.30
$NO_2^- + NO_3^-$				
Interaction	1	7.28	0.73	0.42
Condition	1	7.96	0.62	0.45
Scrobicularia	1	8.26	1.34	0.28
Si				
Interaction	1	7.69	0.20	0.67
Condition	1	7.76	0.57	0.47
Scrobicularia	1	8.64	0.17	0.69
NH_4^+				
Interaction	1	6.70	1.64	0.24
Condition	1	7.81	1.01	0.35
Scrobicularia	1	7.80	0.66	0.44
PO_4^{3-}				
Interaction	1	7.68	0.002	0.97
Condition	1	7.77	13.98	**0.006**
Scrobicularia	1	8.62	0.11	0.75

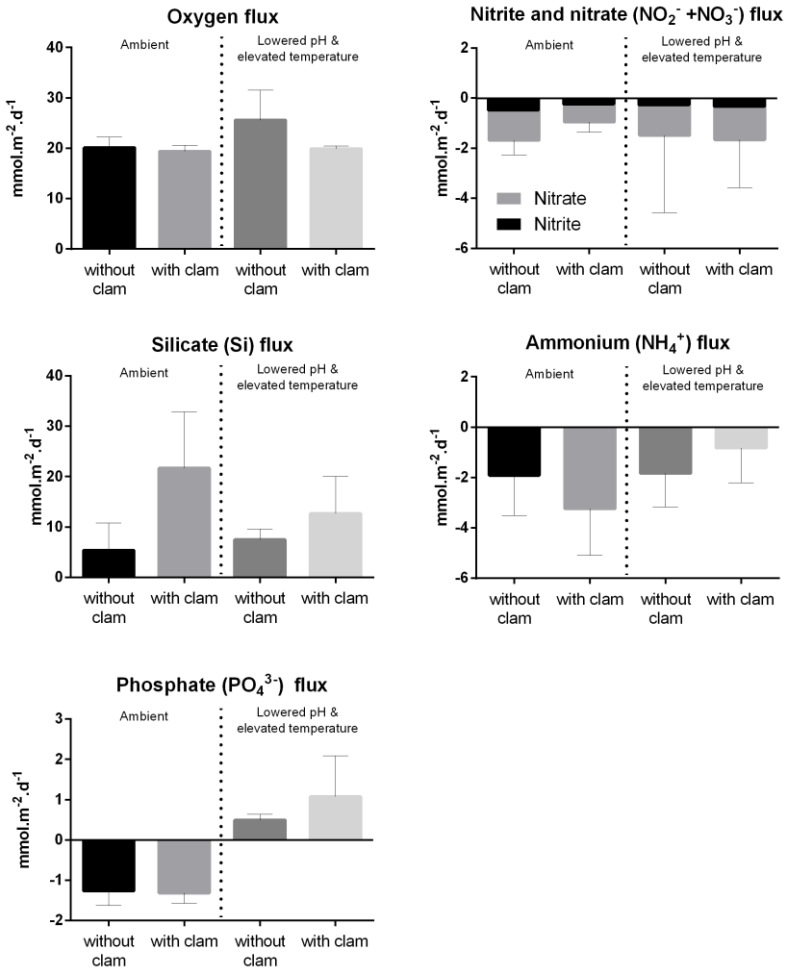

Figure 4A.5 Average fluxes (± SE) of oxygen, nitrite and nitrate, silicate, ammonium and phosphate in the four treatments. Clam = *Scrobicularia plana*.

Chapter 5

General discussion:

Marine ecology in a high pCO_2 world

The overall aim of the thesis was to investigate the mechanisms driving the future functioning and diversity of marine soft-sediment ecosystems with an emphasis on bivalves, specifically under the decline in pH and the increase in temperature predicted for the next 80 years (Collins et al., 2013). This was achieved through a series of laboratory experiments that (1) provided insights into the behavioural and physiological responses of selected benthic key species (chapter 2 and 3) and (2) unravelled how changes in these responses alter the species' contribution to ecosystem functioning (chapter 4) under multiple interactive stressors (acidification and warming of seawater).

The behavioural and physiological consequences of ocean acidification and warming on two key benthic tellinid bivalve species (*Cerastoderma edule* and *Scrobicularia plana*) are presented in chapter 2 and 3, respectively (Table 5.1). Chapter 2 demonstrates that survival of cockles remained high after 6 weeks incubation under a high pCO_2 (lowered pH and elevated temperature) condition. However, the condition index of cockles was synergistically reduced under this condition. Respiration rates increased under lowered pH, with highest rates measured when cockles were exposed to the combination of lowered pH and warm conditions. Calcification was negatively affected by lowered pH, while clearance rates increased under warm conditions and were generally lower in lowered pH treatments.

Table 5.1. Overview of different thesis chapters, experimental conditions and analysed biotic and abiotic parameters.

Stressors	Chapter 2	Chapter 3	Chapter 4
pH	●	●	
Temperature	●	●	
Interaction of pH and temperature	●	●	●
Presence key-species			●

Responses analysed	Chapter 2	Chapter 3	Chapter 4
Species level			
Mortality	●	●	●
Condition index	●	●	
Physiology			
❖ Respiration rate	●		
❖ Clearance rate	●		
❖ Calcification rate	●		
Behaviour (event and/or duration)		●	
Community level			
Macrobenthos abundance			●
Marcobenthos diversity			●
Abiotic parameters			
Sediment pigments		●	●
Porewater nutrients			●
Oxygen and nutrients fluxes			●

The cumulative effects of these parameters explained the reduction in the condition index. However, cockles may allocate extra energy to cover their increasing maintenance demand under stressful conditions, leaving less energy for other processes such as activity, growth and reproduction. In contrast, chapter 3 showed no effect of the aforementioned applied stressors on the condition index of *S. plana*, whose behaviour was however altered under both stressors. *S. plana* switched feeding mode from suspension to deposit feeding under acidic and warmer conditions, most likely (1) to limit the intake of low-pH

seawater into the body and (2) to cope with the higher metabolism due to elevated temperature.

In order to increase the ecological relevance of our study (see section 5.1.1), we included an intertidal community-based study (chapter 4). In chapter 4 (Table 5.1), we examined the effect of different ocean scenarios (current, future) by analysing the combined effects of abiotic and biotic factors on an intertidal macrobenthic community in the presence/absence of an intertidal key species (i.e. S. *plana*). Lowered pH (7.6) and elevated temperature (20 °C) led to a lower macrobenthos abundance compared to the current pH (8.1) and ambient temperature (17 °C) after 4 weeks of incubation, particularly when *S. plana* was absent. Our results suggest that macrobenthos responses to lowered pH and elevated temperature are taxon-specific. The gastropod *Hydrobia ulvae* and juvenile bivalves were the most vulnerable, while Oligochaeta were least affected by future ocean scenario. The abundance of *H. ulvae* and juvenile bivalves under the future ocean scenario was particularly lower, when *S. plana* was absent. Three dominant polychaete species showed very distinctive responses, with the abundance of *Heteromastus filiformis* being positively affected by *S. plana* irrespective of the climate scenario, whereas the opposite pattern was found for *Hediste diversicolor*. The abundance of *Pygospio elegans* was reduced under the future ocean scenario especially

when *S. plana* was absent. These results illustrate that in addition to direct effects of climate change (e.g. mortality) on species, populations and communities, indirect effects via changes in the ecosystem interaction network can also rapidly induce strong changes in soft sediment habitats.

In the following sections, the limitations and considerations of the experimental designs in the context of marine ecology will be addressed. Next, the need for insights into different levels of biological organisation will be elaborated. Thereafter, the fitness consequences of living in a high pCO_2 ocean for bivalve populations using preliminary experimental findings and literature will be discussed. Finally, the interaction of ocean warming and acidification with other environmental changes is presented, e.g. food availability and host-parasites relationships, and future challenges for the research will be considered.

5.1 Good practices for ocean acidification and warming research

The experimental work presented in this thesis covered the responses from species to community level under multiple stressors, providing valuable insights into the capability of an organism or a community of coping with changing environmental conditions. The following subsections cover methodological considerations including the ecological

relevance and interpretability of our experiments, the need to consider different spatio-temporal scales, the avoidance of pseudoreplication, and accuracy of laboratory setting and measurements.

