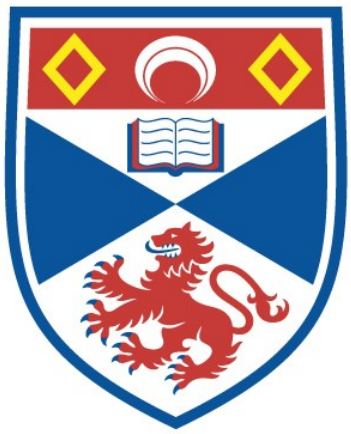
QUANTIFICATION OF THE EFFECTS OF OCEAN ACIDIFICATION ON BENTHIC FORAMINIFERA

Luis Fabricio Guamán Guevara

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Quantification of the effects of ocean acidification on benthic foraminifera

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Abstract

The global ocean has experienced an alteration of its seawater chemistry due to the continuing uptake of anthropogenic carbon dioxide (CO_2) from the atmosphere. This ongoing process called Ocean acidification (OA) has reduced seawater pH levels, carbonate ion concentrations (CO_3^{-2}) and carbonate saturation state (Ω) with implications for the diversity and functioning of marine life, particularly for marine calcifiers such as foraminifera.

The vulnerability of this ubiquitous calcifying group to future high pCO_2 /low pH scenarios has been assessed naturally and experimentally in the last decades. However, little is known about how benthic foraminifera from coastal environments such as intertidal environments will respond to the effects of OA projected by the end of the century.

This research aimed to quantify the effects of OA on a series of biological parameters measured on the benthic foraminifera *Elphidium williamsoni* and *Haynesina germanica* through a laboratory-based experimental approach where future scenarios of a high CO_2 atmosphere and low seawater pH were explored.

Experimental evidence revealed that survival rates, test weight and size-normalized weight (SNW) of *E. williamsoni* were negatively affected by OA. Whereas *H. germanica* was positively affected (i.e. enhanced growth rates) showing a species-specific response to OA at 13°C. However, the combined effect of OA and temperature (15°C) reduced survival and growth rates for *Elphidium williamsoni* and *Haynesina germanica*.

Test morphology (i.e. test surface and feeding ornamentation) of live *E. williamsoni* and *H. germanica* were severely affected after 6 weeks by OA, negatively influencing the uptake of ^{13}C-labelled diatoms of *Navicula sp.*, notably for *E. williamsoni*.

Test dissolution rates were enhanced by OA and negatively affected foraminiferal morphology of recently dead assemblages with implications for net accumulation and preservation. These results imply that the long-term storage of inorganic carbon and cycling of carbon in coastal benthic ecosystems will be considerably altered by future OA.

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Chapter 1. Introduction

1.1 Coastal Areas

Coastal zones account for approximately 10% of the ocean area and include coastal waters and the adjacent shorelands. These marine areas comprise a wide range of ecosystems such as intertidal areas, salt marshes, wetlands, mudflats, mangroves forests, coral reefs, sandy beaches and rocky shores (Lalli & Parsons 1997; Mitra et al. 2014; Parsons et al. 2016). Although these coastal areas are considered as some of the most productive and biologically diverse in the world (Solan et al. 2004); due to their proximity to land, the ecosystem services provided by these marine habitats are strongly influenced by anthropogenic factors such as eutrophication, overfishing, pollution and habitat destruction (Solan et al 2004; Lalli & Parsons 1997; Halpern 2008; Lopes et al 2015; Brouwer et al. 2016). In addition, these areas are also potentially vulnerable to global threats such as global warming, sea level rise and ocean acidification (OA) that might have additional consequences for these marine ecosystems (Morton et al. 2011; IPCC 2013; Strong et al. 2014; Parsons et al. 2016). For instance, notable changes in biological productivity caused by the alteration of biogeochemical cycling of carbon and nutrients, biodiversity reduction and loss of natural habitats are expected as direct responses of coastal habitats to these environmental factors (Solan et al. 2004).

These multiple stressors might have the potential for synergistic, additive or cumulative effects on coastal environments (Boyd & Hutchins 2012). This environmental complexity supports the need for laboratory experiments to unravel the effects of changes in environmental factors (e.g. temperature and seawater pH) and their

associated impact on marine ecosystems and productivity on local and global scales (Murray 2006; Horton & Murray 2007).

1.2 Ocean Acidification

Anthropogenic activities such as fossil-fuel combustion, deforestation, agriculture, industrialization, cement production and changes in land-use have caused a steady increase in atmospheric CO_2 concentrations since industrial revolution times. The subsequent absorption of part of this CO_2 by the ocean (~30% of total CO_2 emissions) has changed seawater chemistry through a process known as ocean acidification (OA) (Caldeira & Wickett 2003; Sabine 2004; Guinotte & Fabry 2008; Keul et al. 2013). As a result of this process, seawater pH, carbonate ion concentration [CO_3^{2-}], and saturation state (Ω) with respect to carbonate minerals have been declining (Gattuso et al. 1998; Langdon et al. 2000; Caldeira & Wickett 2003; Feely et al. 2004; Sabine 2004; Raven et al. 2005).

Since the pre-industrial period until the present time, the concentration of CO_2 in the atmosphere has been steadily increased from 280 parts per million (ppm) to exceed 400 ppm by volume (ppmv) (Raven et al. 2005; Guinotte & Fabry 2008; Betts et al. 2016). However, model predictions (IS92a CO_2 emission scenario) by the Intergovernmental Panel on Climate Change (IPCC) suggest that these levels may exceed 1000 ppmv by the end of the year 2100 unless considerable reductions in future CO_2 emissions occur. Hence, estimated rates of increase in CO_2 concentrations in the atmosphere over the next centuries may be 100 times faster than the maximum rate observed in at least the past 650,000 years (Raven et al. 2005; Siegenthaler et al. 2005).

Although the reduction in the ocean pH is mainly linked to atmospheric CO_2 uptake, the atmospheric deposition of other chemicals such as nitrogen, and sulphur can also change the surface ocean chemistry (Guinotte & Fabry 2008; Gattuso & Hansson 2011). However, these alterations account for only a small proportion of the acidification compared to the anthropogenic CO_2 uptake by the ocean (Doney et al. 2007; Gattuso & Hansson 2011).

It is estimated that the current seawater pH value has already dropped by 0.1 pH units over the last two centuries (Orr et al. 2005). This implies an increase in both hydrogen ion concentration [H^+] and its corresponding acidity levels in seawater of approximately 30% in comparison with preindustrial values. Projected ocean pH values indicate an additional drop of 0.3–0.4 and 0.77 pH units by the year 2100 and 2300, respectively (Gattuso et al. 1998; Caldeira et al. 2003; Orr et al. 2005; Raven et al. 2005; Caldeira et al. 2007; Feely et al. 2008; Guinotte & Fabry 2008; Gattuso & Hansson 2011; Tyrrell 2011; IPCC 2013). Changes in the natural seawater pH levels projected for the year 2100 may represent an event not recorded on Earth's history in at least the past 20 million years (Feely et al. 2004).

1.3 Carbonate system in an acidifying ocean

Through the continuous air-sea gas exchange, atmospheric CO_2 is taken up by the upper ocean to form carbonic acid (H_2CO_3) with an immediate dissociation to form bicarbonate ions (HCO_3^-) and carbonate ions (CO_3^{2-}) (see Equation 1.1). The equilibria between carbonate species in the ocean are briefly detailed as follows (see Equation 1.1) (Zeebe & Wolf-Gladrow 2001; Tyrrell 2011):

$$CO_{2\,(aq.)} + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+ \qquad \text{(Eq. 1.1)}$$

In seawater, as the concentrations of H_2CO_3 and CO_2 (aq.) are really small, the sum of their concentrations is often represented as $[CO_2]$ (see Equation 1.2).

$$[CO_2] = [CO_2\ (aq.)] + [H_2CO_3] \qquad \text{(Eq. 1.2)}$$

Where brackets represent total stoichiometric concentrations.

Thus, the concentrations of the three dissolved carbonate species account for: $[HCO_3^-]$ (>86.5%), followed by $[CO_3^{2-}]$ (13%) and $[CO_2]$ (~0.5 %) (Zeebe & Wolf-Gladrow 2001). As a consequence of the equilibria among the three carbonate species, seawater is weakly buffered with respect to changes in hydrogen ion concentration $[H^+]$.

The three carbonate species are collectively referred to as Dissolved Inorganic Carbon (DIC) (Tyrrell 2011; Gattuso & Hansson 2011). Most of the CO_2 absorbed by the ocean over the last two centuries not only has increased the DIC content of seawater but also has increased the release of more H^+ while lowering both pH and $[CO_3^{2-}]$ availability (Raven et al. 2005; Tyrrell 2011). Thus, under future scenarios of an increase in CO_2 (aq.) concentrations in seawater, a higher H^+ will consume more $[CO_3^{2-}]$ available to form immediately $[HCO_3^-]$ diminishing the pH buffering capacity of surface ocean (Raven et al. 2005). Current $[CO_3^{2-}]$ already exhibits a reduction in the availability of approx. 30%, and it is estimated that this amount will increase in the next centuries more likely affecting the productivity of the oceans through a decrease in $CaCO_3$ production (Gattuso & Hansson 2011).

The process of formation and dissolution of carbonate minerals are strongly related to marine photosynthesis and respiration (organic matter oxidation). To some extent, all processes together control the seawater pH and atmospheric CO_2 concentrations (see Equation 1.3) (Erez 2003):

$$\underset{\text{Dissolution}}{\leftarrow} \quad \underset{\text{Respiration}}{} \quad \underset{\text{Photosynthesis}}{\rightarrow} \quad \underset{\text{Biomineralization}}{}$$

$$Ca^{2+} + 2HCO_3^- \Leftrightarrow CaCO_3 + CO_2 + H_2O \Leftrightarrow CaCO_3 + CH_2O + O_2 \qquad \text{(Eq. 1.3)}$$

The formation of carbonate minerals (calcification) takes place when the reaction moves to the right and is commonly described by Eq. 1.3. The calcification process is nearly all biogenic, and in the absence of photosynthesis, the biomineralization releases CO_2 back to the atmosphere. Thus, a potential decline in calcification rates may increase the CO_2 storage capacity in the upper oceans exerting a negative feedback on atmospheric CO_2 levels with ultimate opposing effects on the marine carbon cycle (Riebesell et al. 2000; Feely et al. 2004). In contrast, carbonate dissolution process takes place through a reverse reaction in Eq. 3 (moving to the left side of the equation). This dissolution of carbonate minerals occurs mainly in the deeper water and on the ocean floor (Erez 2003).249

Seawater is in an equilibrium state when the saturation state of carbonate minerals (Ω) is equal to 1; dissolution of $CaCO_3$ minerals occurs in undersaturated seawater when Ω is lower than 1, whereas inorganic precipitation takes place in supersaturated seawater at Ω greater than 1 (Gattuso & Hansson 2011; Tyrrell 2011). Calcite, aragonite and magnesium calcite (Mg-calcite) are the most important precipitated carbonate minerals produced by marine calcifying organisms (Gattuso & Hansson 2011; Orr et al. 2005), and based on their structure, aragonite is more easily dissolved due to its higher solubility product (K^*_{sp}) and lower saturation state compared with calcite ($\Omega_{ar} < \Omega_{ca}$).

The availability and stability of carbonate minerals are affected by the concentration of $CO_{2\,(aq.)}$ which in turn is partially determined as a function of temperature. Thus,

calcifiers from colder and more acidic waters due to a greater amount of dissolved CO_2 may be more affected than calcifiers from warmer waters (Guinotte & Fabry 2008).

The calcium carbonate saturation state (Ω) for carbonate minerals is the determining factor in the kinetics of precipitation or dissolution of $CaCO_3$, and it is defined as the ratio between the observed ion product and the expected ion product when the solution is in equilibrium with particular calcium carbonate mineral (see Equation 1.4):

$$\Omega = [Ca^{2+}] \times [CO_3^{2-}] / K^*_{sp} \quad \text{(Eq. 1.4)}$$

Owing to $[Ca^{2+}]$ and K^*_{sp} being relatively constant through the oceans, variations of Ω are driven mainly by $[CO_3^{2-}]$. K^*_{sp} represents the solubility product of a specific carbonate mineral phase at *in situ* temperature, salinity and pressure (Zeebe & Wolf-Gladrow 2001; Dueñas-bohórquez et al. 2011)

In general, an important reduction in both $[CO_3^{2-}]$ and calcium carbonate saturation state (Ω) may have deleterious consequences mainly for marine biota that rely on biogenic carbonate minerals to build up their skeletons (Orr et al. 2005).

1.4 Evidence of the effect of OA on marine organisms and environments

Certainly, the amount of atmospheric CO_2 absorbed by the ocean has helped mitigate global climatic impacts (Sabine 2004; Fabry et al. 2008; Dissard et al. 2010; Gattuso & Hansson 2011). However, this process of CO_2 uptake has changed the carbonate chemistry of seawater causing a wide range of effects on marine environments and associated biota, particularly on marine organisms with $CaCO_3$ structures. The ecological concern is that the rate at which the ocean chemistry has changed since the

last two centuries will make it more difficult for calcifiers to be efficiently adapted to cope with these changes over the next centuries (Guinotte & Fabry 2008).

Although the effects of OA are much better understood, there is still a limited understanding of the implications of OA across different marine ecosystems (Benjamin S. Halpern, Shaun Walbridge, Kimberly A. Selkoe et al. 2008). Most of the evidence to date about the effects of OA comes from *in-situ* and laboratory experiments on calcifying organisms from two different trophic levels: primary producers (e.g. coccolithophores, coralline algae, etc.) (Kuffner et al. 2008; Beaufort et al. 2011; Porzio et al. 2018), and secondary consumers (e.g. corals, foraminifers, pteropods, mussels and oysters) (Moodley et al. 2000; Ries 2011; Feely et al. 2004; Maier et al. 2012; Allison et al. 2010; Allison et al. 2011; Keul et al. 2013; Kuroyanagi et al. 2009; Khanna et al. 2013; van Dijk et al. 2017). The ultimate findings from these studies are, in some cases, markedly different from each other due to the natural variability among individuals, developmental stage, taxonomic groups, species, communities, ecosystems and experimental exposure time (Raven et al. 2005; Zeebe et al. 2008; Bernhard et al. 2009; Keul et al. 2013; Kroeker et al. 2013).

In general, previous research, although with some exceptions exhibiting a positive or no influence of OA on survival, growth and calcification of marine organisms (Iglesias-Rodriguez et al. 2008; Ries et al. 2009; Kroeker et al. 2010; Kroeker et al. 2011; Hikami et al. 2011; Rodolfo-Metalpa et al. 2011; Vogel & Uthicke 2012; Kroeker et al. 2013; McIntyre-Wressnig et al. 2013; McIntyre-Wressnig et al. 2014; Connell et al. 2017; Doubleday et al. 2017), the decline in calcification efficiency due to a reduction in calcite and aragonite saturation states (Ω) may be the most notable biological response

of marine organisms and ecosystems to adverse effects of OA (Riebesell et al. 2000; Caldeira & Wickett 2003; Orr et al. 2005; Andersson et al. 2008; Maier et al. 2012; Kroeker et al. 2013)**.**

For instance, with a decreased calcification in corals and coral reef communities, the coral distribution and its reef framework may be considerably reduced resulting in a decline of the overall productivity of reef environments (Gattuso et al. 1998; Kleypas et al. 2001; Leclercq et al. 2002; Kleypas et al. 2006; Jokiel et al. 2008). As the calcification process in corals also depends on other factors such temperature, water depth, light and nutrients, any alteration in calcification rates in corals should not be exclusively linked to a single environmental forcing such as low pH levels and its corresponding low [CO_3^{2-}] (Kleypas et al. 2006).

In other organisms also with carbonate exoskeletons such as pteropods and foraminifera, a reduced calcification rate may make them particularly vulnerable to erosion (both biological and physical) and dissolution processes (Orr et al. 2005; Raven et al. 2005).

Laboratory experiments on coccolithophores, corals and foraminifera have already shown a reduction in calcification up to 50% when the atmospheric CO_2 concentration was set to two-fold the pre-industrial values (from 280 to 560 ppm CO_2) (Feely et al. 2004; Raven et al. 2005; Guinotte & Fabry 2008). This decrease in calcification rates may continue to decline in pteropods, corals (warm-water corals) and some calcareous algae possessing aragonite as the main carbonate mineral in their skeletal structures due to aragonite being more easily dissolved than calcite.

Under projected scenarios of a high CO_2 world, a reduction in calcification rates and an increase of dissolution rates may considerably affect the competitive fitness of some calcareous species. This also could lead to a shift in natural communities (e.g. benthic communities), resulting in an ultimate loss of calcareous species all over the world and giving more ecological and evolutionary competitive advantage to organism whose skeletal structures are limited in mass or made of materials other than $CaCO_3$ (non-calcifying organisms) (Bambach et al. 2002; Raven et al. 2005; Fabry et al. 2008; Kuffner et al. 2008; J. Ries 2011). This fact may reduce the structural complexity of coastal marine communities by reducing their biodiversity, biological interactions and productivity (Agostini et al. 2018).