5.1.1 Ecological relevance and interpretability of the experimental approaches

To accurately predict responses of marine organisms and ecosystems to global change, multiple approaches (e.g. field and laboratory work) are necessary in order to address all the potential permutations of change involved (Boyd et al., 2018). There are 5 identified approaches which each have their own advantages and limitations in predicting the biological responses to environmental changes (summarised in Table 5.2), and there is no single best method (Boyd et al., 2018).

In situ studies (e.g. modern observation, paleo and modern proxies) allow scientists to investigate species and ecosystem responses to changing environmental conditions by considering interactions of biotic and abiotic factors in a natural environment. However, it is extremely challenging to control other confounding factors that complicate the interpretation of data (Riebesell and Gattuso, 2015), as well as to quantify the underlying mechanisms or processes of single species in the natural environment. On the other hand, laboratory experiments

(e.g. micro- and mesocosms) measuring individual species and community responses in a controlled and isolated manner can be particularly useful (chapter 2 and 3). However, information obtained from single-species experiments is difficult to translate into the natural environment without the knowledge of species interactions or interactions with other environmental factors (Höss and Williams, 2009).

Although both natural (field) and more controlled conditions (laboratory) have their own advantages and disadvantages, nevertheless, there is a way forward in linking between species physiological responses and community/ecosystem impacts by co-designing appropriate approaches, e.g. upscaling from species responses and downscaling from community/ecosystem level (Boyd et al., 2018). Information obtained at the community level could facilitate the identification of those physiological response mechanisms that prevail in the complex ecosystems. In turn, improved knowledge of physiological mechanisms can subsequently be used as a guideline to design and conduct a more complex experiment involving community assemblage (Boyd et al., 2018, e.g. chapter 4, Dashfield et al., 2008). Hence, information obtained from higher hierarchical levels (e.g. community, ecosystem) can be extrapolated more precisely to the ecological context in order to fill the knowledge gap (e.g. species interactions and responses to other environmental

factors), allowing to make accurate and precise predictions of these complex processes (Benton et al., 2007).

5.1.2 Different spatio-temporal scales

Understanding spatial and temporal patterns and scales remain a central challenge in the field of ecology (Levin, 1992). The analysis of ecological causes and the prediction of consequences of global change requires the integration of phenomena that occur at different spatio-temporal and organisational scales (Levin, 1992). Both of our study species (i.e. *C. edule* and *S. plana*) have wide geographical distributions (Malham et al., 2012; Santos et al., 2011), including different *in situ* pH (chapter 2; Schade et al., 2016) and temperature ranges (Bazaïri et al., 2003; Compton et al., 2007; Hughes, 1971; Rygg, 1970). These different spatial scales are highly beneficial for field studies across gradients of acidification and warming that provide interesting *in situ* information and can be complementary to the insights from laboratory studies. For example, studies have translated laboratory experiments and modelled predictions of ocean acidification into field conditions at volcanic vents (Calosi et al., 2013; Hall-Spencer et al., 2008), which provide realistic insights into how shallow marine communities will respond to low pH conditions in the future.

Table 5.2 Advantages and limitations of five scientific approaches used to improve our understanding of the effects of environmental drivers on marine organisms. Manipulative microcosm experiments are frequently used in a controlled manner to investigate responses of single species or small communities; mesocosm experiments are larger-scale experiments used to manipulate entire marine communities. Paleo-ceanography studies the past natural climate shift (Paleo-Proxies), Modern natural environments are used as proxies of particular anthropogenic change processes, e.g. acidification resulting from natural CO_2 vents; Modern observations record extended spatial or temporal aspects of global environmental change. Table modified from Boyd et al. (2018).

Approaches and examples	Advantages	Limitations	Reference
Manipulative experiments (Microcosms)	• Many highly controlled and targeted treatments • Extensive replication and statistical power possible	• Few species • Limited ecological relevance	(Wernberg et al., 2012)
Manipulative experiments (Mesocosms)	• Include many species interactions and capture indirect effects • High ecological relevance	• Expensive and logistically difficult (especially for multiple drivers, long-term studies) • Few replicates possible, low statistical power	(Alsterberg et al., 2013)
Paleo-proxies (Paleocene-Eocene Thermal Maximum)	• Natural analogs for anthropogenic change • Investigate globally or regionally integrated ecosystem impacts	• Emergence and extinction slower than anthropogenic change scenarios • Low temporal and taxonomic resolution	(Gibbs et al., 2016)
Modern proxies (Natural CO_2 vents)	• Natural analogs for anthropogenic change • Large, observable signals and ecosystem responses	• Driver combinations differ from future scenarios • Recruitment from outside vent systems	(Hall-Spencer et al., 2008)
Modern observations (Ocean time-series)	• Detailed records over relevant timescales of change • Extensive biological, chemical and physical supporting data sets	• Limited spatial resolution • Climate variability can obscure long-term trends (low signal:noise ratio)	(Rivero-Calle et al., 2015)

Ocean acidification and warming are global scale phenomena that occur gradually and over a longer period of time (Rhein et al., 2013). Acquisition of more accurate predictions of the ecological consequences of these phenomena requires an improved knowledge of longer term response mechanisms that moderate the vulnerability of species to a changing environment (Form and Riebesell, 2012). However, the majority of the currently available evidence on ocean acidification and warming is based on short-term perturbation experiments, typically ranging from days to weeks (Kroeker et al., 2013), without considering response mechanisms such as acclimation and adaptation (Hernroth et al., 2011; Pörtner, 2008), behavioural or physiological compensatory mechanisms associated to food availability and/or seasonal variability (Kroeker et al., 2011; Seibel et al., 2012). This is because long-term experimental studies can be very challenging in terms of technical or logistic constraints as well as financial limitations which often restrict the duration and scale of experiments.

In this PhD thesis, studied species and communities were incubated in manipulated seawater for a duration ranging from 4 to 6 weeks, which is considered as short-term incubations. One of the concerns about short-term laboratory incubations is that the short exposure times may be insufficient to allow potential acclimation and evolutionary adaptation under a changing

environment (Stockwell et al., 2003). For example, a study demonstrated that calcification rates of the cold-water coral *Lophelia pertusa* decreased under low pH after 8 days but increased after 6 months of incubation due to acclimation (Form and Riebesell, 2012). Another study showed that the behaviour and growth (i.e. bioturbation and bioirrigation) of the intertidal polychaete *Alitta virens* were altered only after an 18-month incubation under high pCO_2 and elevated temperature conditions (Godbold and Solan, 2013). These laboratory observations illustrate that compensatory mechanisms of some species require extended time to manifest. On the other hand, long-term incubation at sub-optimal laboratory conditions can invoke changes in condition/fitness (e.g. chapter 2) as well as other behavioural (e.g. Bibby et al., 2007: predator avoidance) or physiological responses (e.g. Wood et al., 2008: muscle depletion). One way to account for these feedback mechanisms is to prolong the duration of experiments by including the seasonal fluctuation (e.g. Gazeau et al., 2014). However, the prolongation increases the probability of including other processes (e.g. reproduction) that have the potential to alter the outcome of the result. For example, species may respond differently to acidified warmer seawater in different seasons of the year or life cycle stage (Byrne and Przeslawski, 2013) and different interactions may take place. Therefore, experimental

studies need to be designed optimally in order to effectively access the temporal scale of the parameters of interest.

5.1.3 The need to avoid pseudoreplication

Conducting an appropriate, valid analysis of experimental data is one of the fundamental components in science. Pseudoreplication is frequently linked to weak experimental designs (Kroodsma et al., 2001). According to Hurlbert (1984), pseudoreplication is defined as "the use of inferential statistics to test for treatment effects with data from experiments where either treatments are not replicated (though samples may be) or replicates which are not statistically independent". Avoiding pseudoreplication can be extremely difficult due to the complexity of the statistical procedures needed (Millar and Anderson, 2004).

The treatments presented in this PhD thesis are pseudoreplicated in the experimental designs. According to Cornwall and Hurd (2016) and Hurlbert (1984), the experimental design of the tank array in chapter 2 is categorised as clumped segregation with interdependent replicates within treatment, where seawater from a single manipulation tank flowed into three experimental aquaria/units of each treatment and circulated back to the manipulation tank. Whereas in chapter 3 and 4, the design of our experiment tank arrays represents isolation segregation, where

experimental units were isolative segregated in a manipulation tank of each treatment (Cornwall and Hurd, 2016; Hurlbert, 1984). We are aware that our experimental designs are violating the principle of interspersion and this may consequently undermine the confidence in accurately reporting our results. However, as Hurlbert (1984) mentioned "despite errors of design or statics", the results "nevertheless contain useful information". Therefore, we took into account two precautionary steps in analysing, reporting and concluding our findings.

Firstly, we genuinely reported the details of our experimental procedures and designs (e.g. layout and numbers of tanks) stating how treatments were created and the number of "replicates" present in each treatment. These details with respect to replication and randomisation are very important to allow scientists to compare results of different studies (Table 1 in Cornwall and Hurd (2016)). Secondly, it is demonstrated that the problem of pseudoreplication can be eliminated by taking into account the structure of the randomness inherent in the data. Thus, proposing some appropriate model-based remedies, including mixed-effects modelling and generalised linear mixed models, exist (Millar and Anderson, 2004). In most of the cases, we chose to perform statistical analyses with linear mixed-effects models and generalised linear mixed-effects models that take into account the non-independence of these data, allowing

random intercepts for aquaria/tank identity, in order to accurately report our findings. Alternatively, we could have repeated the experiment many times and treat the mean as the experimental unit, or to take the mean of the examined unit's response over a series of repeated measures (Cornwall and Hurd, 2016). Such repeated measurements were not performed due to time constraints.

5.1.4 Accuracy of laboratory setting and measurements

This section discusses some other issues related to our experiments and proposes some good laboratory practises for future research e.g. the measurement of seawater total alkalinity (TA) and the regulation of a manipulated parameter (i.e. pH).

There were differences in the seawater TA values among the three experiments; specifically, the TA in chapter 2 was very high in comparison to chapters 3 and 4. Since the laboratory is not located near the sea, the seawater from North Sea origin (filtered through a sand filter) was delivered multiple times during the course of this PhD thesis. Hence we expected some differences in the seawater TA, since TA varies seasonally in the North Sea (Hoppema, 1990). However, this does not explain the high TA values in chapter 2. We think that the high TA values in chapter 2 might be due to the difference in measurement method used. The seawater TA in chapter 2 was measured using a Mettler

Toledo G20 compact titrator, whereas the TA from chapters 3 and 4 was measured with a HydroFIA TA (CONTROS Systems & Solutions GmbH). Low accuracy of TA in chapter 2 prompted us to search for alternative methods, which is why in the subsequent chapters we chose to measure TA using a high-accuracy HYdroFIA TA titrator in collaboration with the Flanders Marine Institute (VLIZ).

Furthermore, seawater pH in chapter 2 (Figure 2A.1) seems to rise steadily over the course of the experiment. One-way ANOVA shows that there was a time effect (day) on the pH of control pH treatments. As we discovered that regulation of seawater pH using pH in NBS scale did not help to stabilise the pH throughout the experiment (i.e. chapter 2, Figure 2A.1), we decided to use an external pH meter to intercalibrate against the pH sensor in the IKS manipulation system in the subsequent chapters (e.g. Figure 3A.2 , 4A.1 and A.1). Furthermore, TRIS buffer solution was used to convert pH in NBS scale to pH in total scale (e.g. Figure 3A.2 , 4A.1 and A.1). The use of pH in total scale is highly recommended and it is part of good practises for ocean acidification research (Riebesell et al., 2011).

5.2 The need for insights at different levels of biological organisation

Biological communities are created by complex interactions between organisms with other species as well as their environment (Bolker et al., 2003). Therefore, it is necessary to assess different levels of biological organisation such as individuals, populations, communities and ecosystems in an integrated approach to better understand their response to changing environments. In this thesis, behavioural and physiological responses of bivalves (chapter 2 and 3) were investigated to assess impacts of ocean acidification and warming in a multi-dimensional approach. These different responses are relevant because behavioural modification is predominantly the first response by animals to environmental change (Tuomainen and Candolin, 2011), whereas physiological performance represents an important determinant of a species' tolerance towards environmental change (Doney et al., 2012). Both of these changes will in turn affect species interactions and ecological processes (Candolin and Wong, 2012; Doney et al., 2012). The obtained information from the population responses (chapter 3) can thus translate into a more complex community-based approach (chapter 4). Stressors such as ocean acidification and warming can modify the behaviour, density, and distribution of populations via interspecific interactions

(Dashfield et al., 2008; Hale et al., 2011). These interspecific interactions sum up the important connections between animal behaviour and physiology, environmental change, and the ecological processes that govern marine ecosystems (Gaylord et al., 2015).

In the following section, effects at the individual level from this thesis will be presented, with the overall findings on the conditional, behavioural and ecophysiological response of *C. edule* and *S. plana* to ocean acidification and warming (chapter 2 and 3) being summarised and set into context in Figure 5.1 and Figure 5.2. The relative pelagic trophic interaction strength associated to the clearance rate of *C. edule* was added to Figure 5.1, to demonstrate the consequences of changes on the primary productivity and recruitment success (i.e. survival of bivalve larvae during their initial pelagic life stage in the ecosystem, Newell and Koch, 2004; Thrush et al., 1996). Note that the lower condition index in the combined treatment may reduce the population fitness at the longer term and thus influence biotic interaction strengths at the population level.

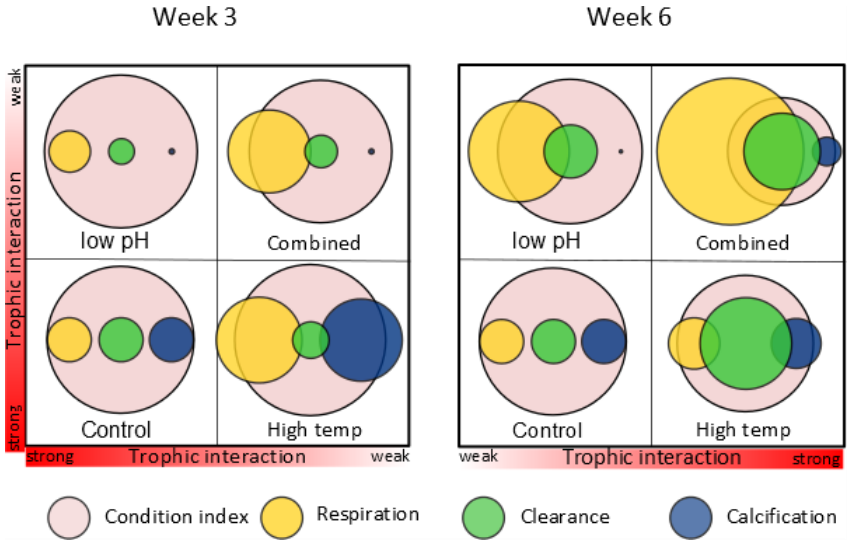

Figure 5.1 Summary of the conditional and ecophysiological responses to ocean acidification and warming of *Cerastoderma edule*, at different times of exposure (week 3 and 6), with the corresponding relative pelagic trophic interaction strength associated to the changes in the cockles' clearance rate. The radius of each circle represents the relative measured value compared to the control in the respective week.

On the other hand, behavioural changes of *S. plana* under different levels of pH and temperature can influence both pelagic and benthic trophic interaction, as changes in deposit feeding of *S. plana* can influence trophic interactions within the benthic community, while change in suspension feeding can affect the pelagic trophic interactions (see below). Since our experiment did not quantify the strength of such trophic interactions, they are not included in Figure 5.2.

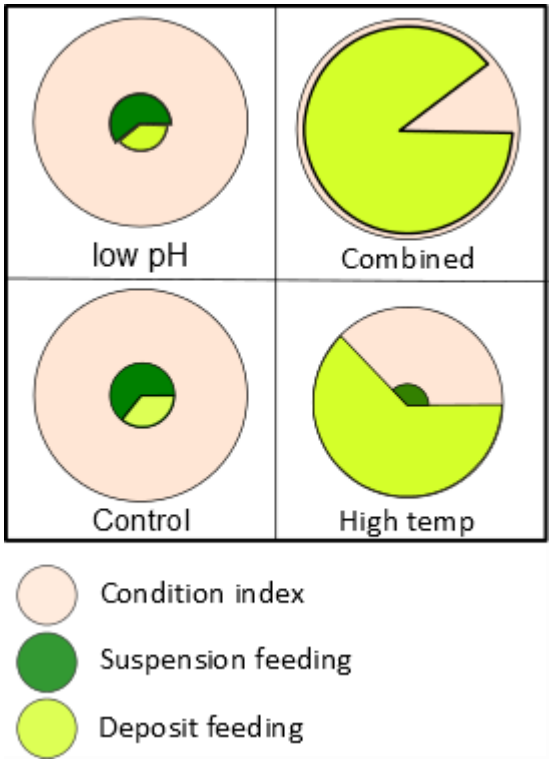

Figure 5.2 Summary of the conditional and behavioural responses of *S. plana* to ocean acidification and warming after 3 weeks of incubation. The radius of each circle represents the relative measured value compared to the control. The degree of the feeding circle (i.e. suspension and deposit) represents the frequency of the respective feeding modes, whereas the area of the feeding circle represents the total time spent on feeding.