Furthermore, a reduction in surface biogenic $CaCO_3$ production due to higher $CaCO_3$ dissolution rate may cause a substantial decline in the net rate of $CaCO_3$ accumulation and burial on deep seafloor and shallow waters sediments. The long-term implications of reduced calcification rates by planktonic and benthic calcifiers associated with future ocean chemistry changes may affect substantially the amount of material available for the sedimentation of organic matter and $CaCO_3$, with an ultimate impact on marine carbon cycle (Wolf-Gladrow et al. 1999; Zeebe & Wolf-Gladrow 2001; Marshall et al. 2013)**.**

1.5 Benthic communities and optimal environmental conditions

The vertical distribution of benthic communities in the marine sediment is highly stratified, with species-specific distribution patterns (Barnes & Hughes 1988; Raven et al. 2005). The distribution, abundance and ecological activities of benthic organisms can

be naturally limited to specific sediment layers depending on their tolerance to the existing environmental conditions (e.g. oxygen concentration, pH, etc.).

The higher density of organisms occurs within the upper layers of sediments (few centimetres depth) where pH levels, the oxygen and nutrients supply are not limiting factors for their ecological and biochemical activities (Alve & Bernhard 1995; Raven et al. 2005). Considering the uppermost layers of sediments possess pH levels closer to the overlying seawater (Silburn et al. 2017), surface benthic species with high diversity and abundance but with a relatively restricted mobility and distribution (e.g. foraminifera) (Gross 2000) may be more susceptible to slight changes in seawater pH driven by OA in comparison to other active motile groups (e.g. metazoans) (Raven et al. 2005; Bernhard et al. 2009). Some species of metazoans with high motility (e.g. burrowers) can reside in microhabitats created deeper in the sediments to tolerate low pH, the oxygen and nutrients limitation from surroundings. Thus, these large benthic organisms are adapted to displace continuously through a naturally strong geochemical gradient of approximately one unit of pH within the first 30 cm of unmixed sediments (Fenchel & Riedl 1970; Raven et al. 2005).

Studies of sediments off the UK coast indicate that natural changes in pH of 0.5-1.0 units can also take place within the first cm of sediment, with impacts on benthic organisms which are not yet fully understood (Ostle et al. 2016). These natural conditions may drastically change as ongoing OA has the potential to affect ecosystems and biogeochemical processes driven by benthic communities (e.g. foraminifera) altering their diversity, ecological structure and function (Raven et al. 2005).

1.6 Introduction to Foraminifera

Foraminifera (Order Foraminiferida, Supergroup Rhizaria) are one of the most diverse groups of heterotrophic unicellular eukaryotes protists comprising around 10,000 extant species and tens of thousands of fossil taxa (Vickerman 1992; Flakowski et al. 2005). The foraminiferal cell body, called test (also referred to as shell), comprises of one or more chambers whose composition consists of particles cemented together (agglutinated), forming an organic matrix or $CaCO_3$ (mainly calcite over aragonite) (Gooday 2003; Barras 2008). Thus, foraminifera have been subdivided based on their test's characteristics: agglutinated, porcellaneous or hyaline (Murray 2006).

This group of calcifying organisms possesses a relatively short lifespan with an estimated duration of life ranging from 3 months up to 2 years (Murray 1983; Murray 1991; Barras 2008). Their complex life cycles usually involve an alternation of sexual and asexual generations. Under intense environmental conditions, this cycle can be altered and asexual reproduction may be more common (Goldstein 1999; Gooday 2003; Jones 2014). This biological feature of generations changes may explain the distinct morphological dimorphism observed in tests of many species, including large fossil forms (Sen Gupta 1999).

The size of foraminifera depends on the taxa, but generally, their size (test diameter) is likely to range from 38 µm to 1 cm or longer (Wolf-Gladrow 1999; Murray 2006; Barras 2008; Uthicke et al. 2013).

Foraminifera possess a retractile cytoplasm and a network of a granulo-reticulose pseudopodia inside of their test (Gooday 2003; Barras 2008; Jones 2014). Pseudopodia extend outside through the apertures and perforations in the test, and their principal

functions are to provide attachment, locomotion, protection, building and structuring the test (Kitazato 1988; Goldstein 1999; Gooday 2003; Barras 2008; Jones 2013). In addition, pseudopodia are also highly efficient at food-capturing and contribute largely to the role foraminifera have in decomposing and recycling high rate of organic carbon within marine habitats (Bernhard & Bowser 1992; Bowser 2002; Mojtahid et al. 2011).

Geographically, foraminifera are considered as ubiquitous groups and their wide modern distribution include marine and fresh waters (Archibald et al. 2003; du Châtelet et al. 2004). However, nearly all the foraminifera groups are constrained to marine environments with a supremacy of foraminiferal taxa in benthic habitats (99%) (Barras 2008). Depending on their life strategies, foraminiferal planktonic and benthic communities can be found from deep-sea to coastal environments, frequently forming the major component of meiofaunal biomass (Bernhard & Bowser 1992; Gooday et al. 1992; Moodley et al. 2000; Murray 2006; Mojtahid et al. 2011).

1.7 Benthic Foraminifera

From the estimated number of 10,000 of extant foraminiferal species (Vickerman 1992), in comparison with planktonic species, benthic species account for the majority of the modern foraminifera group and possess a much longer geological record (Sen Gupta 1999). Benthic foraminifera highly contribute to the benthic communities biomass and diversity, mainly in the deep ocean (Gooday et al. 1992; Moodley et al. 2000; Heip et al. 2001). On a global scale, benthic foraminifera contributes with one-third of the estimated total annual $CaCO_3$ produced by foraminifera (Schiebel 2002; Langer 2008) and their estimated contribution in shallow waters range from 5 to 30% of carbonate production (Langer 2008).

In shallow and deep waters, benthic foraminifera are vertically distributed from the uppermost layer up to 35 cm deep in the sediment (Moodley & Hess 1992; Bernhard 1993). The shell morphology of some benthic foraminifera species is related to the vertical microhabitats they usually inhabit such as epifaunal or shallow (upper 2 cm of sediments), semi-infaunal and deep infaunal (below the upper 2 cm of sediments) (Corliss 1991; Moodley & Hess 1992; Linke & Lutze 1993; Loubere et al. 1995)

This benthic group of calcifiers plays a fundamental role in the biogeochemical cycle due to their ability to degrade large amounts of organic matter in the surface sediments. Their major contribution in the carbon cycle and $CaCO_3$ cycling is through the production of skeletons with either high-Mg calcite or low-Mg (calcification) (Moodley et al. 2002; Habura et al. 2005; Nomaki et al. 2005; Mojtahid et al. 2011; Prazeres et al. 2015).

1.7.1 Trophic dynamics and diet of benthic foraminifera

Given their transitional trophic position between microbes and macrofauna, the trophic interactions of predation or competition of foraminifera with other species depend exclusively on their feeding strategies such as carnivory, parasitism, bacterivory, cannibalism and symbiosis (Goldstein 1999; Gooday 2003; Jones 2014). Thus, they consume organic carbon mainly from diatoms; however, depending on their habitat and the prevailing environmental conditions, they can also feed on cyanobacteria, flagellates, algae, algal-derived detritus, other prokaryotes, metazoans and bacterial communities (Bernhard & Bowser 1992; Gooday et al. 1997; Mojtahid et al. 2011; Lei et al. 2014). In all feeding strategies, benthic foraminifera use actively their pseudopodia to capture and incorporate all types of organic carbon sources available

(Lipps 1983; Vickerman 1992; Austin et al. 2005). However, detailed information on foraminiferal feeding behaviour is limited to one calcareous intertidal species such as *H. germanica*. This benthic species actively uses tooth-like test ornamentations (tubercles) located on the apertural region to crack frustules of large benthic diatom *Pleurosigma angulatum*, consequently removing and ingesting all organic contents (i.e. chloroplasts) via continuous use of pseudopodia networks (Austin et al. 2005). In general, diatom-derived chloroplasts usually remain active up to several weeks within the cytoplasm of benthic foraminifera such as *H. germanica* (Lekieffre et al. 2018; Jauffrais et al. 2018).

Benthic foraminifera are the main food source for many metazoans living on the seafloor. This foraminiferal ingestion by other microorganisms can be either as a selective process carried out by polychaetes, gastropods, nematodes and isopods or as an indirect process accomplished by fish and other sediment filtering species (Murray 2006; Nomaki et al 2008). This indirect or unselective predation on foraminifera, mainly on juvenile specimens, may be one of the main factors of the high mortality rate and low standing crops values observed in foraminifera populations (Cearreta 1988).

1.7.2 Controlling factors

Foraminiferal spatial and temporal distribution as well as abundance, morphology, diversity, growth, reproduction and isotopic composition are mainly controlled by biological (i.e. predation, nutrients, food (organic matter) availability, etc.) and abiotic factors such as seawater temperature, salinity, exposure rates, oxygen conditions, water depth, pH and [CO_3^{2-}] gradient, and sediment grain size (substrate) (Hohenegger et al. 1989; Gross 2000; Gooday 2003; Mendes et al. 2004; Kuroyanagi et al. 2009; Geslin et

al. 2011; Reymond et al. 2011; Saraswat et al. 2011; Lei et al. 2014; Pettit et al. 2015; Brouwer et al. 2016; Eder et al. 2016; Enge et al. 2016).

Relative influence of some specific environmental parameters can vary depending on the habitat; this will play a major role in the control of distribution and density of benthic foraminiferal assemblages. For instance, in the deep sea, the predator-prey relationships with other primary and secondary consumers may influence in the abundance and diversity of foraminiferal which are not limited only by nutrient and oxygen availability (Murray 2006; Nomaki et al. 2008; Nomaki et al. 2009). In the case of intertidal zones, factors such as intertidal vegetation type, phosphate and organic carbon content, proximity of the open sea, percentage of mud, salinity and tidal exposure (tidal elevation) may strongly influence the foraminiferal distribution and density (Armynot du Châtelet et al. 2009).

1.7.3 Foraminiferal growth and calcification process

Foraminiferal growth is usually referred to as a change in biomass, test weight, volume, test size or number of newly deposited chambers throughout their life cycle (Austin 2003; Reymond et al. 2011; Jones 2014; Briguglio & Hohenegger 2014; Eder et al. 2016; van Dijk et al. 2017). Naturally, the growth rates may vary among species and also between their life stages (e.g. juveniles and adults). For instance, as the metabolism is weight-dependent, in early developmental stages, smaller specimens have higher metabolic requirements in comparison with adults specimens (Mahaut et al. 1995; Heip et al. 2001). Hence, young specimens are able to grow rapidly with a subsequent decrease in their growth rate as individuals become adults. At this stage and under

favourable environmental conditions, individuals may reach their maximum size as well as their reproductive maturity (Murray 1983; Austin 2003).

In general, the intermittent production of new chambers is directly linked to the biological process of calcification. Two mechanisms of calcification have been described, and in both, the formation of a specific space is fundamental to reach the ions concentration required for this biological process:

1. In the intracellular process observed in the Miliolid group (porcelaneous, imperforate), intracellular vesicles accumulate the precipitated calcite crystals, then these calcifying compartments are transported to the site of chamber formation where the crystals are released and assembled (Bentov & Erez 2006).

2. *in situ* precipitation observed in calcite-radial foraminifera (e.g. hyaline, lamellar and perforated species) starts when pseudopodia partially isolate the individual from their surroundings by creating a specific space covered by a thin organic matrix with the shape of the new chamber. Subsequently, $CaCO_3$ is precipitated around this organic layer which acts as a template for biomineralization. Ultimately, the incorporation of dissolved essential elements such as Ca^{2+}, CO_3^{2-} and Mg^{2+} from the surrounding seawater facilitate the formation process of their $CaCO_3$ tests (calcite or aragonite) (Goldstein 1999; Erez 2003; Bentov & Erez 2006; Mojtahid et al. 2011; Mewes et al. 2014).

Although the foraminiferal growth process is intrinsically controlled by genetic factors, changes in environmental factors might strongly alter calcification and growth processes (Eder et al. 2016). Thus, the foraminiferal capacity to elevate the intracellular pH at the site of calcification by one unit above the surrounding seawater pH will be affected by

future decreasing pH levels, and as a response to such unfavourable environmental conditions, foraminifera may need to invest additional energy to calcification resulting in less energy for other vital metabolic processes. This 'trade-off' may impact considerably on growth rate, calcification efficiency, fitness of the organism and ultimately affecting the global inorganic carbon cycle (Raven et al. 2005; de Nooijer et al. 2009; Dueñas-bohórquez et al. 2011).

1.7.4 Ecological importance as a bioindicator

Benthic foraminifera are widely recognized as remarkable ecological indicators due to their narrow ecological tolerance levels and their corresponding high sensitivity to environmental changes (du Châtelet et al. 2004; Schönfeld et al. 2012; Strotz 2015; Brouwer et al. 2016). The environmental disturbances can be recorded on their $CaCO_3$ tests during their short life cycle before becoming microfossils that are remarkably well preserved in the deep-sea and coastal sedimentary deposits (Gooday 2003).

Initially, these outstanding characteristics have made it possible for foraminifera to be used as a tool (proxy) for palaeoenvironmental reconstructions of past sea-level changes, nutrients, pH, and temperature (Horton et al. 1999; Hintz et al. 2004; Ries 2011; Berkeley et al. 2014; Martínez-Botí et al. 2015). Furthermore, many laboratory studies have also revealed the importance of using living foraminifera as an experimental bioindicator to observe ecological changes when environmental drivers such as salinity, temperature, oxygen, and pH are manipulated (Alve & Bernhard 1995; Kuroyanagi et al. 2009; Allison et al. 2010; Allison et al. 2011a; R. Saraswat et al. 2015; Khanna et al. 2013; van Dijk et al. 2017; Wukovits et al. 2017).

These applications have been used as an analogous tool to further palaeoenvironmental interpretations of foraminiferal fossil assemblages (Berkeley et al. 2007; Benjamin P. Horton & Murray 2007; Berkeley et al. 2014), and also to estimate future impacts of increased seawater surface temperature (global warming) and decreasing seawater pH (ocean acidification) on coastal marine ecosystems and their productivity as mentioned below.

1.7.5 Evidence of acidified ocean and its impact on benthic foraminifera

Over the last decade, multiple studies have assessed the effects of changes in seawater chemistry on benthic foraminifera inhabiting different ecosystems, demonstrating that the biological response of foraminifera to OA varies both among and within foraminiferal species (also referred to as a species-specific response).

In general, most studies, with some exceptions exhibiting a positive or no influence of low pH on calcification, growth, survival, fitness (Hikami et al. 2011; Vogel & Uthicke 2012; McIntyre-Wressnig et al. 2013; McIntyre-Wressnig et al. 2014), show that projected declining pH can strongly influence biometric and morphological features of foraminiferal test (e.g. thickness, size/diameter, weight, functional feeding structures, etc.) with an ultimate effect on the growth and net calcification rates and biomass of benthic foraminifera, especially in shallow water areas (Kuroyanagi et al. 2009; Allison et al. 2010; Allison et al. 2011; Fujita et al. 2011; Haynert et al. 2011; Hikami et al. 2011; Khanna et al. 2013; Haynert et al. 2014; Prazeres et al. 2015). However, Keul et al. (2013) emphasized that foraminiferal growth rates and size-normalized weight (SNW) were affected mainly by low [CO_3^{2-}] rather than high CO_2 concentrations in seawater and low pH.

Much of this research has focused on the use of benthic foraminifera from coral reef habitats (Kuroyanagi et al. 2009; Engel et al. 2015; Prazeres et al. 2015; Vogel & Uthicke 2012; Briguglio & Hohenegger 2014; Fujita et al. 2011). However, other biological responses of foraminifera from non-reef habitats to OA are less understood, such as how future reduced net calcification rates and growth can impact on foraminiferal survival, distribution, abundance and community composition. New insights into this topic have been provided from habitats with a natural gradient of calcium carbonate saturation and pH. Thus, assemblages of calcareous species naturally found at pH 8.19 shifted to agglutinated species at pH 7.7 (Pettit et al. 2015). Despite this reduced pH level, foraminiferal calcareous species are able to calcify to maintain the integrity of their tests made of low-magnesium calcite, but at a low rate (Bentov & Erez 2006; Pettit et al. 2015).

Generally, the potential disappearance of one calcareous species may be directly linked to high shell dissolution rates, combined with reduced calcification rates as direct consequences of low pH levels/ high CO_2 concentrations (Wootton et al. 2008; Haynert et al. 2011). Moreover, these environmental studies have confirmed that the potential shift in benthic foraminiferal composition driven mainly by OA will be highly beneficial to non-calcifying species in long-term (Dias et al. 2010; Fabricius et al. 2011; Khanna et al. 2013; Pettit et al. 2015).

These potential modifications in ecosystem structure and function, as well as the energy flow via a shift in trophic dynamics, may alter the carbon cycling and ecosystem productivity of different environments (Widdicombe & Spicer 2008; Wootton et al. 2008; Blackford 2010; Khanna et al. 2013; Kroeker et al. 2011; Nagelkerken & Connell 2015). However, before such drastic changes take place in modern oceans, a progressive

process of ecological succession should precede species disappearance; thus, in some cases, the prevalence of some benthic calcareous species over other co-occurring calcareous species is likely to be observed.

Therefore, new insights into the ecological mechanisms by which early foraminiferal succession processes are generated are urgently needed. This includes identifying, the time required for benthic organisms to display notable changes in multiple biological parameters (e.g. survival, test morphology, growth, calcification, feeding efficiency and carbon uptake); the optimal target species to be assessed; and the synergistic or additive effects of multiple stressors (e.g. OA and increased temperature) on benthic foraminifera are still required.

As coastal habitats exhibit extreme diel and seasonal fluctuations in temperature (Helmuth et al. 2002; Wukovits et al. 2017), high variability in pCO_2/pH values (Cai & Wang 1998; Wootton et al. 2008; Miller et al. 2009; Hofmann et al. 2011) and intense daily cycle of inundation and exposure (e.g. tidal flats) (Joye et al. 2009), it is more likely that coastal communities are already experiencing pH levels as low as those values projected for the open ocean until the year 2100 (Ceballos-Osuna et al. 2013) .

Consequently, resident benthic communities from nearshore habitats may be more susceptible to future changes in the ocean carbonate chemistry as a function of increased atmospheric CO_2 (Wootton et al. 2008; Hofmann et al. 2011; Andersson et al. 2015). Physiologically, the combination of these factors may drastically reduce the level of tolerance of coastal communities to future high atmospheric CO_2 and low pH scenarios (Hofmann et al. 2011), ultimately altering the abundance and diversity, food web-dynamics and the biogeochemical processes (e.g. nutrient fluxes, carbon sink, etc.)

driven by benthic communities (e.g. foraminifera) in coastal habitats such as intertidal mudflats.

Finally, although over the last decades OA has been increasingly recognised as a global threat affecting marine ecosystems (Capstick et al. 2016; Caldeira & Wickett 2003) and great progress on the knowledge of ecological responses to future increased CO_2 concentrations in the ocean has been achieved; new studies should focus on multiple species from other coastal environments apart from coral reef habitats. For instance, the information available on the effects of OA on foraminiferal species from intertidal flats is limited (Khanna et al. 2013; Khanna 2014). Hence, it is still crucial to devote research on integrated laboratory-based and field-based studies on multiple biological parameters of foraminiferal species from intertidal mudflats potentially affected by future high CO_2/low pH scenarios.

Intertidal benthic foraminifera can notably contribute up to 84% of the total biomass of protozoan observed in intertidal flats areas (Lei et al. 2014). Their ecological role in nutrient fluxes, carbon cycle, nitrogen cycle, aerobic and anaerobic organic matter remineralization in sediments (Geslin et al. 2011; Cesbron et al. 2016; Wukovits et al. 2017) and the capacity to respond and preserve environmental changes in their structures (du Châtelet et al. 2004; Schönfeld et al. 2012; Strotz 2015; Brouwer et al. 2016; Wukovits et al. 2017) render this benthic group relevant for further studies on their biological responses to environmental disturbances by future ocean acidification and warming scenarios.

This will allow us to more accurately predict the ecological impact of the future decline in pH values driven by OA on the sediment-associated-biodiversity and ecosystem function of coastal oceans and inform on potential mediation or mitigations strategies.

1.7.6 Target intertidal benthic foraminifera in this research

Due to the ability of live intertidal foraminiferal species to exhibit an immediate response to abrupt environmental changes occurred during a single sampling period (Milker et al. 2015; Wukovits et al. 2017), two dominant and co-occurring benthic foraminifera species found on local intertidal cohesive sediment in the Northeast of Scotland have been selected for this experimental research to identify their multiple biological responses to future high CO_2 concentrations/low pH scenarios.

Selected foraminiferal species *Elphidium williamsoni* (Williamson) and *Haynesina germanica* (Ehrenberg) are common heterotrophic hyaline species well-adapted to brackish environments with extremely changing physicochemical conditions in temperate regions, particularly in the Northeast Atlantic area.

They inhabit extremely euryhaline and eurythermal habitats such as tidal flats, tidal drainage channels, tidal salt marsh, marsh ponds and small pools (Alexander & Banner 1984; Cearreta 1988; Müller-Navarra et al. 2016)**.** These two foraminiferal species are denominated as kleptoplastic benthic species due to their ability to sequester/incorporate chloroplasts mainly from diatoms (Knight & Mantoura 1985; Goldstein et al. 2004; Austin et al. 2005; Jauffrais et al. 2016; Cesbron et al. 2017; Lekieffre et al. 2018).

In general, their maximum abundance is found in the uppermost oxygenated layers of the sediment (upper half or 1 cm). These epifaunal species are also highly dominant in

live and dead assemblages of foraminifera of intertidal habitats (Murray 1983; Cearreta 1988; Austin 2003; Müller-Navarra et al. 2016), especially in British coastal brackish waters (Alve & Murray 1994).

Ecological studies have also described some other biological features for each species:

1.7.7 *Elphidium williamsoni*

This species exhibits a seasonal variability in dominance and abundance associated particularly to seasonal variations in food availability. The seasonal peak of abundance and dominance generally occurs between April and July (Swallow 2000; Austin 2003; Benjamin P. Horton & Murray 2007; Benjamin P Horton & Murray 2007) but it may vary with geographical location within the Northeast Atlantic, and through natural inter-annual variability (Murray 1983; Swallow 2000).

The seasonal reproduction of *E. williamsoni* generally starts its in April, reaching its adult maximum size in wintertime (Murray 1991). This benthic species has been found in habitats with *in-situ* temperature, salinity and pH ranged from -2 to 34°C, 4 to 36 ‰ and 7.6 to 8.2 units, respectively (Murray 1983; Alexander & Banner 1984; Müller-Navarra et al. 2016). At an experimental temperature of 10°C, *E. williamsoni* exhibited growth rates of up to 14µm/day (Austin 2003).

Benthic foraminifera *E. williamsoni* have been used mainly in studies related to general ecology (Murray 1983; Alexander & Banner 1984; Swallow 2000; Austin 2003; Horton 2015; Müller-Navarra et al. 2016); in studying effects of seawater pH and calcification rate (Allison et al. 2010; Allison et al. 2011a); for reconstruction of past sea level (Horton et al. 1999; Benjamin P. Horton & Murray 2007); general and advanced

taxonomy (Buzas et al. 1985; Pillet et al. 2013; Darling et al. 2016; Roberts et al. 2016), taphonomy (Alve & Murray 1994; Murray & Alve 1999) and ocean acidification impacts on morphology (Khanna 2014). In particular, there is no evidence describing the biological responses (via multiple biological parameters) of *E. williamsoni* to future declining seawater pH and calcium carbonate concentrations and increased temperature.

1.7.8 *Haynesina germanica*

This species displays a seasonal variability in dominance and abundance associated mainly with seasonal fluctuations in food availability rather than temperature and/or salinity variations (Cearreta 1988; Austin 2003). Likewise, the growth of *H. germanica* also depends on the amount of available food (Cearreta 1988).

The seasonal peaks of abundance may occur in different months depending on the local environmental conditions prevailing in a specific geographical area in the Northeast Atlantic (Cearreta 1988; Swallow 2000; Austin 2003; Horton & Murray 2007). In general, the higher occurrence of *H. germanica* was observed in spring and autumn and lower abundance values during winter and summertime (Cearreta 1988). These seasonal fluctuations of this species may also be altered in sites with a high content of organic matter and high presence of heavy metals. The massive abundance of *H. germanica* in polluted areas has been used as a proxy of environmental health status (i.e. deterioration of coastal ecosystems) (Cearreta 1988; du Châtelet et al. 2004; Pati & Patra 2012).

Specimens of *H. germanica* have been found in habitats with *in-situ* temperature, salinity and pH ranged from -2 to 34°C, 4 to 40.01‰ and 7 to 8.5 units, respectively (Alexander & Banner 1984; Cearreta 1988; Müller-Navarra et al. 2016). The experimental optimum temperature observed for reproduction of this foraminiferal

species ranged from 12 to 20°C (Cearreta 1988), However, the maximum density of individuals experimentally observed occurred at 12°C (Goldstein & Alve 2011).

Benthic foraminifera *H. germanica* have been used mainly in studies related to general ecology (Alexander & Banner 1984; Swallow 2000; Alve & Murray 2001; Horton 2015; Seuront & Bouchet 2015; Cesbron et al. 2016; Müller-Navarra et al. 2016), including feeding mechanisms (Austin 2003; Austin et al. 2005); carbon and nitrogen uptake (Wukovits et al. 2017; Lekieffre et al. 2018), taphonomy (Alve & Murray 1994; Murray & Alve 1999; Berkeley et al. 2007) ocean acidification impacts on morphology (Khanna et al. 2013; Khanna 2014), reconstruction of past sea level (Horton et al. 1999; Benjamin P. Horton & Murray 2007) and general and advanced taxonomy (Buzas et al. 1985; Pillet et al. 2013). In particular, there is no new evidence describing the biological responses (via multiple biological parameters) of *H. germanica* to future declining seawater pH and calcium carbonate concentrations and increased temperature.

1.8 Aims of Research

The aims of this research are to quantify the effects of ocean acidification on the intertidal benthic foraminifera *Elphidium williamsoni* (Williamson) and *Haynesina germanica* (Ehrenberg) through a laboratory-based experimental approach. Each chapter of the thesis is designed to address a specific aim and research question/hypothesis:

1. Determine the initial effects of short-term high CO_2 concentrations and low pH in seawater on survival, growth/calcification and taphonomic processes (e.g. dissolution) of the benthic foraminifera *E. williamsoni* and *H. germanica* (**Chapter 3).**

Hypothesis One: OA will negatively affect survival rate, test size and weight, growth/calcification rate, morphology and post-mortem dissolution (**Chapter 3).**

2. Determine multiple biological responses of *E. williamsoni* and *H. germanica* exposed to short-term high CO_2/ low pH levels and concomitant low carbonate ion concentrations [CO_3^{2-}] (**Chapter 4).**

Hypothesis: Multiple biological parameters will help determine that foraminiferal growth and calcification of the benthic foraminifera *E. williamsoni* and *H. germanica* are adversely affected as a result of short-term exposure to high CO_2 concentration seawater (**Chapter 4).**

3. Estimate the feeding efficiency and carbon (^{13}C) uptake by benthic foraminifera exposed to short-term high CO_2 concentrations and low pH seawater under laboratory conditions with ^{13}C-labelled food supply (**Chapter 5).**

Hypothesis: Diatom consumption and carbon (^{13}C) uptake by *E. williamsoni and H. germanica* will be substantially affected due to damaged feeding structures as a consequence of short-term exposure to high CO_2 concentrations and low pH in seawater (precondition). This will alter foraminiferal feeding/sequestration mechanisms of primary production and also the subsequent amount of energy transfer within the marine food web **(Chapter 5).**

4. Examine the impacts of a combined effect of elevated temperature and increased CO_2 concentrations on benthic foraminifera growth and calcification (**Chapter 6).**

Hypothesis: A synergistic effect, of combined warmer and more acidic seawater, will reduce growth/calcification in benthic foraminifera (**Chapter 6).**

5. Quantify the influence of **s**hort-term high CO_2/low pH levels on post-mortem dissolution of *E. williamsoni and H. germanica* (**Chapter 7).**

Hypothesis: Enhanced post-mortem dissolution rates induced by high CO_2/ low pH levels will severely affect the morphology of benthic foraminifera *E. williamsoni* and *H. germanica* (**Chapter 7).**

1.9 Thesis Structure

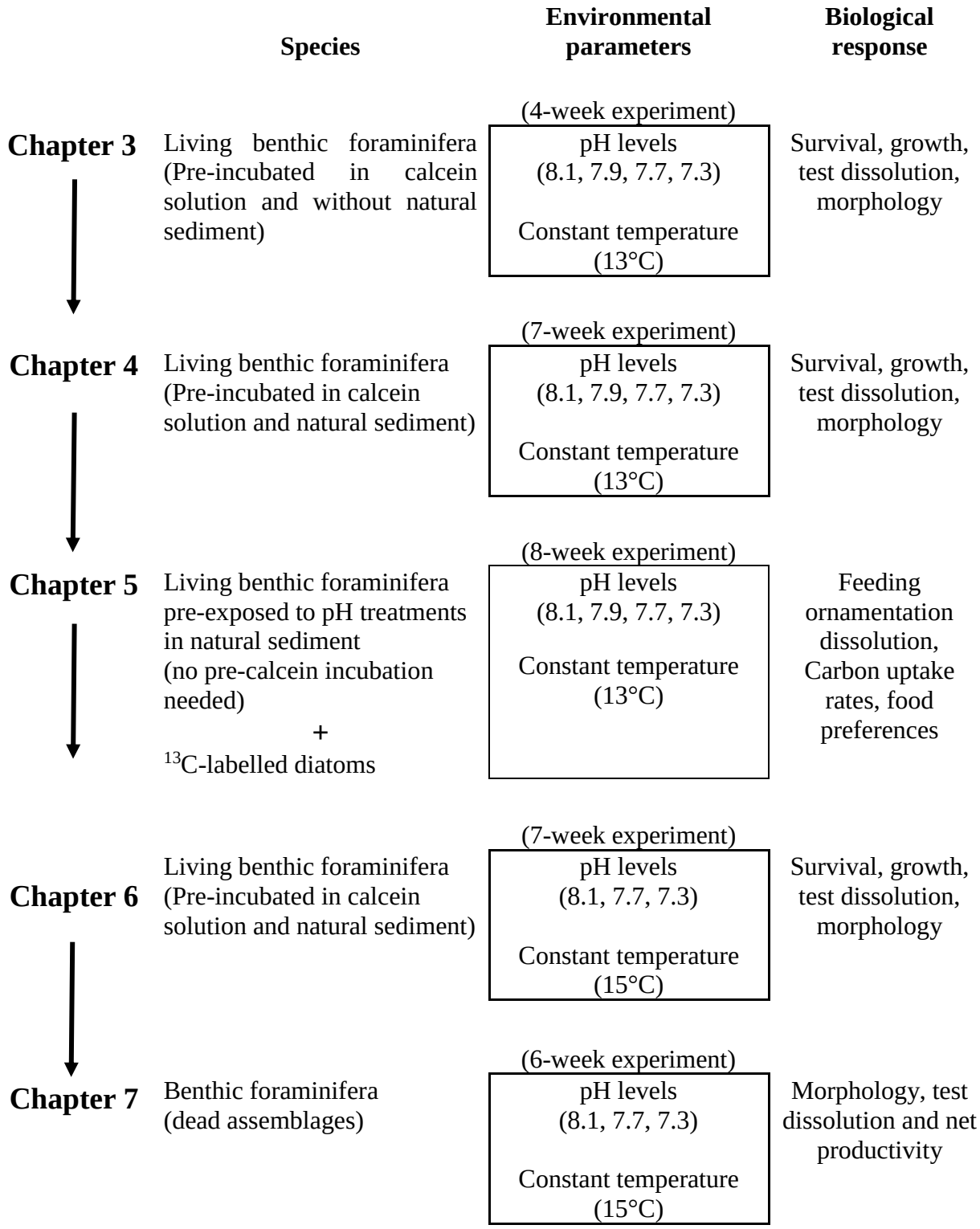

Figure 1.1 Flowchart illustrating the main biological parameters data obtained for each chapter of this thesis.

Chapter 2. Materials and Methods

This chapter details the methodology for the short-term foraminiferal culturing experiments, aimed at addressing the hypotheses detailed in the introduction, and is relevant for all data chapters. As the experimental design differs slightly between experiments, detailed information is provided in the methodology section of each chapter.

2.1 Collection site and sampling

Sediment surface scrapes (~ 1cm depth) containing benthic foraminifera were collected from the local intertidal mudflat in the Eden Estuary, Fife, N.E. Scotland (56°22'N, 2°50 W) during low tide in late April 2015 and in late July for the years 2015, 2016, and 2017 (Fig. 2.1). Foraminifera sampling was performed in months where the relative frequency (%) was equally dominated by *E. williamsoni* and *H. germanica* at high intertidal stations as indicated by Austin (2003). Sediment from the top first centimetre was carefully taken to exclude any underlying anoxic sediment from deeper layers and isolate live foraminiferal specimens as suggested by Murray (2006).