Estuaries are critical transition zones that provide crucial ecosystem functions, e.g. nutrient cycling and primary production, decomposition and regulation of biotic and abiotic fluxes from terrestrial, riverine, and marine environments (Levin et al., 2001). Due to the combination of the inherently low biodiversity and the high relevance for ecosystem functioning of estuarine environments, changes in diversity in these systems are particularly important (Levin et al., 2001). In marine soft

sediments, large organisms play an important role in influencing ecosystem functions and trophic interactions, e.g. by altering hydrodynamics and biogeochemistry within the sediment (Austen et al., 2002; Herman et al., 1999; Levin et al., 2001; Thrush et al., 2006). However, estuarine habitats will be among the most affected by ocean acidification and warming by the end of the century (Collins et al., 2013). Consequently, these environmental changes will affect the behaviour and physiology of large organisms and thus modify their relationship with community and ecosystem processes (Brierley and Kingsford, 2009). In the following, we summarise and extrapolate our findings on the responses of two intertidal tellinid bivalves (*C. edule* and *S. plana*) incubated under lowered pH and elevated temperatures, in the perspective of population, community and ecosystem responses.

There is growing evidence suggesting that ocean acidification and warming have direct impacts on primary production (Hallegraeff, 2010), community structure (Hale et al., 2011; Mevenkamp et al., 2017b), host-parasite interactions (Magalhães et al., 2018), predator-prey interactions (Watson et al., 2014) as well as the behaviour and physiology of marine animals (reviewed in Nagelkerken and Munday, 2016; Parker et al., 2013, e.g. chapter 2, 3). However, there is still a lack of studies on the indirect or cascading effects of these stressors mediated by key species to the ecosystem (but see Alsterberg et al., 2013; Dashfield

et al., 2008). Here, we use our findings combined with previous studies to explain the potential effects of future ocean acidification and warming on intertidal benthic bivalves (*C. edule* and *S. plana*), their relationship to different trophic levels and abiotic factors and concomitant knock-on effects on the functioning of intertidal soft-sediment habitats (Figure 5.3).

Ecosystem functioning can be impacted even before large organisms go locally extinct (Thrush et al., 2006). In coastal soft-sediment habitats, stress-induced changes in organismal behaviour and physiology can affect biodiversity and ecosystem properties e.g. biogeochemistry and primary production (Candolin and Wong, 2012; Doney et al., 2012). Chapter 4, Hale et al. (2011) and Mevenkamp et al. (2017) demonstrate that future ocean conditions can alter the meio- and macrobenthic community diversity and abundance directly and indirectly through changes in biotic interactions (Figure 5.3b). Especially resilient species to ocean acidification and warming could, therefore, play a critical role in mediating these stressful conditions for other benthic organisms in the future intertidal ecosystem.

On the other hand, more vulnerable species such as cockles may experience a deterioration of fitness or condition due to the direct stress effects from high pCO_2 and warmer temperatures (chapter 2) as well as indirect effects e.g. an increase of parasite infection

(Figure 5.3e, for details see 5.4.1), ultimately leading to a collapse of local populations (Figure 5.3f). Consequently, the loss of cockle populations will affect cockle-mediated effects on ecosystem properties, e.g. the control of surface dwelling species, enhancing microalgal biomass (Figure 5.3h). This can further reduce the resilience of estuarine soft-sediment systems towards future environmental change (Beukema and Cadée, 1996; Van Colen et al., 2013), which may cause a potential long-term detrimental change to biodiversity (Beukema et al., 1999; Thrush et al., 2003).

Furthermore, these two bivalves also represents an important food source for oystercatchers and eiders (Figure 5.3i , Beukema and Dekker, 2005). The increase of total siphon exposure time (i.e. deposit feeding) of *S. plana* under future ocean conditions increases the risk of predation by birds, crabs and fish. The damaged tissue is able to regenerate within a few days (Hodgson, 1982), however, extra energy allocation is needed for the repair mechanism which may lower the condition or fitness of *S. plana* over a long period of time. Therefore, the increase of predation risk of *S.plana* together with the future reduction of cockle abundance may have significant consequences for the functioning of estuarine ecosystems at all trophic levels (Figure 5.3g, i, Beukema and Cadée, 1996).

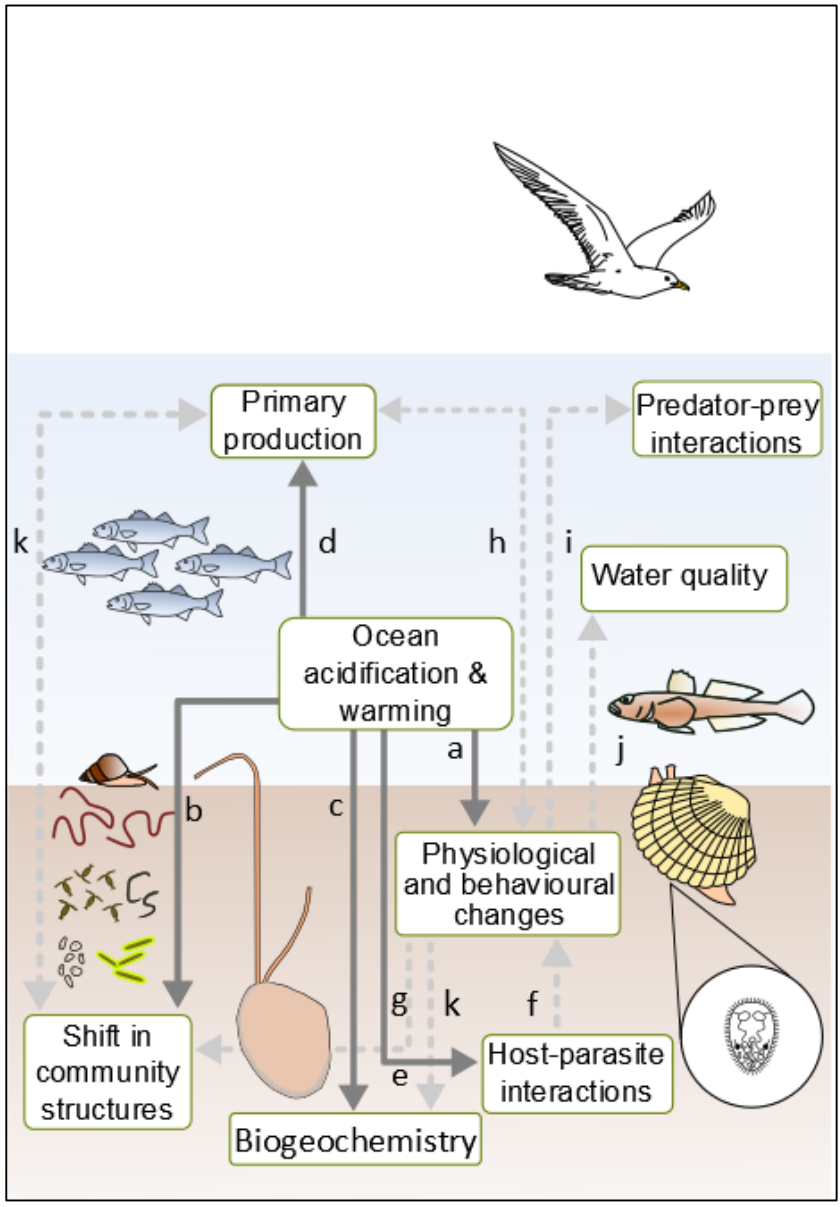

Figure 5.3. Schematic overview of the response of *Cerastoderma edule* and *Scrobicularia plana* to future ocean conditions and concomitant impacts on other trophic levels and abiotic factors in a coastal soft-sediment habitat. Dark grey lines represent direct effects (a-e) while dashed lines indicate indirect effects (f-k) of ocean acidification and warming mediated by behavioural and physiological changes of these bivalves. Note that this figure represents a simplified version of a non-exhaustive list of biotic and abiotic interactions.

Our findings show that future ocean conditions have direct effects on the behaviour and physiology of intertidal bivalves, e.g. a reduction of feeding activity (i.e. suspension feeding, Figure 5.3a). The reduction of feeding activity decreases these species' ability to "top-down" control or prevent eutrophication and harmful algal blooms in their habitats (Figure 5.3j), consequently affecting ecosystem properties such as microbial activity, water quality and primary production (Figure 5.3h, j, e.g. Baker et al., 2015; Lohrer et al., 2004; Mermillod-Blondin et al., 2005, 2004; Newell and Koch, 2004; Thrush et al., 1992). On top of that, the future ocean conditions can alter the microphytobenthos community structure both directly (Figure 5.3d, reviewed in Liu et al., 2010) and indirectly (via the behavioural and physiological changes in macrofauna, Chennu et al., 2015; Hylleberg, 1975), which may influence the productivity of the whole ecosystem (Figure 5.3h). Finally, the future ocean conditions can also influence biogeochemical cycling directly (Braeckman et al., 2014b) and indirectly (Braeckman et al., 2014a). These indirect effects mainly derived from behavioural and physiological changes of large organisms, due to changing of environmental conditions, that may influence the nutrient cycling. The behavioural changes of macrobenthic bioturbators such as *C. edule* and *S. plana* under such conditions have important repercussions for benthic ecosystem functioning.

In summary, *C. edule* and *S. plana* are undoubtedly key species in the intertidal habitats under the current conditions. However, cockles seem to be more vulnerable while *S. plana* appears to be rather resilient to future high pCO_2 warmer conditions. Nevertheless, these consequences of future ocean conditions largely depend on adaptation capacity and tolerance of these species to changing environmental conditions during the different life stages. In what follows, a discussion on the impacts of acidification and warming on bivalve population fitness will be presented with our preliminary results in the light of current knowledge.

5.3 Impacts of acidification and warming on bivalve populations fitness

In this PhD thesis, only adult specimens were studied, while ocean acidification has greater negative impacts on the early-life stages of marine calcifying organisms rather than on juveniles and adults (Parker et al., 2013; Ross et al., 2011). Studies and theory demonstrate that ocean acidification can particularly impact the early-life stages of molluscs because (1) they deposit amorphous calcium carbonate, which is a precursor phase for aragonite and is far more soluble compared to aragonite and calcite, to form their shells and outer skeletons (Orr et al., 2005; Ross et al., 2011; Weiss et al., 2002); and (2) the lack of specialised

ion-regulatory epithelia in sperm, eggs, zygotes and embryonic stages (Melzner et al., 2009). Previous studies reported that the early-life stage development of bivalves was negatively affected by ocean acidification, resulting in e.g. suppressed growth, lowered survival, reduced fertilization and gamete collision as well as malformations (Gazeau et al., 2011, 2010; Shi et al., 2017; Van Colen et al., 2012a; Watson et al., 2009).

Furthermore, as thermal stress can affect all life history stages (Pörtner, 2008), the combination of both stressors (i.e. acidification and warming) may have detrimental effects on the early-life stages of bivalves. Broadcast spawning marine organisms are particularly vulnerable to acidification and warming because fertilisation of gametes and development of planktonic larvae take place in the water column with direct exposure to warmer and acidified seawaters (Ross et al., 2011). Exposing two oyster species (*Crassostrea gigas* and *Saccostrea glomerata*) to acidified and warmer seawater resulted in reductions of fertilization success, embryonic development and larvae size, and increased the number of shell malformations (Parker et al., 2010). Furthermore, the survival, lipid synthesis, growth and development of *Argopecten irradians* and *Mercenaria mercenaria* larvae were significantly lowered under acidified and warmer conditions (Talmage and Gobler, 2011).

Although there is still lack of research on the combined effects of both acidification and warming on the early-life stages of marine bivalves, the prevailing negative effects mentioned above may potentially affect survival and delay the settlement of larvae. In general, a prolongation of the larval life stage is considered to be unfavourable, because it increases the risk of predation in the water column, especially when a delay in settlement is combined with the absence of well-calcified skeletons and shells (Ross et al., 2011). Moreover, smaller larval size can decrease the feeding efficiency of bivalve larvae and lead to mortality due to starvation (Kurihara et al., 2007; Talmage and Gobler, 2011). Consequently, the cumulative effects of lowered pH and elevated temperatures can critically modify the composition and fitness of larvae, cause delays in settlement and reduce larval survival rates in the water column, which may alter recruitment, post-settlement mortality, and ultimately adult bivalve populations (Ross et al., 2011).

While the embryonic/larval development of bivalves is well studied, there is a lack of studies on the consequences of combined ocean acidification and warming on gamete production (but see Shi et al., 2017). Therefore, we conducted a 6-week experiment to quantify gamete production of two bivalve species (i.e. *Cerastoderma edule* and *Limecola balthica*) across different levels of temperature (7 and 10 °C) and pH (7.5 and 8.0)

in a fully crossed design (For material and methods, result see Appendix). Our study shows that there was no significant effect on spawning success of *C. edule*, however, significant effects were found on the spawning success of *L. balthica* (Table A.2 and Figure A.3). Remarkably, no spawning of *L. balthica* was observed under the combined stressors treatment. This indicates that the combination of both stressors may have detrimental effects on the reproduction of *L. balthica* (Figure A.3). In general, more sperm was produced under lowered pH treatments for both species. On the other hand, less eggs were produced under lowered pH treatments for *L. balthica*. This suggests that low pH may enhance polyspermy, at least for *L. balthica*.

5.4 Interaction of acidification and warming with other environmental changes

Interacting environmental factors are necessary to consider because they may influence the results of experiments under a high pCO_2 warmer ocean. Among these factors are host-parasite interactions and habitat energy availability (i.e. food supply) that could potentially influence species performance. The interaction between acidic warmer water and these factors could be beneficial or detrimental to bivalves (MacLeod, 2017; Thomsen et al., 2013) and should thus be integrated into a more climate-driven research.

5.4.1 Host and parasites interactions

At present, the effects of ocean acidification and warming on marine animals and ecosystems have been intensively studied, whilst the effects of these stressors on host-parasite interactions remain largely neglected (Macleod and Poulin, 2012). Parasites (e.g. trematodes) can alter the behaviour, fitness, physiology, reproduction and survival of their host, thus playing a crucial role in regulating host species populations, biodiversity and their contribution to ecosystem functioning (Hatcher et al., 2012; Mouritsen and Poulin, 2002; Poulin and Mouritsen, 2006; Thompson et al., 2005).

As bivalves are particularly vulnerable to ocean acidification (e.g. Gazeau et al., 2014), theory suggests that parasitic infection of bivalves could result in the increase of pathogenicity given that parasites may be more resilient against ocean acidification (Macleod and Poulin, 2012). Studies showed that ocean acidification increases the energy demand for basal maintenance and calcification of marine organisms (chapter 2; Pan et al., 2015). On top of that, parasite infections may further increase the hosts' metabolic demand (Choi et al., 1989; Soudant et al., 2013). Moreover, theory suggests that endoparasites exposed to lowered pH elevate their metabolic energy consumption and subsequently increase the energy demand of the host, forcing the infected host to adapt to the overall energy budget increase

(MacLeod, 2017). Failure to cope with an elevated energy budget and a reduction in food intake (i.e. in cockles, chapter 2) under low pH conditions, would gradually deplete the energy storage of the infected host, eventually causing mortality (MacLeod, 2017). In addition, laboratory experiments have demonstrated that elevated temperature significantly increased the infection of second intermediate hosts (i.e. cockle) by the trematode *Renicola roscovita* (Thieltges and Rick, 2006). Therefore, the combination of acidification and warming may increase the infectivity of cercariae on bivalve species.

The discovery of free swimming cercariae of trematode *Bucephalus minimus* in the spawning experiment enabled the quantification of parasite infection percentage in each treatment (see Appendix for material and methods, results). The infection rate of cockles by *B. minimus* was significantly higher under lowered pH and elevated temperature treatment (83 %) compared to other treatments (Table A.3 and Figure A.4). This suggests that cockles are more vulnerable to parasite infection under the effects of high pCO_2 warm condition. Similarly, a recent study reported that low pH (7.8) increased the infection success of another trematode parasite *Himasthla elongata* on cockles and decreased cockle enzymatic activity particularly in the infected cockles (Magalhães et al., 2018). Reductions of cockle condition and metabolism, and an increase of cellular damages

under low pH (chapter 2, Magalhães et al., 2018) are likely to lower their immunity systems against the parasite infection.