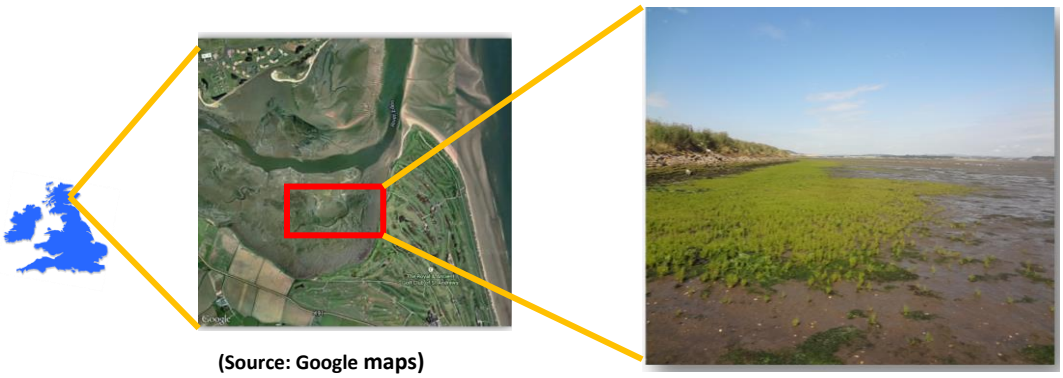

Figure 2. 1 Sampling site, mudflats on Eden Estuary, Fife, UK.

2.2 Identification and abundance of foraminiferal specimens.

On return to the laboratory, all sediment samples were sieved over a set of 63 µm and 500 µm screens to remove the very fine sediment fraction (clay and silt) and macrofauna, and also to retain both juvenile and adult individuals of *E. williamsoni* and *H. germanica.* The sieving process started when sediment samples were continually sprayed with filtered local seawater. The retained sediment fraction was kept to settle in plastic containers for 30-60 minutes prior to use it.

By simple observations of a small amount of this sediment through a stereoscopic binocular microscope, both target living foraminiferal species were easily identified because they showed colourful protoplasm extensively distributed across the entire foraminiferal tests, except in the last chambers (Murray 2006; Kitazato & Bernhard 2014). The selection of foraminifera, and subsequent cleaning of any detritus attached to their tests were carried out using a fine paint-brush. Isolated specimens were counted and transferred to petri dishes pre-filled with filtered seawater (Fig. 2.2). Ice packs were placed under the petri dishes during the isolation process to keep specimens in similar temperatures as they were found during sampling (13-15°C). Petri dishes with foraminifera were immediately left in a temperature-controlled room with a light condition of 12:12-hr light: dark cycle.

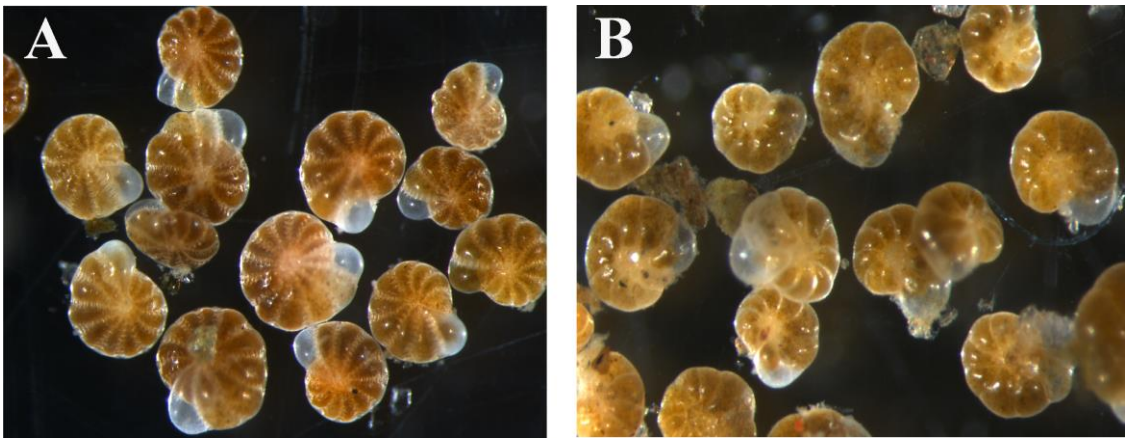

Figure 2. 2 Living assemblages of A) *Elphidium williamsoni* and B) *Haynesina germanica* observed in sediment samples collected from intertidal mudflats on Eden Estuary, Fife, UK.

The naturally intense protoplasm colour has been widely used as an indicator for live (viable) foraminifera (Bernhard et al. 2004; Khanna 2014). The observation of subsequent pseudopodial activity confirmed that those randomly selected individuals were alive prior to being transferred to culture chambers.

2.3 Foraminiferal calcein incubations

The calcein staining method for foraminifera was used to distinguish newly deposited chambers (fluorescent) from pre-existing chambers (non-fluorescent) at the end of calcein incubation period (Bernhard et al. 2004; Dissard et al. 2009; Dissard et al. 2010).

The calcein incubation used in chapters involved the use of living assemblages of foraminiferal specimens of *E. williamsoni* and *H. germanica* placed either in flasks or Tupperware containers with filtered seawater containing 5 mg/L, 10 mg/L or 20 mg/L of fluorescent stain calcein at which foraminiferal specimens were exposed. An independent

line connected to an air pump allowed bubbles of ambient atmospheric air to aerate the containers. The containers were kept inside the temperature-controlled room with a light condition of 12:12-hr light: dark cycle (Fig. 2.3 A). Temperature and length of experimental periods for calcein incubations varied and depended on the specific aims and experimental designs detailed in each chapter.

Seawater plus calcein was changed once a week. In addition to this, fortnightly sampling observations through a fluorescence microscope provided information on incorporation processes of calcein into the new growth of foraminiferal tests as shown in Fig. 2.3 B.

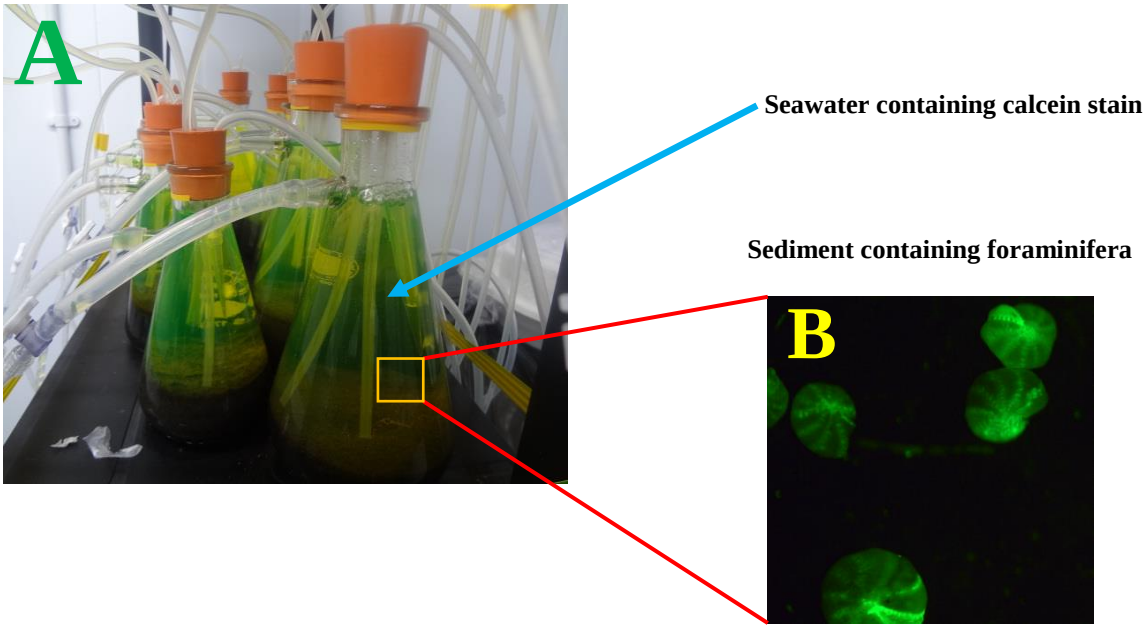

Figure 2. 3 A) One of the seawater recirculating system used for calcein incubation of *Elphidium williamsoni* and *Haynesina germanica* under temperature-controlled conditions with a light condition of 12:12-hr light: dark cycle. B) Specimens of *Elphidium williamsoni* showing the incorporation of calcein into the new growth of foraminiferal test.

When the calcein incubation period was concluded, live specimens were examined again through a fluorescence microscope, and surviving specimens were randomly selected, picked out, cleaned and transferred into foraminiferal culture chambers (Austin 2003; Hintz et al. 2004; Allison et al. 2010; Allison et al. 2011a; Khanna 2014). The latter were subsequently connected to the respective treatments of the CO_2 manipulative mesocosm experiment.

2.4 Culturing chamber setup

Acrylic culture chambers were used to house the foraminiferal individuals throughout the experimental period. These experimental chambers were connected to seawater reservoir tanks through inputs and outputs located on the top and bottom half of each chamber. These connections allowed seawater to be continuously pumped into the chambers from above and below a culture insert. The culture insert was fitted with a polycarbonate membrane (8 μm pore size, diameter 25 mm) and sat in the chamber base (Fig. 2.4). The membrane function is to prevent loss of foraminiferal individuals out of the chambers, and it also allowed them to live under a substrate of 1 mm thick silica added to the membrane in each culturing chamber (Allison et al. 2010; Allison et al. 2011a; Khanna 2014). The silica function simulates the natural foraminiferal habitat of soft sediment.

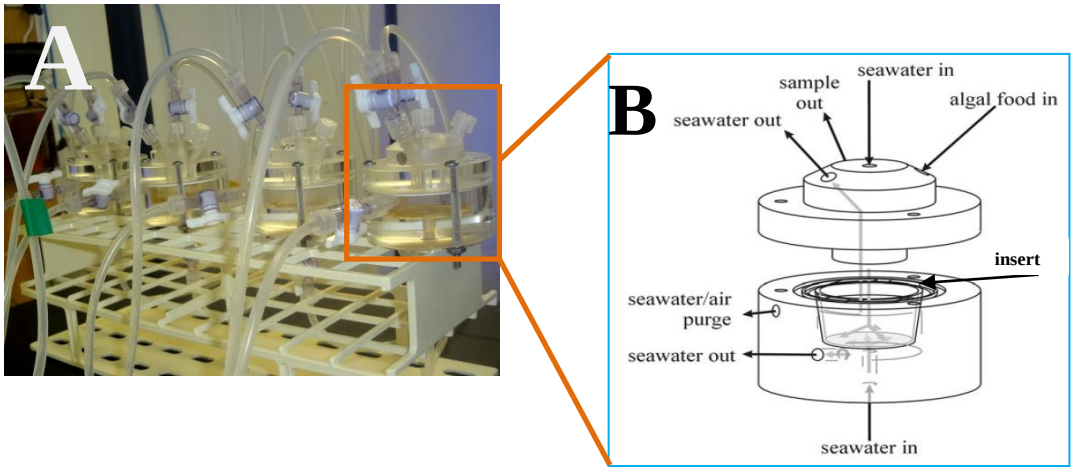

Figure 2. 4 A) Foraminiferal culturing system used for CO_2 experiments. B) Schematic design of a foraminiferal culture chamber indicating seawater flow patterns and culture insert (modified from Hintz et al. 2004).

Four replicate chambers per treatment were subsequently connected to a manipulative mesocosm, with controlled recirculating seawater. Prior to the start of the experimental period, an extra time period of 10 days for acclimation was required to prevent foraminiferal individuals from any acidity shock (Khanna 2014). During the acclimation time, the seawater pH was reduced gradually (0.1 units per day) until each treatment reached its target pH level/pCO_2.

Seawater was continuously recirculated within the system through multi-channel peristaltic pumps via platinum cured silicone tubing with a flow rate of 3 mL min^{-1}. Seawater residence time is 37s inside the culture insert and 6 min below the culture insert (Allison et al. 2010; Allison et al. 2011a; Khanna 2014).

2.5 Carbonate chemistry manipulation system

2.5.1 Experimental setup

Four reservoir tanks of 400 litres of high-density polyethylene (HDPE) were filled with seawater drawn by an HDPE pipe from a shallow (6m deep) site in St. Andrews Bay. This seawater was pre-filtered using 10 µm and 1µm filters and also purified through an ultraviolet water treatment system. Independent lines connected to air pumps bubbled ambient atmospheric air into each of the four 400 L tanks, ensuring the seawater is fully aerated and mixed for several weeks prior to the start of the experimental period.

Subsequently, the seawater pH was continually manipulated (except for the first reservoir tank with ambient seawater) by bubbling air with a known equivalent atmospheric concentration of CO_2 (approx. 600, 1000 and >2000 µatm pCO_2) into 3 reservoir tanks, respectively (Fig. 2.5). Thus, the four selected pH levels of 8.1 (ambient), 7.9, 7.7 and 7.3 mostly represent the range of pH predicted for future scenarios in a high CO_2 world (Gattuso et al. 1998; Caldeira et al. 2003; Orr et al. 2005; Raven et al. 2005; Feely et al. 2008; Guinotte & Fabry 2008; Gattuso & Hansson 2011; Tyrrell 2011; IPCC 2013). Seawater was continually pumped from the 400 L tanks into the culturing chambers of each treatment through peristaltic pumps for several weeks. The length of the experimental periods also varied and depended on the specific aims detailed in each chapter.

Figure 2. 5 Foraminiferal culturing system connected to the controlled recirculating seawater system. From the left reservoir tanks with seawater bubbled with atmospheric CO_2 concentrations of approx. 400 µatm pCO_2/pH 8.1, 600 µatm pCO_2/pH 7.9, 1000 µatm pCO_2/pH 7.7 and >2000 µatm $pCO2$/pH 7.3.

This type of recirculating seawater and the culturing system has previously been used successfully by other researchers conducting pH experiments (Austin 2003; Allison et al. 2010; Allison et al. 2011; Khanna 2013) which is also a modification of another existing recirculating system (Hintz et al. 2004). The entire seawater system was housed in the same temperature-controlled room as used for calcein incubations. The main constant temperature of 13°C, which was selected for most experiments, represents the equivalent of the monthly average seawater temperature historically recorded in Saint Andrews, Fife, for the month of July over the last 100 years (World sea temperature 2014). Seawater temperature of Saint Andrews is the closest meteorological station to the study site where sampling was carried out for the present research.

Probes measuring pH and temperature continually monitored the 4 reservoir tanks via separate modules and a continuous data logging system (IKS Aquastar control system). Additional measurements of pH, temperature, and salinity were recorded manually at fortnightly intervals via additional probes.

2.5.2 Carbonate system parameters

Three replicate seawater samples from each mesocosm were taken throughout the experiment at fortnightly intervals to measure total alkalinity (A_T). These samples were stored in borosilicate glass Labco exetainer vials (12 mL) and poisoned with 50 µL of mercuric chloride ($HgCl_2$). Vials were kept under refrigeration (4°C) prior to analysis at the Scottish Association for Marine Science (SAMS). Total alkalinity (A_T) concentration of the seawater samples collected was analysed using an automatic alkalinity titration kit (Metrohm, Switzerland) at 25°C. The measured values of temperature, salinity, pH (Total) and total alkalinity (A_T) were used to calculate the other carbonate system parameters such as dissolved inorganic carbon (DIC), pCO_2, bicarbonate ions (HCO_3^-), carbonate concentration (CO_3^{2-}) and saturation states of calcite ($\Omega_{Calcite}$) and aragonite ($\Omega_{Aragonite}$) using CO_2sys.xls (version v2.3) (Pierrot et al. 2006) with appropriate solubility constants (Mehrbach et al. (1973), refit by Dickson & Millero, 1987,1989) and KSO_4 (Dickson, 1990)..

2.6 Biological Parameters

After completing the experimental period, all chambers were opened and foraminifera were picked out and transferred into clean petri dishes and washed carefully with distilled water

to remove mainly the excess of silica and food cells. Foraminiferal specimens were individually mounted on 32 holed micro-palaeontological cardboard slides, and each foraminiferal individual was assigned a unique identification number.

Due to the large number of specimens expected to be used, and the characteristics of the mesocosm design that prevented repeat sampling, continuous measurements of biological parameters such as numbers of chambers added, maximum test diameter and test weight were not monitored throughout the experimental periods; hence, no initial measurements were recorded. Instead, biological parameters of live specimens were only recorded at the end of the experimental periods. These measured parameters allowed the estimation of additional parameters such as survival rate, growth rate, test size-normalized weights (SNW), size-weight relationships across different pH conditions.