The infection of *B. minimus* in cockle begins in the digestive gland and gonad (De Montaudouin et al., 2000; Magalhães et al., 2015; Pina et al., 2009). Studies demonstrated that *B. minimus* infection causes castration of cockles and starvation to dead due to the autolysis of the digestive tract (Carballal et al., 2001; Dubois et al., 2009), which ultimately may lead to population decline and local extinctions.

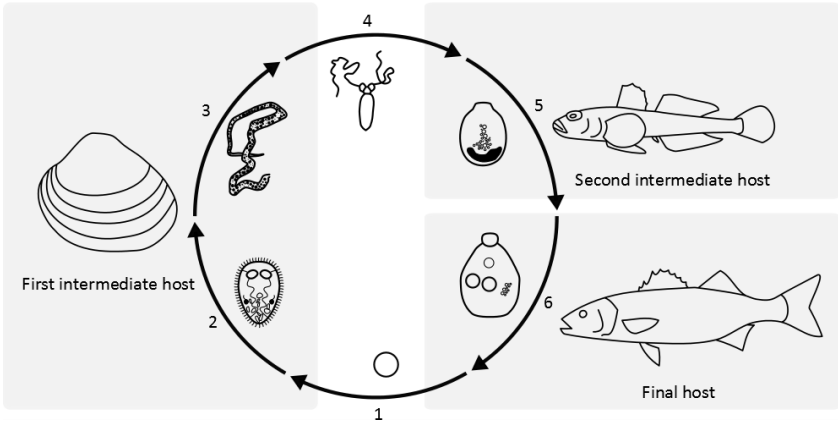

Figure 5.4. Schematic diagram of the life cycle of the trematode *Bucephalus minimus* with different life stages and its respective hosts: 1. egg; 2. miracidium; 3. sporocysts (growth in the first intermediate host *Cerastoderma edule*); 4. free-swimming cercaria; 5. metacercaria (growth inside the second intermediate host *Pomatoschistus* spp.); 6. adult stage (growth in the final host *Dicentrarchus labrax*). Modified from Magalhães et al. (2015).

Overall, our laboratory study shows higher infection rate of *C. edule* by *B. minimus* (61 – 83%) compared to that of the field population (Slikken Van Viane) (10 - 30% (Van den Brink et al., 2010)). The high infection rates may be due to the seawater

circulation system in our laboratory setting, where cockles were constantly exposed to the trematode. Apart of being the first intermediate hosts to *B. minimus*, cockles also serve as the second intermediate hosts for two other parasites (namely *Himasthla* spp. and *Parvatrema minutus*). The infection rates of *Himasthla spp* and *P. minutus* can reach up to 100% and 80% in Slikken van Viane, respectively (Van den Brink et al., 2010). Therefore, enhanced parasite infections under future ocean conditions may further decrease population fitness, ultimately leading to local extinctions.

5.4.2 Food availability

The basics of energy balance in animals show that organisms highly depend on food consumption for activity, development, growth, maintenance and reproduction (Sokolova et al., 2012). This indicates that food availability in the natural habitat could be a decisive factor for organisms to outweigh the negative impacts of stressors such as lowered pH and elevated temperature. For example, studies demonstrated that marine calcifying organisms such as the barnacle *Amphibalanus improvisus*, larvae of the Olympia oyster *Ostrea lurida* and the juvenile blue mussel *Mytilus edulis* were able to withstand the negative effects of high pCO_2 when the food supply was abundant (Hettinger et al., 2013; Pansch et al., 2014; Thomsen et al., 2013).

Concurrent with the above findings, other studies suggested that the future high pCO_2 ocean can affect photosynthesis and growth in micro- and macroalgae (reviewed in Beardall et al., 2009; Wu et al., 2008). However, the effect on phytoplankton or macroalgae production can be either positive (Riebesell et al., 2007; Wu et al., 2008) or negative (Gao et al., 2012; Wu et al., 2008). Moreover, studies showed that warming of seawater causing large-scale shifts in both and global phytoplankton composition e.g. diatoms, dinoflagellates and coccolithophores (Hallegraeff, 2010; Hare et al., 2007; Merico et al., 2004). Hence, cumulative effects of elevated temperature and pCO_2 may either enhance or reduce the food availability in the future ocean, which in turn affects the marine food webs. In addition, these stressors may affect some phytoplankton by shifting their blooming period or inducing poleward migration, creating a mismatch between different trophic levels (Edwards et al., 2004).

5.5 Future research directions

In what follows, I aim at giving some guidance, which one may find useful in his/her endeavour towards better comprehension of this interesting but challenging field of research. This thesis highlights the responses of bivalve species under future high pCO_2 warmer conditions and the subsequent cascading effects on the functioning of intertidal soft-sediment habitats. First, I

would like to encourage those who are new to this research field to follow the "Guide to Best Practises for Ocean Acidification Research and Data Reporting (Riebesell et al., 2011) for seawater manipulation methods; I also recommend to follow the guideline of Cornwall and Hurd (2016) on the use of experimental units in experimental design in order to prevent artefacts associated to inappropriate replication and randomisation.

Since changing environmental conditions can affect the energy balance of an organism (Sokolova et al., 2012), it would be appropriate to quantify energy budgets using methods such as Dynamic Energy Budget models (DEB) or scope for growth. This will improve our knowledge on the potential energy cost of stress responses of an organism under environmental stress. Based on my knowledge, I also suggest to integrate different levels of biological organisation in future research on the impacts of ocean acidification and warming on intertidal benthic ecosystems. The integration of multiple biological levels will undoubtedly improve our understanding of ecological processes from species to community/ecosystem interaction responses.

Most studies on the effects of ocean acidification and warming on marine ecosystems are short-term, which involves only a small fraction of an organism's life span (Pansch et al., 2014). Even longer-term studies often only cover within-generation responses to changing environmental conditions that primarily

reveal physiological acclimation capacities of individual organisms (but see Dupont et al., 2013, 2010b; Parker et al., 2012). However, only transgenerational responses can allow a full assessment of the populations' adaptive capacity to environmental shifts (Sunday et al., 2011). Therefore, future research should consider the importance of "carry-over" effects to assess the cross-generational adaptive capacity of populations to future ocean conditions (e.g. Dupont et al., 2013; Parker et al., 2012).

A more comprehensive, ecosystem-based approach accounting for other important interacting biotic factors e.g. host-parasite and predator-prey relationships (Chivers et al., 2014; Magalhães et al., 2018) which can regulate individuals, populations and communities represents a second important challenge for future research on ocean acidification and warming. These interacting biotic factors have been largely ignored, despite the fact that such relationships are among the most fundamental ecological interactions (MacLeod, 2017). Furthermore, incorporating biomarkers such as fatty acids and stable isotopes can significantly improve our understanding on changes in the food-web structure of intertidal communities. Ideally, laboratory experiments should be conducted in a way that follows the natural variability in the field (e.g. food availability and

seasonality) to realistically predict the impacts of acidification and warming in future oceans.

In addition, it would be interesting to incorporate micro- and meiobenthic communities in order to assess the effects of acidification and warming on these communities and disentangle their relationship with the macrobenthos for a better understanding of ecosystem functioning under future ocean conditions. Finally, field observations are highly recommended in order to verify the ecological relevance of laboratory experiments. The combination of field (e.g. Hall-Spencer et al., 2008) and laboratory studies on single-species or community responses could therefore allow accurate predictions of future ocean conditions.

We, the generation that faces the next century, can add the… solemn injunction, “If we don’t do the impossible, we shall be faced with the unthinkable.”

Petra Kelly

(1947 -1992)

Appendix

Supplementary material to chapter 5

A.1 Material and methods

A.1.1 Collection and incubation of specimen

In spring 2017, *Limecola balthica* and *Cerastoderma edule* were collected at the lower intertidal zone at Paulina tidal flat, Westerschelde estuary (51° 20′ 55.4′′ N, 3° 43′ 20.4′′ E) and Slikken van Viane, Oosterschelde estuary (51° 37′ N, 4° 2′ E), respectively. Specimens and sediments were transported to the research facility within 2 hours. Clams and cockles were randomly allocated to 8 rectangular boxes (40 cm × 30 cm × 18 cm, 4 for each species) which were filled with respective sediments collected from sampling sites. These rectangular boxes were then allocated into four incubation tank systems, submerged in aerated seawater in a laboratory condition of 7 °C, pH 8.1 and salinity of 30, in order to allow acclimation prior to the manipulation of seawater pH and temperature. Specimens were fed once a week with Shellfish Diet 1800 (Reed Mariculture Inc., consisted of 40% *Isochrysis*, 15% *Pavlova*, 25% *Tetraselmis* and 20% *Thalassiosira weissflogii*).

There were two factors in our fully crossed experiment; temperature (2 levels: ambient or elevated) and pH (2 levels: ambient, lowered). Four holding tanks (100 L) were used to store the seawater and pumped from each holding tank to incubation tank (95 cm × 65 cm × 40 cm) and circulated back to the holding

tank through overflowing via silicon tubing. The aeration and manipulation of seawater was conducted in the incubation tanks. To maintain the salinity in the system, 20% of the seawater in each holding tank was replaced with fresh seawater in a weekly basis. The refreshment process took less than 20 min for the entire set-up (i.e. 4 treatments). All incubation tanks were exposed to a 12:12 h light and dark regime. Both species were fed twice per week with 2.5 mL of commercial Shellfish Diet 1800 diluted in 4 L of fresh seawater and distributed evenly to each holding tank.

After 4 days of acclimatisation, elevated temperature treatments were increased by 0.5 °C per day over 6 days until a temperature of 10 °C was achieved. pH manipulation started a day after the temperature manipulation. Lowered pH treatments were decreased 0.1 per day over 5 days until a pH of 7.5 was achieved. These conditions were maintained for 5 weeks (see Table A.*1* and Figure A.1) and at week 6 temperature in ambient/control temperature treatments was increased gradually by 0.5 °C per day over 6 days until 10 °C before inducing them to spawn. The seawater temperature was regulated using temperature heater/chiller controllers (Teco Refrigeration technologies; Model: TK200H), whereas, the seawater pH of lowered pH treatments was manipulated and controlled by IKS Aquastar Industrial control panel coupled with pH glass-electrodes

(compression-proof) via the bubbling of CO_2. On a weekly basis, all pH glass electrodes were 2-points calibrated with IKS GmbH NBS buffer solutions (pH 4.01 and 7.01 at 25 °C). Furthermore, the pH glass electrodes were inter-calibrated using a portable pH/Conductometer (Metrohm; Model: 914) which calibrated on the total scale using TRIS buffer solution of salinity 35 (Dickson, 2010), thus, all the pH measurement values were reported in total scale. Salinity was measured using WTW portable conductivity meter (model: LF320). In addition, 200 mL seawater samples were collected from incubation tanks and filtered through GF/C filter papers for the quantification of total alkalinity (TA). These samples were stored at 4 °C prior to subsequent titration using HydroFIA TA (CONTROS Systems &Solutions GmbH). Prior to the titration, HydroFIA TA was calibrated with CO_2 in Seawater Reference Material (CRM, batch 154). The weekly recorded values of temperature, pH, salinity and TA were used to determine other carbonate chemistry parameters (e.g. partial pressure of carbon dioxide (pCO_2), carbonate and bicarbonate ions (CO_3^{2-} and HCO_3^-) and saturation state with respect to aragonite (Ωa) and calcite (Ωc)) using CO2SYS software (Pierrot et al., 2006) where the thermodynamic constants of Mehrbach et al. (1973) was applied.

Table A.1 The average carbonate chemistry of seawater in four treatments during 5 weeks of incubation: temperature, pH_T, salinity, total alkalinity (TA), partial pressure of carbon dioxide (pCO_2), concentration of bicarbonate and carbonate ion (HCO_3^- and CO_3^{2-}) and saturation state with respect to calcite and aragonite (Ωc and Ωa). The ± values represent standard deviation.

Temperature	7	7	10	10
pH_T	8.0	7.5	8.0	7.5
Temperature (°C)	7.09 ± 0.62	6.99 ± 0.50	9.80 ± 0.57	10.0 ± 0.46
pH_T	7.99 ± 0.02	7.50 ± 0.03	8.01 ± 0.02	7.51 ± 0.04
Salinity	30.7 ± 0.04	30.8 ± 0.05	30.8 ± 0.24	31.1 ± 0.16
TA (µmol.kg^{-1})	2359 ± 13	2359 ± 14	2346.3 ± 10	2375 ± 13
pCO_2 (µatm)	237 ± 14.1	848 ± 108	247 ± 21.2	958 ± 60
HCO_3^- (µmol.kg^{-1})	1925 ± 29	2201 ± 17	1897 ± 32	2210 ± 17
CO_3^{2-} (µmol.kg^{-1})	177.4 ± 7.1	64.3 ± 6.3	183.9 ± 9.1	67.2 ± 5.1
Ωa	2.7 ± 0.11	1.0 ± 0.10	2.8 ± 0.14	1.0 ± 0.08
Ωc	4.3 ± 0.17	1.6 ± 0.15	4.5 ± 0.22	1.6 ± 0.30

A.1.2 Spawning induction of bivalves

Individual specimens were collected from rectangular boxes and placed individually in 150 mL cleaned containers. Specimens were induced to spawn in 0.2 µm filtered seawater (salinity of 30.1) with the addition of the selective serotonin re-uptake inhibitor fluoxetine, followed by a temperature shock of +6 °C (Honkoop et al., 1999). Eggs and sperm produced were identified. Eggs were counted under Leica Microscope M216, whereas sperm concentrations were quantified using optical density at 660 nm. Calibration was done by counting trials using a Beckman Coulter counter (100 µm aperture) to determine the relationship between optical density and sperm concentrations.

During the identification and quantification of the gametes, free swimming cercaria of trematode *Bucephalus minimus* were

discovered (Figure A.2, Annabelle Dairain and Xavier De Montaudouin, personal communication). The discovery enabled the quantification of parasite infection percentage in each treatment.

A.1.3 Statistical analysis

A 3 × 2 contingency analysis was used to examine the difference in gender spawning success percentage (male, female, and non-fertile) between treatments. The effects of temperature (ambient, elevated) and pH (ambient, lowered) on eggs and sperm count per individual were tested using two-way analysis of variance (ANOVA), with individuals being the replicates. Prior to analysis, the normality and variance homogeneity assumptions were checked using Shapiro test and Levene's test. In case of non-normal data, data were Log_{10} transformed to improve normality. Statistical analyses were not performed for sperm production of *L. balthica* and egg production *of C. edule,* due to absence of gametes in a particular treatment, and due to the consumption of eggs by *B. minimus*, respectively Finally, a 2 × 2 contingency analysis was used to compare infected and non-infected *C. edule* by *B. minimus* between each pair of treatments.

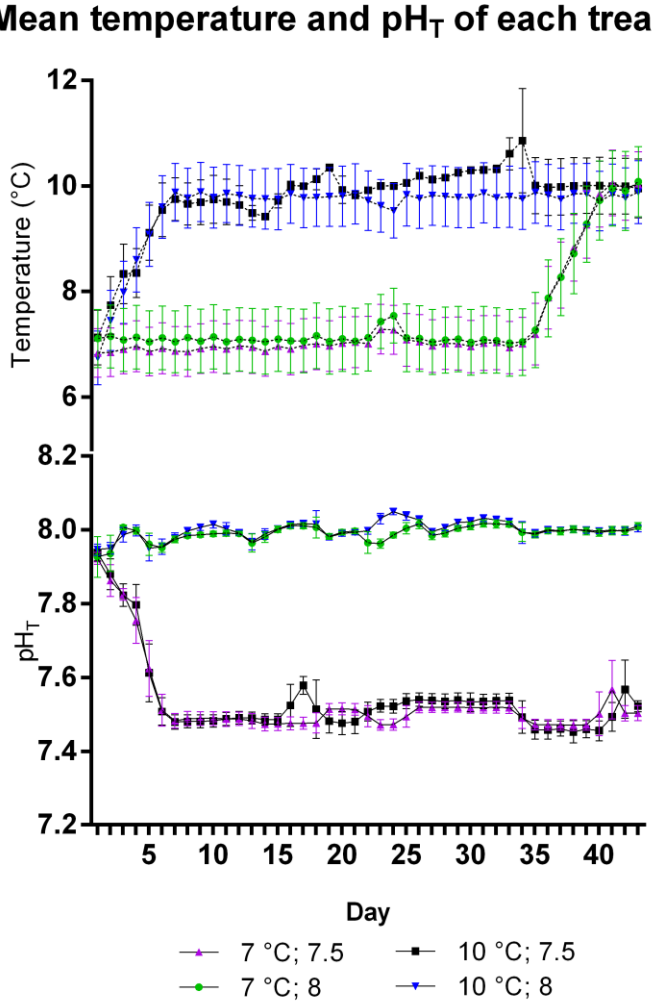

Figure A.1 Variations of temperature and pH in four treatment in 45-day incubation. The experiment started at 6th day and the temperature of ambient/control temperature treatments was elevated 0.5 °C per day from 35th day until 10 °C was achieved prior to spawning induction. Error bars represent standard deviation.