2.6.1 Morphological response

All retrieved tests of target species were observed under a binocular microscope, and any changes or damages to their morphology were recorded. The Morphological responses of live and dead specimens of *E. williamsoni* and *H. germanica* to the experimental pH conditions were classified in three levels according to the physical condition of the tests observed: Level 1= intact test, Level 2 = minor changes, Level 3 = broken test (Fig. 2.6)

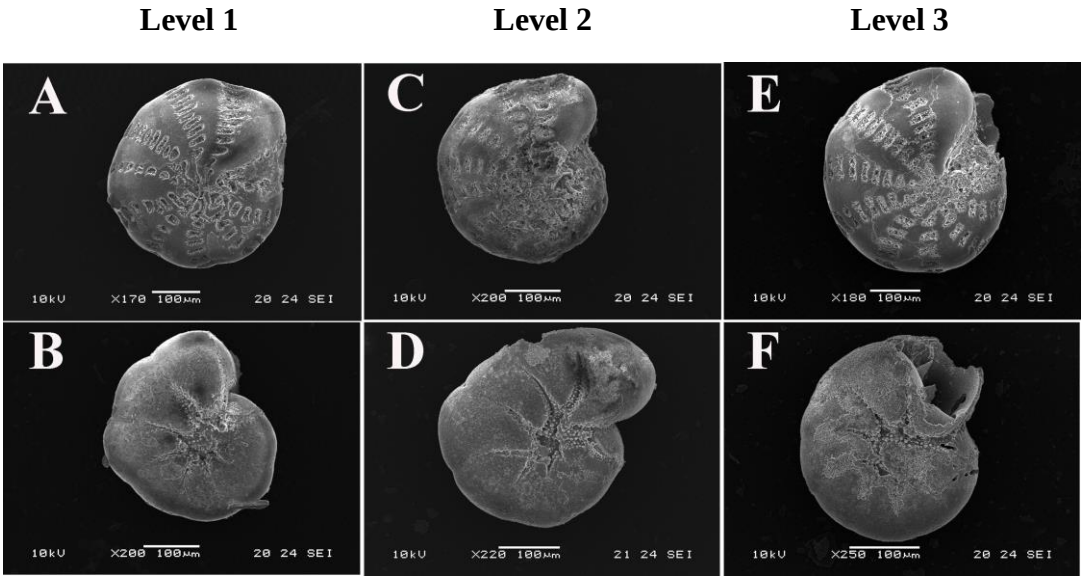

Figure 2. 6 Scanning electron micrographs (SEM) images to illustrate the levels of morphological responses displayed by *Elphidium williamsoni* (A, C & E) and *Haynesina germanica* (B, D & F) exposed to different CO_2/pH conditions. Level 1= intact test, Level 2 = slightly dissolved test, Level 3= broken test.

2.6.2 Newly deposited chambers

The observation and counting of the newly formed chambers added after calcein incubations were carried out under a Nikon® epifluorescence microscope. New chambers precipitated in the last whorl were easily recognized due to the lack of fluorescent colour compared with the pre-existing fluorescent chambers (Bernhard et al. 2004). This characteristic non-fluorescent colour (Fig. 2.7 A & B) is because they grew in seawater without exposure to calcein-labelling (Allison et al. 2010; Allison et al. 2011a; Khanna 2014). Only individuals that showed clear evidence of one or more new chambers deposited during the experimental period (post-fluorescent growth) were referred to hereafter as 'live'

individuals (Fig. 2.7 A & B). This criterion was applied to discern recently active growth within the experimental environment and exclude any 'dead' specimens from the further analyses. These 'dead' individuals showed only fluorescent chambers (calcein labelling) indicating that no growth occurred during the experiment.

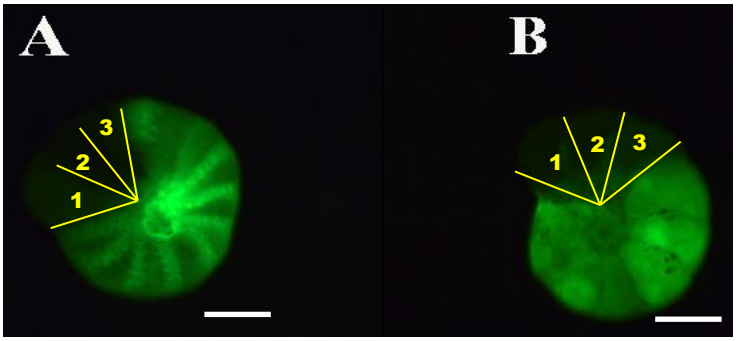

Figure 2. 7 Image of live specimens of A) *Elphidium williamsoni* and B) *Haynesina germanica* displaying new chambers (darker shaded sections on the test) deposited after an experimental period of 31 days at different pH conditions. Chambers precipitated in the last whorl are equal to 3 in each specimen above. White scale bar represents 100 µm.

2.6.3 Survival rate

Survival rate (SR) was calculated as a percentage of the total number of surviving individuals compared with the total number of individuals retrieved at the end of the experiment for each treatment. For each foraminiferal species, all specimens retrieved were included in the survival rate estimate regardless of their potential morphological responses to OA (e.g. intact test or broken last chamber). In addition, when possible, mortality rate (%) directly linked to an OA treatment effect was calculated by subtracting the total mortality (%) observed in each pH condition from mortality observed at ambient condition (pH 8.1).

2.6.4 Maximum test diameter

Measurements of maximum test diameter (µm) of each individual specimen were measured by using an eyepiece graticule incorporated in a binocular microscope and calibrated with a micrometre scale. Each unit of the graticule at the magnification used represented 13.99 µm.

2.6.5 Dry test weight

Measurements of dry test weight (µg) of each individual specimen were obtained by employing a microbalance (Sartorius M2P Microbalance, with a precision of ±1µg). This device was tested over several days in a controlled trial to reduce the error associated with any changes either in temperature, pressure or air flow in the air-conditioned weighing room. Subsequently, using a pre-weighed aluminium capsule, each foraminifer was individually weighed three times on three different days and its overall average weight was used for further analyses.

2.6.6 The shell size-normalized weight (SNW)

The shell size-normalized weight (SNW) was estimated by dividing recorded measurements of dry test weight (µg) of each specimen by its maximum test diameter (µm). This parameter (see Equation 2.1) was calculated across different culture conditions as a good indicator of density and test thickness in response to changes in carbonate concentrations [CO_3^{2-}]. This parameter helps to remove any influence of foraminiferal test size on weight (Keul et al. 2013; Marshall et al. 2013; Mewes et al. 2014).

$$SNW_{specimen} = \frac{\text{dry test weight } (\mu g)_{specimen}}{\text{maximum test diameter } (\mu m)_{specimen}} \quad \text{(Eq. 2.1)}$$

2.6.7 Foraminiferal growth rate

The average foraminiferal growth rate in each pH treatment was estimated based on the number of newly formed chambers added throughout the experiment. Thus, average numbers of chambers deposited for all live individuals in each culture condition were divided by the total number of experimental days (including days in acclimation period) to estimate average new growth across treatments.

2.7 Foraminiferal feeding

2.7.1 Selection of algae species for experiments

Algae species, *Dunaliella tertiolecta*, *Rhodomonas salina* and the diatom *Navicula sp.* were used as main food sources to feed the benthic foraminifera. *Dunaliella tertiolecta* and *Rhodomonas salina* have been used in several previous laboratory and *in-situ* experiments (Nomaki et al. 2005; Nomaki et al. 2006), and *Navicula sp.* is a common representative of natural phytoplankton observed in the sediment from Eden Estuary where dominant foraminiferal species were collected. *Navicula sp.* was also selected for the experiment because it has been previously found impaled on feeding structures of benthic foraminiferal species (Austin et al. 2005; Khanna et al. 2013).

In all experiments carried out, benthic foraminiferal species potentially utilized at least one algae species as a carbon source. Preferences for any particular algae are not discussed

here; however, it is believed that by providing different organic matter sources, the mortality rates of foraminiferal specimens were considerably reduced due to avoidance of starvation periods.

2.7.2 Feeding process

During the calcein incubation and throughout all manipulative CO_2 experiments, the foraminifera were fed weekly with ~10μL/cm^2 of each of the algae *Dunaliella tertiolecta* and *Rhodomonas salina* (typically 1×10^7 cells ml^{-1}) (Allison et al. 2010; Allison et al. 2011a; Khanna 2014). The use of the diatom strain *Navicula sp.* was limited to ^{13}C feeding experiment (Chapter 5). Algae species were axenic clones cultured at 20°C and maintained in seawater containing F/2 medium. They were provided by the Culture Collection of Algae and Protozoa (CCAP) at SAMS. Algae were harvested in 50 ml centrifuge tubes, concentrated by centrifugation and kept frozen (-20°C) until feeding time at weekly intervals. Concentrated algal solutions were defrosted prior to use for foraminiferal feeding.

In the manipulative mesocosm, peristaltic pumps were switched off during the feeding procedure for 2 hours to allow algae to settle and also to avoid loss of this food material by resuspension when the system was restarted (Allison et al. 2010; Allison et al. 2011a; Khanna 2014). The feeding procedure itself involves using a syringe to add the algae to the culturing chambers through one of the free ports. All foraminifera in all chambers were fed at approximately the same time each week.

2.8 Scanning Electron Microscopy (SEM) images

Selected live specimens with intact tests from each treatment were mounted onto SEM stubs using double-sided adhesive tabs, after being measured and weighed. An Emscope® SC 500 sputter coater was used to coat specimens with a thin layer of gold. Specimens were imaged with Jeol JSM-35CF SEM (Chapters 3-4) (Austin et al. 2005; Khanna et al. 2013; Khanna 2014) and Jeol JSM-5600 SEM (Chapters 6-7). A current of 15 mA at 1.2 kV was applied for 3-5 minutes (Khanna et al. 2013). Images recorded by SEM were used to estimate the morphological response on benthic foraminiferal tests that may be severely compromised due to short-term exposure of high CO_2/low pH levels.

2.9 Statistical Analysis

Shapiro-Wilk and Levene tests were applied to measured variables to verify the normality and homogeneity of Variance, respectively. Murray (2006) suggested the use of ANOVA (parametric analysis) as a first approach to determine potential difference between control and treatments when the assumptions of normality and homogeneity were matched ($p > 0.05$). However, non-parametric tests were also conducted since the assumptions of normality and homogeneity for datasets of some experiments were not matched ($p < 0.05$).

A one-way ANOVA or Kruskal-Wallis rank sum test was performed to establish whether the maximum diameter, weight of tests or the number of deposited chambers changed in response to the different pH treatments for each experiment.

A post-hoc Tukey test or Dunn's-test for multiple comparisons of independent samples was applied following ANOVA or Kruskal-Wallis test to estimate significant difference among pH treatments. Experimental pH conditions were used as a fixed factor. The null hypothesis implied that there was no significant difference between the control treatment (ambient) and the other experimental treatments. When the relationship between maximum diameter (size) and weight was investigated, data of both measured variables were log transformed, and the slopes of linearized functions of each treatment were compared using a Student's test (Prazeres et al. 2015).

All statistical analyses were carried out applying packages and functions of the R statistical language and environment R 3.1.2 (R Development Core Team. 2014).

Chapter 3. The effects of short-term high CO_2 concentrations and low seawater pH on survival, growth/calcification and taphonomic processes (e.g. dissolution) of the benthic foraminifera *E. williamsoni and H. germanica.*

3.1 Introduction

Test production by benthic foraminifera through the biomineralization process (calcification) is a key factor in determining the amount of carbonate incorporated in sediments of coastal marine environments (Hallock 1981); including intertidal habitats (Berkeley et al. 2007). These biogenic carbonate production and sedimentation rates are seasonally and spatially variable (Goineau et al. 2015), and are largely controlled by physical, chemical and biological factors (Jorissen & Wittling 1999; Benjamin P Horton & Murray 2007; Li et al. 2014). In addition, these factors also influence preservation degrees of recently dead foraminifera in the sedimentary record through geochemical processes commonly known as early diagenesis (Berkeley et al. 2007). The latter is considered as one of the most important taphonomic processes (e.g. *post-mortem* test destruction), altering continuously coastal foraminiferal fauna composition (Cummins et al. 1986; Berkeley et al. 2007; Berkeley et al. 2014).

The natural preservation pathways for foraminifera are clearly mediated by chemical reactions and differ remarkably between foraminiferal species due to their test composition. For instance, for agglutinated species, after death, chemical test degradation is accompanied by microbial activities to immediately oxidise organic particle-originated cements allowing subsequent disintegration of foraminiferal specimens within first centimetres of sediment

(Lipps 1983; Diz & Francés 2009). However, in some cases, despite this organic degradation, the remaining parts are preserved for a much longer period in marine sediments due their high sedimentation rates and low sensitivity to specific environmental conditions, rendering agglutinated species more dominant in foraminiferal fauna composition (Alve 1991; Pettit et al. 2013; Pettit et al. 2015; van Dijk et al. 2017). This type of oxidative reaction combined with a permanent supply of organic matter derived from primary producers onto surface sediments notably increase the CO_2 production and carbonic acid, thereby modifying the O_2 concentrations, calcium carbonate saturation states, pH porewater and dissolution rates (Jonas 1997; Marshall et al. 2008; Fink et al. 2017), ultimately altering the integrity of tests of living and dead calcareous species (Berkeley et al. 2007). Some of destructive features observed in calcareous tests have been widely described for natural and experimentally acidified waters, and include fractures, pitting, boring, scratching (Alve 1991; Shroba 1993; Haynert et al. 2011; Haynert et al. 2012; Haynert et al. 2014; Khanna et al. 2013; Khanna 2014).

Considering the imminent impact of atmospheric CO_2-driven climate change (e.g. OA, sea level rise and warming) on coastal marine ecosystems; natural interactions between foraminiferal population dynamics (e.g. production and loss rates) and environmental stressors (e.g. high CO_2 concentrations, pH, salinity, oxygen, etc.) will certainly change. Thus, some *in-situ* and laboratory studies have revealed that under future high CO_2/low pH scenarios, calcification/growth rate, survival, reproduction process, biomass or test morphometric features (e.g. test weight, size/diameter or functional structures) of benthic foraminifera may be severely affected (Le Cadre et al. 2003; Kuroyanagi et al. 2009;

Allison et al. 2010; Reymond et al. 2011; Saraswat et al. 2011; Khanna et al. 2013; Haynert et al. 2014; Prazeres et al. 2015).

Although great progress on the biological responses of 'live' foraminifera to OA has been made; to date, the information available on the effects of OA on living foraminiferal species from intertidal mudflats is still limited (Khanna et al. 2013; Khanna 2014), and yet an understanding of how taphonomic processes such as enhanced post-mortem dissolution may influence coastal foraminiferal fauna composition is relatively poor developed.

Therefore, the aim of this study is devoted to conducting a short-term CO_2 laboratory-based experiment on multiple biological parameters such as survival rate, test size and weight, growth/calcification rate, morphology changes and post-mortem dissolution of two dominant benthic foraminifera *E. williamsoni* and *H. germanica.* Undoubtedly, these new experimental studies improve our understanding of potential ecological responses of foraminiferal fauna to OA (direct effects) and associated taphonomic processes (indirect effects) which may cause a decline in test production and preservational quality of foraminifera in the sedimentary record of coastal environments such intertidal mudflats.

3.2 Materials and Methods

3.2.1 Field sampling and isolation of target foraminifera

Surface sediment scrapes from the top first centimetre were collected in late April 2015 at a low tide from the high intertidal mudflats of the Eden Estuary, N.E. Scotland (Fig. 2.1, Chapter 2).

Target benthic foraminifera *E. williamsoni* and *H. germanica* were identified, cleaned of any detritus attached to their tests, and finally picked out using a fine paint-brush. Isolated specimens were counted and placed in petri dishes pre-filled with filtered seawater and were immediately kept in a temperature-controlled room at 13°C with a light condition of 12:12-hr light: dark cycle until calcein incubation started.

Detailed information on sampling, foraminiferal identification and isolation is outlined in Chapter 2, Sections 2.1 and 2.2.

3.2.2 Foraminiferal calcein incubation

For this experimental design, calcein incubation was carried out without the use of natural sediment, thus approximately 4600 isolated 'live' foraminiferal specimens of *E. williamsoni and H. germanica* were placed in a plastic container with filtered seawater containing 5 mg/L of the fluorescent stain calcein. This container was continually aerated through an independent line connected to an air pump and kept inside the temperature-controlled room at 13°C for 8 weeks (Fig. 3.1 A). Filtered seawater with calcein was changed once a week. Foraminiferal specimens were fed with algae once a week.

Fortnightly sampling observations through a fluorescence microscope provided information on incorporation processes of calcein into the new growth of foraminiferal tests (Fig. 3.1 B).

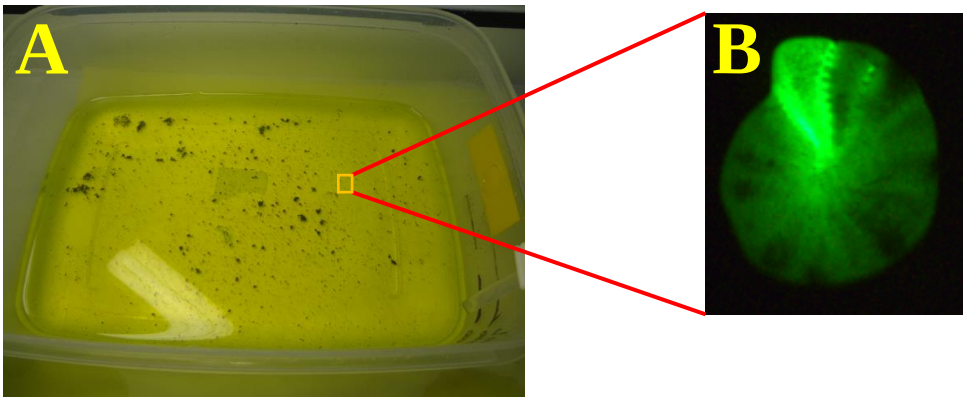

Figure 3. 1 A) Calcein incubation of *Elphidium williamsoni* and *Haynesina germanica* under temperature-controlled conditions of 13°C for 8 weeks with a light condition of 12:12-hr light: dark cycle. B) Specimen of *Elphidium williamsoni* showing the incorporation of calcein into the new growth of foraminiferal test.