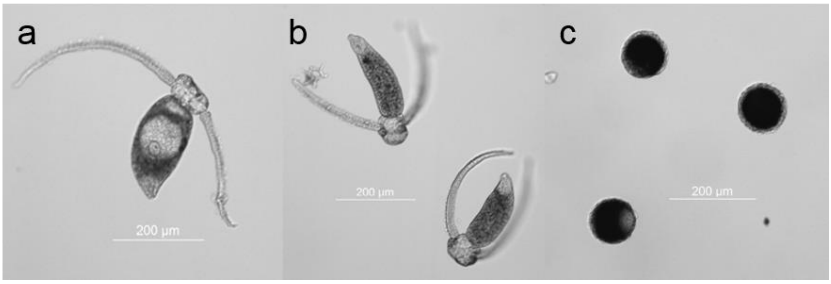

Figure A.2 Free-swimming cercaria of the trematode *Bucephalus minimus* (a, b) and *Cerastoderma edule* eggs (c) were observed during the reproduction experiment. Ingestion of eggs by *B.minimus* is shown in (a).

A.2 Results

3 × 2 contingency analyses show no significant effect of temperature and pH on spawning success percentage of *C. edule*, however, we found significant effects on spawning success of *L.* balthica (Table A.2 and Figure A.3). Our result shows that the spawning success of *L. balthica* was lower under lowered pH treatments particularly in female *L. balthica*. No spawning of *L. balthica* was observed under the combined lowered pH and elevated temperature treatment (Figure A.3a, c, e).

There was no significant effect of temperature and pH on the sperm count for both species; however, in general, more sperm was produced under lowered pH treatments. On the other hand, less eggs were produced under lowered pH treatments for *L. balthica*. There was no data on the number of eggs for cockle as we discovered free-swimming cercaria of the trematode *Bucephalus minimus* consuming the eggs (Figure A.2a).

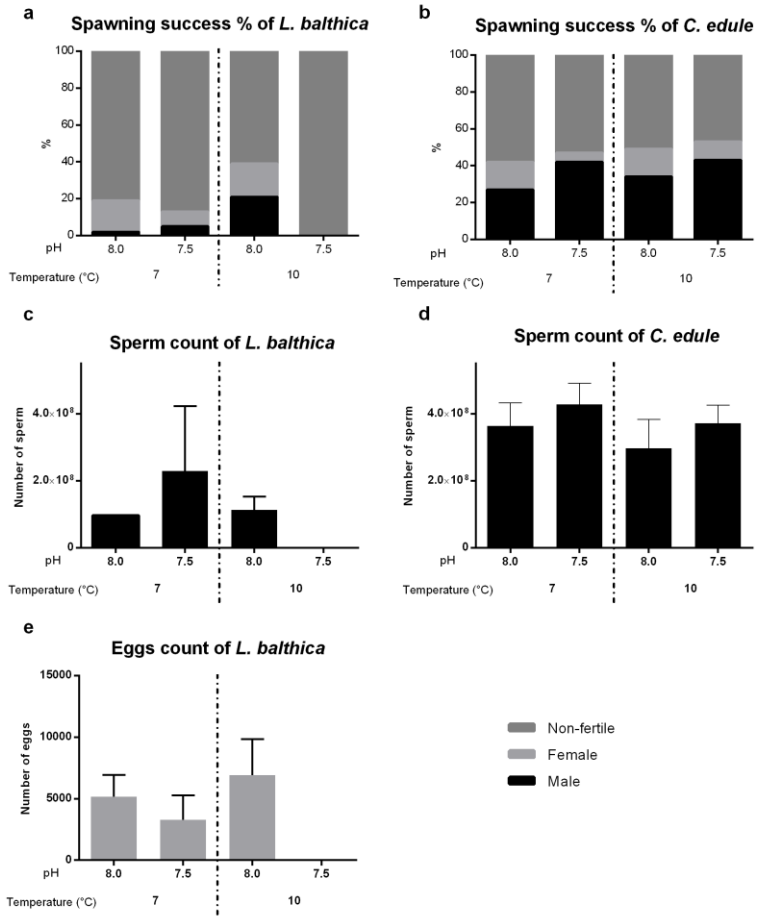

Figure A.3 Spawning success percentage, sperm and eggs count of *Cerastoderma edule* and *Limecola balthica*. Error bars represents standard errors.

Furthermore, our results show a significantly higher percentage of infected cockles under lowered pH and elevated temperature treatment (83 %) compared to control (71 %), lowered pH (61 %), and elevated temperature (63 %) treatments (Table A.3 and Figure A.4), indicating that cockles are more vulnerable to

parasite infection under the combined effects of lowered pH and elevated temperature.

Table A.2 3 × 2 contingency analyses comparing spawning success of *Limecola. balthica* (male, female and non-fertile) between each pair of treatment, $\chi 2$critical = 5.99. Significant results are in bold.

Treatment	Lowered pH	Elevated temperature	Lowered pH-elevated temperature
Control	$\chi 2_2$ = 7.67 P = **0.02**	$\chi 2_2$ = 0.5 P = 0.77	$\chi 2_2$ = 8.51 P = **0.01**
Lowered pH	-	$\chi 2_2$ = 5.48 P = 0.06	$\chi 2_2$ = 14.0 P < **0.001**
Elevated temperature	-	-	$\chi 2_2$ = 11.2 P = **0.004**

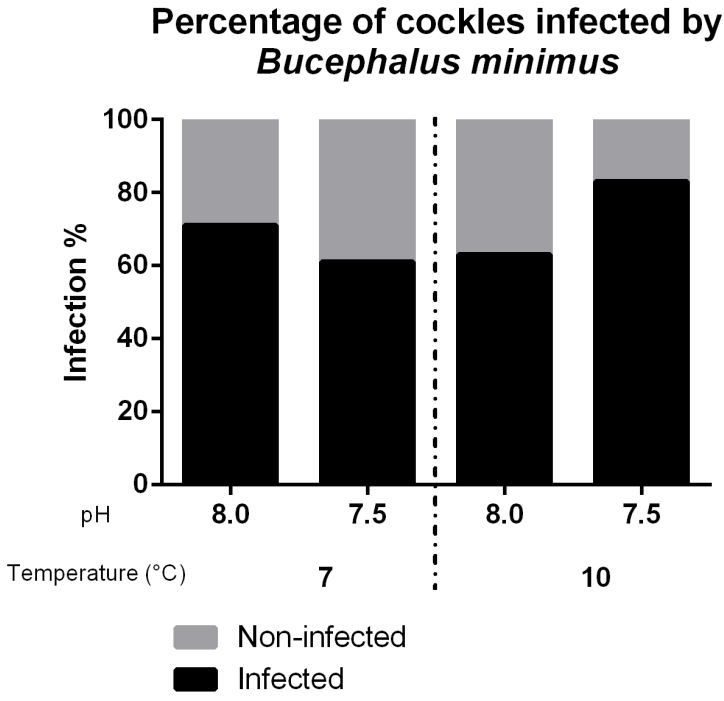

Figure A.4 Infection percentage of cockles by trematode *Bucephalus minimus* in four treatments.

Table A.3 2 × 2 contingency analyses comparing infected and non-infected cockles between each pair of treatments, $\chi 2_{critical} = 3.84$. Significant results are in bold.

Treatment	Lowered pH	Elevated temperature	Lowered pH-elevated temperature
Control	$\chi 2_2 = 0.48$ P = 0.49	$\chi 2_2 = 0.02$ P = 0.88	$\chi 2_2 = 3.94$ P = **0.047**
Lowered pH	-	$\chi 2_2 = 0.28$ P = 0.60	$\chi 2_2 = 6.68$ P = **0.01**
Elevated temperature	-	-	$\chi 2_2 = 4.31$ P = **0.04**

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