When the calcein incubation period was concluded (8 weeks), viable ('live') specimens were examined again through a fluorescence microscope, and surviving specimens were randomly selected, cleaned of detritus attached, and transferred into foraminiferal culture chambers. The latter were subsequently connected to the respective treatments of the CO_2 manipulative mesocosm experiment, as detailed in Chapter 2, Section 2.2.

3.2.3 Experimental conditions

The total number of surviving individuals collected after calcein incubation included 1760 specimens of *E. williamsoni* and 80 specimens of *H. germanica*. 110 specimens of *E. williamsoni* and 5 specimens of *H. germanica* were randomly selected and placed in each

culturing chamber containing already silica onto the polycarbonate membrane insert, as detailed in Chapter 2, Section 2.4.

Each fraction (115 specimens of both species) corresponds to the initial number of specimens placed into each of the 16 culture chambers set up for this experiment. Four culturing chambers (replicates) were used for each pH treatment and these were subsequently connected to a manipulative mesocosm, beginning with an acclimation period and the subsequent CO_2 experiment.

Foraminiferal culturing chambers were maintained for 31 days in a controlled recirculating seawater system within the temperature-controlled room at 13°C, with a 12:12-hr light: dark cycle. This time period included 10 days of acclimation. The remaining 21 days corresponded to the short-term experiment itself with different pH conditions: pH~8.1 (ambient), pH 7.9, pH 7.7 and pH 7.3 (see more detailed information on culturing chamber and carbonate chemistry manipulation system in Chapter 2, Sections 2.4 and 2.5, respectively).

3.2.4 Rose Bengal staining method

After concluding the experimental period, all culture chambers were opened, and membrane inserts were removed and placed in sterile 6-tissue culture plates prior to being stained with Rose Bengal solution for 7 days. Rose Bengal dissolved in ethanol was prepared according to the technique described in Schönfeld et al. (2012). The foraminiferal specimens were then picked out and transferred to clean petri dishes and washed with distilled water to remove any excess staining solution and silica. This staining method

usually makes it easy to distinguish 'live' and dead organisms by observing protoplasm-containing 'live' specimens (Bernhard 2000; Bernhard et al. 2006). Living/recent living organisms showed intensely red-stained protoplasm whereas dead individuals showed either white empty test or spotty stained protoplasm (Austin 2003).

The number of 'live' specimens using Rose Bengal stain was later compared with the number of 'live' specimens estimated by the count of specimens with newly added chambers (calcein fluorescence). This comparison allowed a test of whether or not the Rose Bengal method leads to an overestimation in the number of 'live' foraminifera compared to the calcein (growth) method.

As noted above, in addition to the Rose Bengal method, another criterion for the identification of 'live' foraminifera was applied at the end of the experiment. This method consisted of identifying those individuals under fluorescence microscope that show at least one new chamber deposited during the experimental period. The two different methods for the identification of surviving specimens were compared when survival rates were estimated.

3.2.5 Biological parameters

For each specimen of both species of benthic foraminifera, the following biological parameters were recorded: the morphological response to low pH levels, the number of new chambers deposited, the maximum test diameter and test weight. Survival rates and growth rates were estimated as detailed in Chapter 2, Section 2.6.

3.2.6 Post-mortem dissolution via weight-size relationship of 'live' and recently dead assemblages

The potential post-mortem dissolution effect on *E. williamsoni* and *H. germanica* was assessed via change in the weight-size relationship of 'live' and recently dead assemblages exposed for 31 days to different pH conditions (e.g. pH~8.1 (ambient), pH 7.9, pH 7.7 and pH 7.3) as detailed in Chapter 2 and 3.2.

3.2.7 Statistical Method

All statistical analyses were run in the statistical programme R 3.1.2 (R Development Core Team. 2014) and all of the steps are explained in Chapter 2, Section 2.9.

3.3 Results

3.3.1 Composition of dominant foraminiferal species from field samples

Foraminiferal assemblages of field samples showed that both target species were the two most dominant in abundance in comparison to other foraminiferal species; however, *E. williamsoni* exhibited a much higher number of living individuals (10-20 living specimens/cm^3 of wet sediment) than *H. germanica* (1-3 living specimens/cm^3 of wet sediment). Hence, living specimens of both foraminiferal species were used for this experimental research.

3.3.2 Seawater carbonate chemistry

Measurements of seawater carbonate parameters taken on a fortnightly basis indicate that these parameters were constant throughout the entire time period of 31 days for the CO_2 experiment (Table 3.1).

Table 3. 1 Seawater measurements taken from the experimental carbonate chemistry manipulation system. Values account for mean ± SD, n = 2.

Measured parameters					**Calculated parameters**					
Treatment	**pH (Total)**	**Temp (°C)**	**Salinity (ppt)**	**A_T (µmol/Kg)**	**DIC (µmol/kg)**	**pCO_2 (µatm)**	**HCO_3^- (µmol/kg)**	**CO_3^{2-} (µmol/kg)**	**$\Omega_{Calcite}$**	**$\Omega_{Aragonite}$**
pH 8.1 (ambient)	8.10 ± 0.01	13.03 ± 0.10	32.90	2376.32 ± 83.16	2152.45 ± 76.37	368.07 ± 16.83	1976.01 ± 69.48	161.67 ± 8.21	3.90 ± 0.20	2.49 ± 0.13
pH 7.9	7.92 ± 0.02	13.03 ± 0.10	33.1 ± 0.09	2355.18 ± 30.00	2212.53 ± 34.44	589.74 ± 39.63	2076 ± 35.38	112.07 ± 4.13	2.70 ± 0.10	1.72 ± 0.06
pH 7.7	7.72 ± 0.02	13.13 ± 0.05	33.1 ± 0.18	2324 ± 47.90	2253.26 ± 44.60	962.76 ± 33.51	2141.52 ± 41.88	73.26 ± 3.75	1.77 ± 0.09	1.13 ± 0.06
pH 7.3	7.32 ± 0.02	13.3 ± 0.09	32.86 ± 0.10	2408.41 ± 55.00	2466.79 ± 54.36	2640 ± 140.88	2329.92 ± 52.20	31.76 ± 2.25	0.77 ± 0.05	0.49 ± 0.03

3.3.3 Morphological response

The three levels of morphological changes were observed in tests of 'live' and recently dead assemblages of *E. williamsoni* as a potential response to the experimental pH conditions (Fig. 3.2).

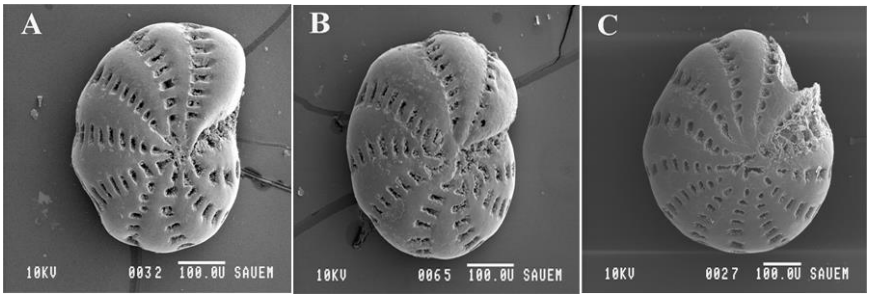

Figure 3. 2 Scanning electron micrographs (SEM) images to illustrate the morphological changes in tests of *Elphidium williamsoni* observed across pH conditions. Morphological response levels were: A) **Level 1**= intact test, B) **Level 2** = minor changes and C) **Level 3** = broken test. White scale bar represents 100 µm.

The highest numbers of broken tests were found at pH 7.3 followed by pH 7.7, 7.9 and pH 8.1 (ambient), respectively, whereas the highest numbers of intact tests were observed at pH 7.9, pH 7.7, pH 7.3 and pH 8.1 (ambient). The highest numbers of specimens with minor alterations were found at pH 8.1 (ambient), pH 7.7, pH 7.9 and pH 7.3, respectively (Fig. 3.3). Despite these observations, there are no obvious trends related to the pH effect on foraminiferal morphology.

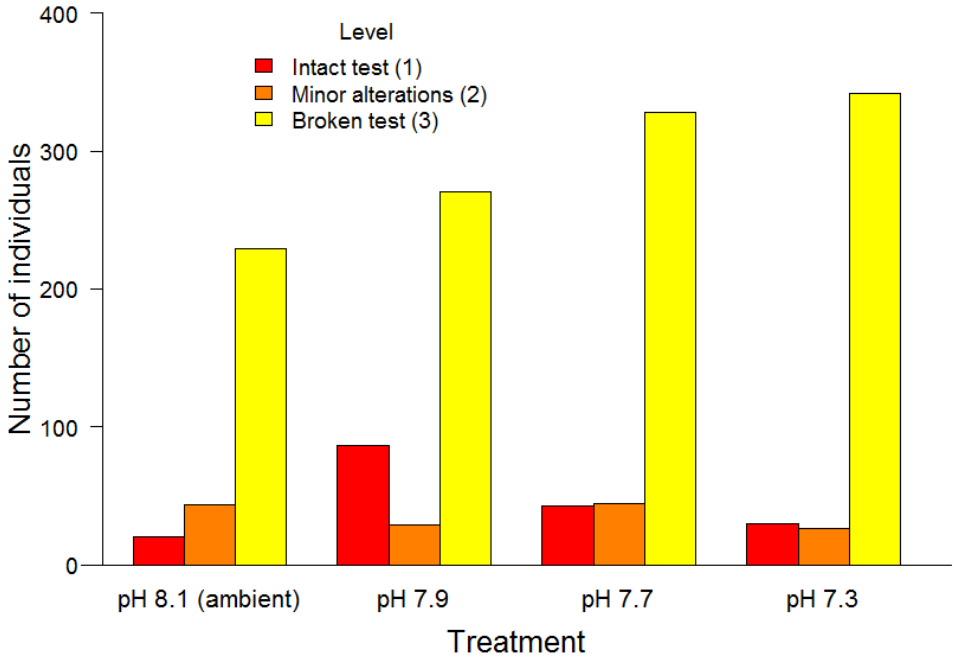

Figure 3. 3 Number of individuals ('live' and recently dead) with morphological changes observed in tests of *Elphidium williamsoni* as a potential response to experimental pH conditions. Morphological response levels were: A) **Level 1**= intact test, B) **Level 2** = minor changes and C) **Level 3** = broken test.

3.3.4 'Live' and recently dead foraminiferal individuals and survival rate

At the end of the experiment, specimens from all culturing chambers were collected and only 1498 individuals of *E. williamsoni* and 14 individuals of *H. germanica* were retrieved. This was a loss of 262 and 66 individuals for both species, respectively (Table 3.2). Lost specimens are most likely to be attributed to migration out of the culturing chambers into the reservoir tanks due to sporadic changes in pressure inside the experimental chambers.

Survival rate (SR) calculated based on Rose Bengal and post-fluorescent growth (new chambers deposited) are shown in Table 3.2.

Table 3. 2 Survival rates (SR) of *Elphidium williamsoni* and *Haynesina germanica* cultured for 31 days under different pH condition: pH~8.1 (ambient), pH 7.9, pH 7.7 and pH 7.3.

	Total number of individuals					
Species and pH conditions	**Start of the experiment**	**End of experiment**			**Survival rate (%)**	
		Retrieved/ Analyzed	**Alive (Rose Bengal)**	**Alive (post-fluorescent growth)**	**Rose Bengal**	**post-fluorescent growth**
E. williamsoni						
pH 8.1 (ambient)	440	294	3	12	0.7	4.1
pH 7.9	440	386	4	23	0.9	6.0
pH 7.7	440	418	15	11	3.4	2.6
pH 7.3	440	399	4	8	0.9	2.0
H. germanica						
pH 8.1 (ambient)	20	8	2	1	10.0	12.5
pH 7.9	20	2	1	0	5.0	0.0
pH 7.7	20	1	0	0	0.0	0.0
pH 7.3	20	3	1	0	5.0	0.0

Due to the low numbers of 'live' individuals of *H. germanica* retrieved at the end of the experiment, these specimens were removed from any further analysis. Henceforth, all subsequent measurements were only based on the remaining specimens of *E. williamsoni.*

3.3.5 Distribution by foraminiferal size class

Recently dead and 'live' specimens of *E. williamsoni* sorted into size class in each experimental treatment are shown in Fig. 3.4. A normal distribution was expected due to the randomness in sampling when individuals were placed into experimental chambers prior to the start of the experiment. However, the use of the bandwidth of 25 µm as a size

interval may have caused a size distribution with multiple peaks, despite all data showing a single well-defined maximum diameter (Fig. 3.4).

Recently dead individuals of a specific size class can be estimated by subtracting 'live' individuals from the total number of specimens observed in the size distribution chart (Fig. 3.4).

In general, the greatest number of recently dead individuals was found in the size class of 400-425 µm. The highest mean maximum test diameters were found in the treatment at a pH of 7.9 followed by the treatment at a pH of 7.7 and pH 8.1 (ambient), respectively. The lowest maximum test diameter observed was in treatment at a pH of 7.3 (Fig. 3.4).

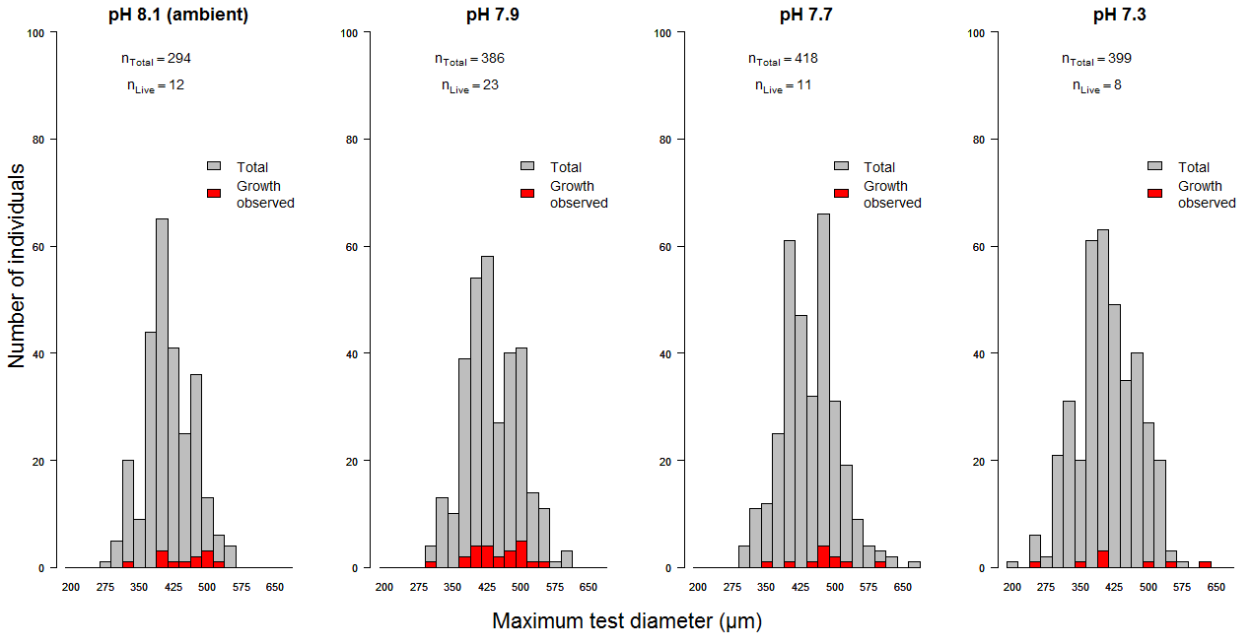

Figure 3. 4 The total number of individuals of *Elphidium williamsoni* sorted into size classes after being collected at the end of the experimental period in each culture condition (pH 8.1 (ambient), pH 7.9, pH 7.7 and pH 7.3). The individuals analysed (n_{Total}) and 'live' specimens (n_{Live}) observed are shown in grey and red, respectively. Bandwidth for each size class was 25 µm.

3.3.6 Size of 'live' foraminiferal individuals

Size classes greater than 400 µm in all treatments had the lowest number of 'live' specimens. Individuals cultured at a pH of 7.9 showed the largest number of surviving specimens (n_{Live} = 23) followed by the treatments of a pH of 8.1 (ambient) and pH 7.7, respectively. In contrast, individuals cultured at a pH of 7.3 showed the lowest number of surviving specimens (n_{Live} = 8) (Fig. 3.5).

The largest specimens were found in the treatment at a pH of 7.7 followed by the treatment at a pH of 8.1 (ambient), pH 7.9 and pH 7.3, respectively (Table 3.3).

Table 3. 3 Maximum test diameter (µm) of 'live' *Elphidium williamsoni* cultured under different experimental pH conditions: pH~8.1 (ambient), pH 7.9, pH 7.7 and pH 7.3.

Measured parameter	pH conditions	Min.	Max.	Mean	Standard deviation (1σ)	Standard error of mean	n
Maximum test diameter (µm)	**8.1 (ambient)**	335.8	545.6	460.5	57.7	16.6	**12**
	7.9	321.8	559.6	457.4	58.8	12.3	**23**
	7.7	363.7	615.6	488.4	65.5	19.7	**11**
	7.3	265.8	643.5	445.9	118.6	41.9	**8**

Shapiro-Wilk and Levene tests verified the normality (p = 0.4615) and homogeneity (p = 0.7227) of the maximum test diameter dataset. A one-way ANOVA indicates that there is no statistically significant effect of pH treatments on foraminiferal size (p = 0.575).

The Tukey test (pairwise comparison) also indicates no significant differences between pH treatments (Fig. 3.5).

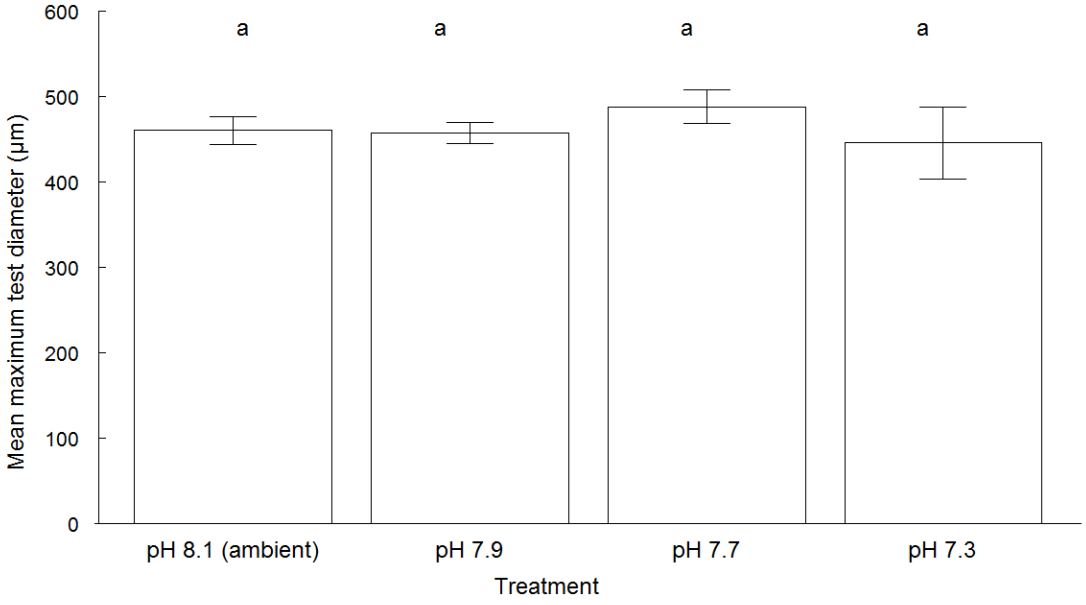

Figure 3. 5 Mean values (± standard error) of maximum test diameter (µm) for 'live' *Elphidium williamsoni* cultured at different pH conditions. Significant differences are indicated by different letters above bars at $p < 0.05$, otherwise, treatments with similar letters (i.e. **a**) correspond to the same group where no significant differences are observed between groups according to the Tukey's test.

3.3.7 Foraminiferal weight of 'live' specimens

The heaviest mean test weights were found in the treatment at a pH of 7.7 followed by the treatments at a pH of 8.1 (ambient) and pH 7.3, respectively. In contrast, the lightest weights were observed at a pH of 7.9 (Table 3.4).

Table 3. 4 Test weight of 'live' *Elphidium williamsoni* cultured under different experimental pH conditions: pH~8.1 (ambient), pH 7.9, pH 7.7 and pH 7.3.

Measured parameter	pH conditions	Min.	Max.	Mean	Standard deviation (1σ)	Standard error of mean	n
Test weight (µg)	**8.1 (ambient)**	5.0	24.0	14.3	5.6	1.6	**12**
	7.9	6.0	22.0	12.9	6.8	1.0	**23**
	7.7	6.0	24.0	15.6	5.4	1.6	**11**
	7.3	4.0	26.0	14.0	4.7	2.4	**8**

Shapiro-Wilk and Levene tests verified the normality (p = 0.1972) and homogeneity (p = 0.8869) of the test weight dataset. One-way ANOVA indicates there is no statistically significant effect of pH treatments on foraminiferal weight (p = 0.602). The Tukey's test (pairwise comparison) also indicates no significant difference between treatments (Fig. 3.6).

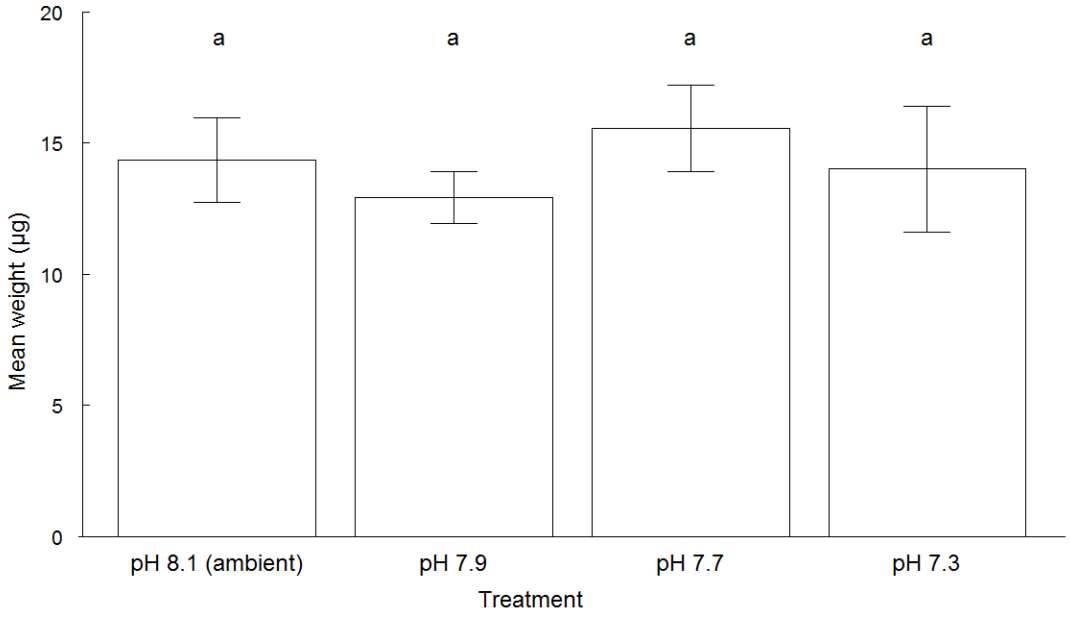

Figure 3. 6 Mean values (± standard error) of weight (µg) for 'live' *Elphidium williamsoni* cultured at different pH conditions. Significant differences are indicated by different letters above bars at $p < 0.05$, otherwise, treatments with similar letters (i.e. **a**) correspond to the same group where no significant differences are observed between groups according to the Tukey's test.

3.3.8 Newly deposited chambers and growth rate

Large differences in the number of new chambers added by 'live' specimens were observed within and between treatments (Table 3.5). The number of new chambers averaged per treatment was used to calculate the mean growth rates (chambers day^{-1}). The days of culturing used were 31 which included 10 days of acclimation and 21 days of experiment.

Table 3. 5 Number of chambers added and growth rate of 'live' *Elphidium williamsoni* cultured under different experimental pH conditions: pH~8.1 (ambient), pH 7.9, pH 7.7 and pH 7.3.

Measured variables	pH conditions	Min.	Max.	Mean	Standard deviation (1σ)	Standard error of mean	n
Number of chambers added	8.1 (ambient)	1.00	3.00	1.41	0.67	0.01	**12**
	7.9	1.00	7.00	2.35	1.77	0.01	**23**
	7.7	1.00	9.00	3.46	2.98	0.03	**11**
	7.3	1.00	4.00	2.38	1.18	0.01	**8**
Growth rate (chambers day^{-1})	8.1 (ambient)	0.03	0.10	0.05	0.02	0.01	**12**
	7.9	0.03	0.23	0.08	0.06	0.01	**23**
	7.7	0.03	0.29	0.11	0.10	0.03	**11**
	7.3	0.03	0.13	0.08	0.04	0.01	**8**

Individuals cultured at a pH of 8.1(ambient) mainly showed an addition of one or two chambers, with the exception of one specimen with 3 chambers added. The average number of chambers added was of 1.42 ± 0.67; suggesting an average growth rate of 0.046 ± 0.022 chambers day^{-1} (Fig. 3.7 and Table 3.5).

Most of the specimens cultured at a pH of 7.9 also showed an addition of one or two chambers over the experiment. However, some specimens added more than 3 chambers; the maximum number of new chambers was 7. The average number of chambers added was 2.35 ± 1.77; suggesting an average growth rate of 0.076 ± 0.057 chambers day^{-1} (Fig. 3.7 and Table 3.5).

Individuals cultured at a pH of 7.7 showed an addition of chambers in a range between 1 and 9. This treatment showed both the highest variability in chambers addition and growth rate compared with the ambient treatment (pH 8.1). The average number of chambers added was 3.46 ± 2.98; suggesting an average growth rate of 0.111 ± 0.096 chambers day^{-1} (Fig. 3.7 and Table 3.5).

Specimens cultured at a pH of 7.3 showed an addition of chambers in a range between 1 and 4. The average number of chambers added was 2.38 ± 1.18, suggesting an average growth rate of 0.077 ± 0.038 chambers day^{-1} (Fig. 3.7 and Table 3.5).

The highest mean growth rates were found in the treatment a pH of 7.7, followed by the pH 7.9, and pH 7.3 treatments, respectively. In contrast, the lowest mean growth rate was observed at a pH of 8.1 (ambient) (Fig. 3.7 and Table 3.5). Shapiro-Wilk and Levene tests verified the normality (p = 2.349 x 10^{-8} and homogeneity (p = 0.01066) of growth rates. Despite the greater variability in chamber addition rate of the pH 7.7 treatment, a Kruskal-Wallis test indicates that there is no significant difference between all the treatments (p = 0.2359). Dunn's-test, a post-hoc (pairwise comparison) test also indicates no significant differences between treatments.

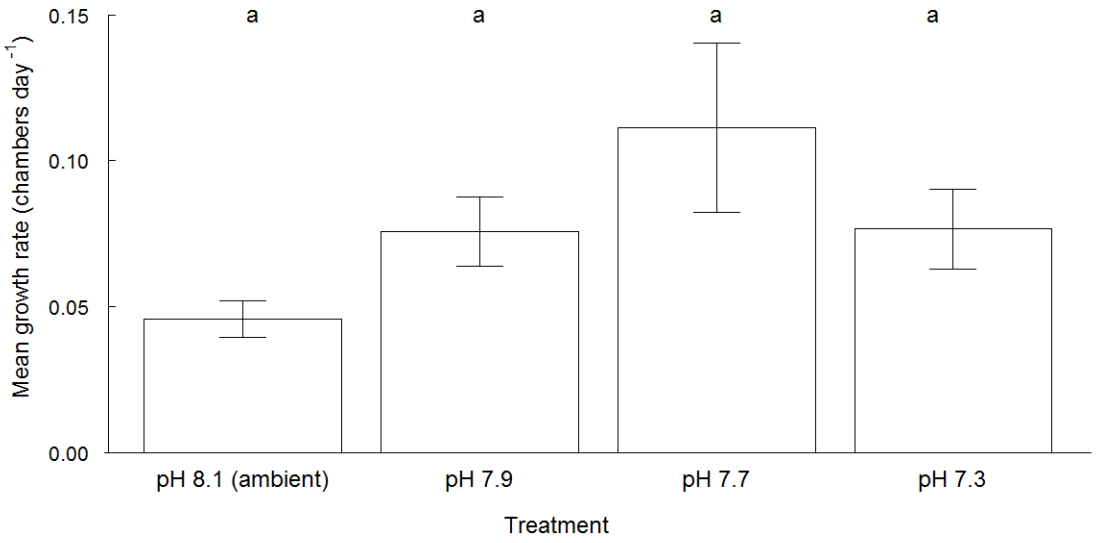

Figure 3. 7 Mean values (± standard error) of the growth rate (chambers day^{-1}) for *Elphidium williamsoni* cultured at different pH conditions for an experimental period of 31 days. Significant differences are indicated by different letters above bars at $p < 0.05$, otherwise, treatments with similar letters correspond to the same group where no significant differences are observed between groups according to the Dunn's-test.

3.3.9 Size-weight relationship of 'live' specimens

The comparison of the slopes of linearised functions for size-weight regression relationships of 'live' *E. williamsoni* showed no statistically significant difference across pH treatments ($p > 0.05$) (Fig. 3.8 and Table 3.6).

Table 3. 6 Comparison between slopes of the linearised function defining the relationship between maximum diameter and test weight for 'live' *Elphidium williamsoni* cultured under different experimental pH conditions: pH~8.1 (ambient), pH 7.9, pH 7.7 and pH 7.3.

Comparison between slopes	Std. Error	t-value	*p*-value
pH 8.1 (ambient) x pH 7.9	0.000554	0.559	0.944
pH 8.1 (ambient) x pH 7.7	0.000643	0.63	0.922
pH 8.1 (ambient) x pH 7.3	0.000531	1.32	0.555
pH 7.9 x pH 7.7	0.000555	-0.171	0.998
pH 7.9 x pH 7.3	0.000420	-0.932	0.788
pH 7.7 x pH 7.3	0.000532	-0.557	0.944

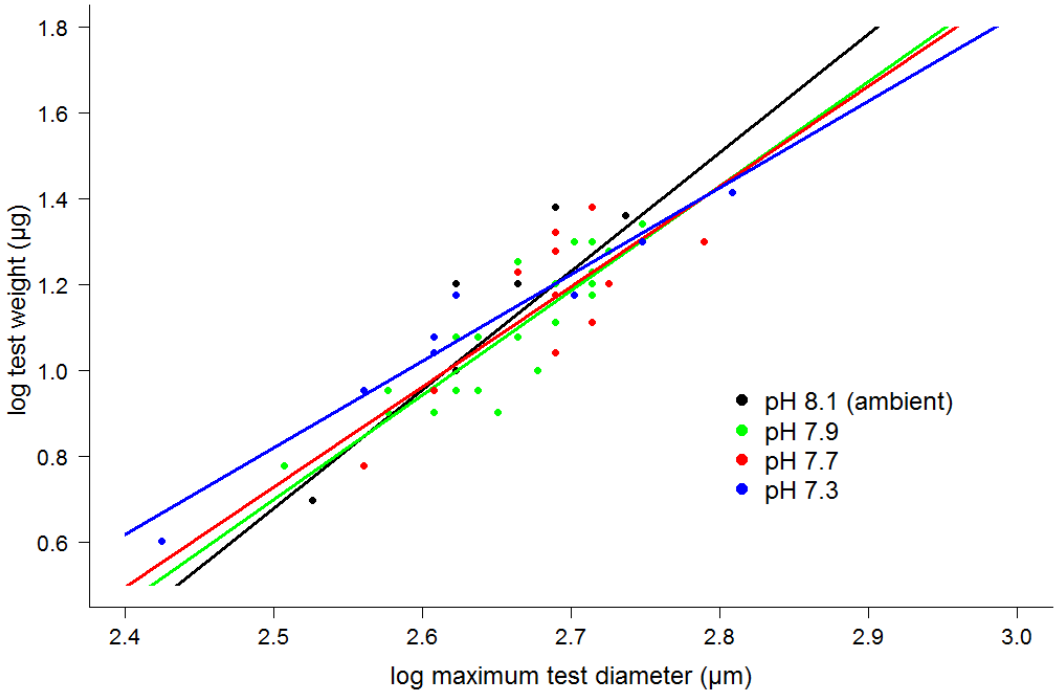

Figure 3. 8 Regression lines of the relationship between test weight and maximum test diameter of 'live' *Elphidium williamsoni* cultured under different experimental pH conditions: black (ambient: pH 8.1/ ~380 µatm CO_2); green (pH 7.9/ ~600 µatm CO_2); red (pH 7.7/ ~1000 µatm CO_2) and blue (pH 7.3/ ~2400 µatm CO_2).

3.3.10 SEM observations of morphological response of 'live' *E. williamsoni*

SEM images of intact tests of 'live' *E. williamsoni* showed considerable morphological differences between pH treatments. These observations indicated a progressive alteration of the foraminiferal morphology (test) when individuals were exposed to high CO_2 concentrations/low pH for 31 days. The most remarkable features observed on the test surface are the presence of cracks and signs of dissolution on individuals exposed to the low pH levels (Fig. 3.9, D-H). Specimens cultured at a pH of 7.7 and 7.3 clearly displayed dissolution around the apertural region, notably on apertural teeth (Fig. 3.9, F & H). In addition, the outermost chambers of foraminiferal specimens cultured at a pH of 7.7 and pH 7.3 (Fig. 3.9, E & G) displayed large and irregular septal bridges and sutures with clear signs of corrosion in comparison to those cultured at a pH of 8.1 and pH 7.9, which exhibited smooth surfaces and regular shapes of these structures (Fig 3.9, A & C).

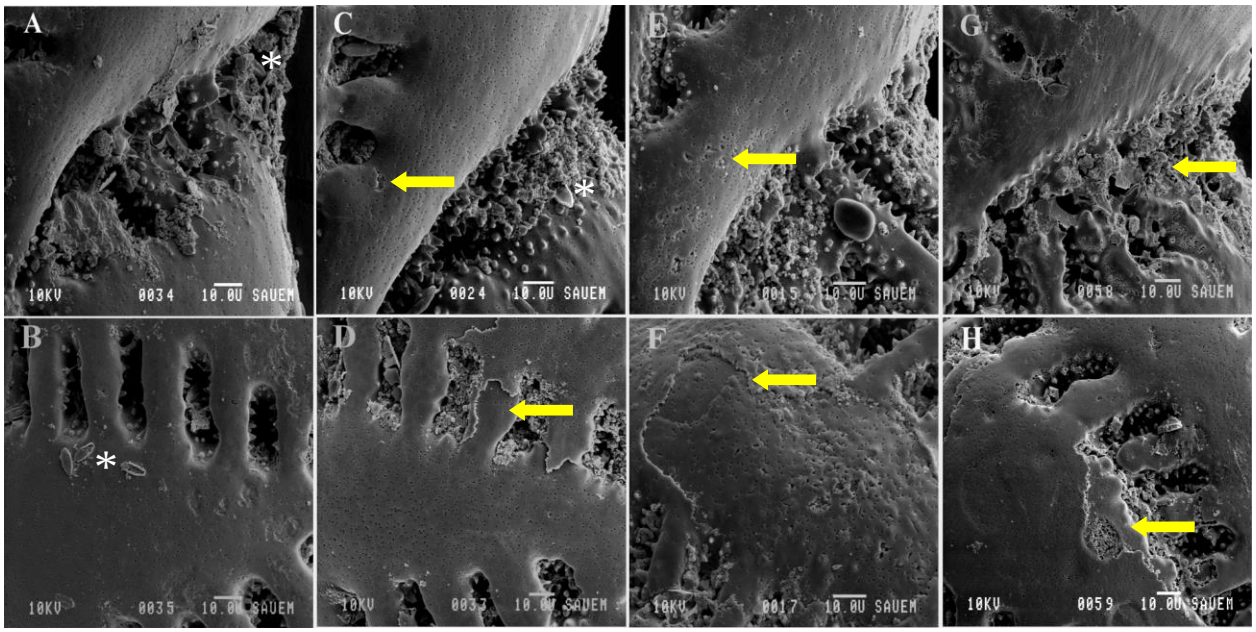

Figure 3. 9 Scanning electron micrographs (SEM) of 'live' *Elphidium williamsoni* cultured at pH 8.1 (A & B), pH 7.9, (C & D), pH 7.7 (E & F) and pH 7.3 (G & H)**.** (A) A side view of the apertural region showing numerous teeth and tubercles. A frustule of the diatom *Navicula sp*. and organic detritus are visible on feeding structures. (B) A side view of the smooth test surface of specimen A. (C) A side view of the apertural region, showing numerous teeth and tubercles with frustules of the diatoms *Navicula sp*. (D) A side view of the smooth test surface with slight signs of dissolution on the septal bridges of specimen C. (E) A side view of the apertural region, where slight signs of dissolution and cracking are observed. Teeth and tubercles are still sharp. (F) A side view of the test surface of specimen E affected by dissolution and cracking processes. (G) A side view of the apertural region showing a reduction in the number of teeth and tubercles. Dissolution and cracking processes are clearly observed on septal bridges and sutures. (H) A side view of the test surface of specimen G, showing several test wall layers, septal bridges and sutures affected by dissolution and cracking processes. (*) White asterisks show the presence of diatom *Navicula sp*. ←Yellow arrows show areas affected by dissolution. White scale bar represents 10 µm.

3.3.11 Post-mortem dissolution effect via size-weight relationship between 'live' and recently dead specimens

To evaluate the effect of OA on the size-weight relationship of *E. williamsoni* cultured at different pH treatments, data of both measured variables were log transformed, and the slopes of linearized functions of 'live' and recently dead specimens were compared for each pH treatment using a T-test (Table 3.7). The comparison of the slopes showed that test weight is positively related to maximum test diameter across treatments. Despite slight differences between slopes ('live' and recently dead) within each treatment (Fig. 3.10 and Table 3.7), no significant differences were observed ($p > 0.05$) except for the treatment at a pH of 7.3 ($p = 0.042$) (Fig. 3.10 and Table 3.7).

Table 3. 7 Comparison between slopes of the relationship between maximum diameter and test weight for 'live' and recently dead *Elphidium williamsoni* cultured under different experimental pH conditions: pH~8.1 (ambient), pH 7.9, pH 7.7 and pH 7.3. All *p*-values in bold indicate a statistically significant difference between slopes compared.

pH conditions	Intercept	Slope	Std. Error	t-value	*p*-value
'Live' specimens					
8.1 (ambient)	-14.37	2.77	0.57	4.88	< **0.001**
7.9	-12.41	2.44	0.29	8.39	< **0.0001**
7.7	-11.76	2.34	0.62	3.78	< **0.01**
7.3	-9.73	2.02	0.19	10.57	< **0.0001**
Recently dead specimens					
8.1 (ambient)	-12.90	2.51	0.09	29.05	< **0.001**
7.9	-14.97	2.83	0.08	36.45	< **0.001**
7.7	-14.05	2.67	0.06	41.48	< **0.001**
7.3	-12.63	2.44	0.08	29.91	< **0.001**
Comparison between slopes of 'live' and recently dead specimens					
8.1 (ambient)			0.58	0.45	0.653
7.9			0.30	-1.30	0.196
7.7			0.62	-0.53	0.597
7.3			0.21	-2.04	**0.042**

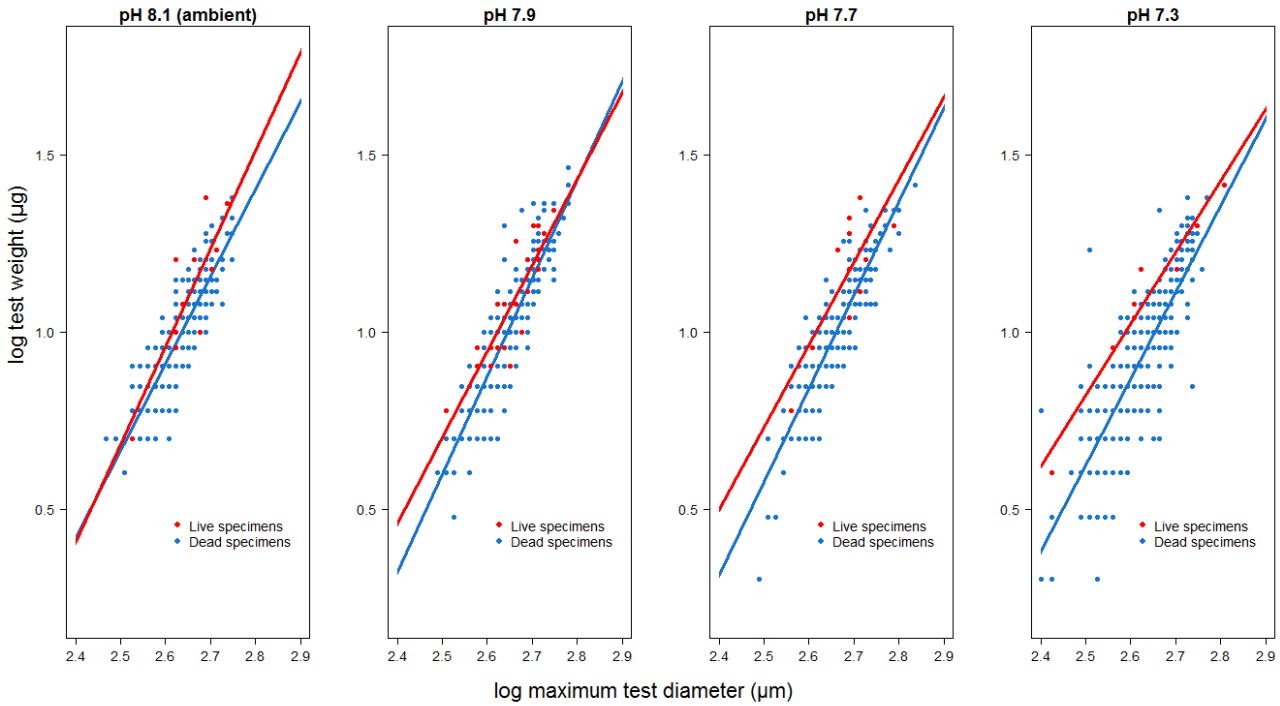

Figure 3. 10 Regression line of the relationship between maximum test diameter and test weight for 'live' (red) and recently dead (blue) *Elphidium williamsoni* cultured under different experimental pH conditions: pH~8.1 (ambient), pH 7.9, pH 7.7 and pH 7.3.

3.4 Discussion

In this study, the dominance of *E. williamsoni* and *H. germanica* over other living benthic foraminiferal species was clearly observed in sediment samples collected from the high intertidal mudflats of the Eden Estuary. This supports previous findings (Austin 2003) on the co-occurrence and dominance of both species throughout the year at a nearby study site but with the only difference that in this current research, *E. williamsoni* was highly dominant over *H. germanica.* The average abundance of specimens per cm^3 observed in wet samples over repeated field trips confirmed *E. williamsoni* as the dominant species. This natural dominance of *E. williamsoni* in samples taken from mudflats on the Eden Estuary in late April 2015 may have coincided with the reproduction season for this particular foraminiferal species, just after the peaks of benthic diatom biomass during March and April as found by Austin (2003).

3.4.1 Survival rate

Survival rate estimated through both the Rose Bengal and the chamber addition methods showed an extremely low percentage of surviving individuals after a 31 day-experiment (Table 3.2). Although the number of surviving specimens varied markedly between the two methods to distinguish between 'live' and dead specimens, the chamber addition criterion was selected over the Rose Bengal method, which tended to over or underestimate 'live' specimens (Bernhard 2000; Bernhard et al. 2006) (Table 3.2). Adoption of chamber addition criterion allowed a larger number of individuals to be analysed because the post-fluorescent growth was easily recognised under a fluorescence microscope (Bernhard et al.

2004). Furthermore, this method avoided an inaccurate selection of individuals weakly stained with Rose Bengal and provided a better estimate of 'live' specimens (Bernhard 2000; Bernhard et al. 2006).

As one might expect, in this study, the lowest number of surviving specimens of *E. williamsoni* was observed at the lowest pH treatments (Fig. 3.4 and Table 3.2).

The number of specimens of *H. germanica* used at the start of the experiment and the subsequent extremely low survival rate did not allow further study of the response of this species to OA.

3.4.2 Foraminiferal maximum test diameter and test weight

As the maximum test diameter of 'live' individuals of *E. williamsoni* ranged from approx. 250 to 650 µm across all pH treatments, juvenile specimens (<100 µm) were not observed at the end of the experimental period. This fact indicates that reproduction events were unlikely to have occurred during the experimental period across all pH treatments. Foraminiferal species may show some reproduction events when they are experimentally exposed to elevated pCO_2. For instance, *Amphistegina gibbosabosa* exhibited reproduction under high CO_2 levels during the first week of the experimental period; this fact was potentially a stress response to the sudden change in environmental conditions (McIntyre-Wressnig et al. 2013).

In this study, both mean maximum test diameter and mean test weight were not significantly affected by high CO_2/low pH conditions (Fig. 3.5 and 3.6, respectively). This fact may indicate that foraminifera grew in size and weight at similar rates regardless of the

pH condition over the short-term duration of the experiment. In addition, factors such as the low number of surviving specimens retrieved at the end of the experimental period and the high variability in size and weight of each 'live' individual of *E. williamsoni* observed within each experimental condition may have affected the estimation of the mean values for size and test weight. Potentially, a much longer exposure time is required for foraminifera to be affected by elevated pCO_2 in order to observe changes in size or weight, ultimately producing smaller tests, thinner chambers and lighter tests similar to those observed in other studies (Kuroyanagi et al. 2009; Allison et al. 2010; Prazeres et al. 2015).

3.4.3 Growth rate

Although foraminiferal growth is commonly estimated based on continuous measurements of biometric parameter such as size or weight (Austin 2003; Kuroyanagi et al. 2009; Haynert et al. 2011; Haynert et al. 2014; Prazeres et al. 2015); in this study, due to experimental restrictions, the growth rate of *E. williamsoni* could not be calculated by counting the number of chambers added throughout the experimental period as continuous measurements (Austin 2003). Instead, the growth rate was estimated through the chamber addition method which required a calcein incubation before the start of experiments as suggested by other studies (Bernhard et al. 2004; Allison et al. 2010; Khanna 2014); in this set-up, newly deposited chambers were easily identified.

No significant difference was found in the number of new chambers added by *E. williamsoni* across pH treatments (Fig. 3.7). These results are similar to those found by Allison et al. (2010) where no significant difference was observed in the number of

chambers deposited by *E. williamsoni* cultured at 15°C for 8 weeks at similar pH levels (8.1 and 7.6).

In contrast, Khanna (2014) using a similar experimental set-up design for a CO_2 manipulative mesocosm found a significant difference in the average number of chambers added by *E. williamsoni* cultured at 15°C for 6 weeks at pH values of 7.7 and 7.3. Estimations of the chamber addition rate (based on the dataset available in Khanna (2014)) suggest an average growth rate of 0.042 (pH 7.7) and 0.099 (pH 7.3) chambers day^{-1}. Furthermore, Austin (2003) experimentally found that *E. williamsoni* was able to grow up to 0.10 chambers day^{-1} at pH ~8.1 (ambient) and 10°C. These mean growth rates found by Austin (2003) and Khanna (2014) are in some cases higher than those found in this study for pH ~8.1 (0.05 chambers day^{-1}), pH 7.7 (0.10 chambers day^{-1}) and pH 7.3 (0.04 chambers day^{-1}) (Fig. 3.7 and Table 3.5).

These contrasting findings may be due to multifactorial effects such as the low number of surviving specimens, the high variability in the number of chambers added by each 'live' individual, limited exposure time of specimens to pH conditions and the temperature at which the foraminifera were cultured.

3.4.4 Growth via the size-weight relationship of 'live' specimens

'Live' *E. williamsoni* exhibited an unchanged response to OA when the size-weight relationship was assessed across pH treatments (Fig. 3.8 and Table 3.6). By using only this relationship, the results from the current research were similar to those observed in specimens of a large benthic foraminifera from coral environment *Marginopora vertebralis*

exposed to similar pH conditions (8.1, 7.9, 7.7 and 7.6) for an experimental period of 30 days at 25°C (Prazeres et al. 2015).

E. williamsoni and *M. vertebralis* differ in the coastal environment they come from and also in their test composition, with low-Mg calcite and high-Mg calcite tests, respectively (Bentov & Erez 2006; Prazeres et al. 2015). However, the two species have exhibited a similar biological (biometric) response to induced OA. Generally, two foraminiferal species with a difference in the carbonate mineral precipitated, biomineralization mechanisms and test morphology should show species-specific responses to lowering pH (Prazeres et al. 2015).

3.4.5 Evidence of post-mortem dissolution via size-weight relationship

It was anticipated that any change in the positive correlation between the size and weight of foraminiferal tests would be reflected in the slope of this relationship (Prazeres et al. 2015). These changes may imply that 'live' specimens are generally heavier than recently dead specimens of the equivalent test diameter. In this research, significant changes in the slopes of both 'live' and recently dead specimens were only observed when specimens were exposed to pH 7.3. This provides the initial evidence of a post-mortem dissolution effect induced by OA in a relatively short experimental period of 31 days where (Fig. 3.10 and Table 3.7).

For the remaining pH treatments, although the relationship between size and weight for both 'live' and recently dead specimens did not change significantly, it was assumed that pH treatments may have caused at least a certain level of corrosion and dissolution on

foraminiferal tests but at lower rates than at pH 7.3. Here, this fact is partially supported by *E. williamsoni* morphological responses to OA (e.g. high number of broken tests at lowest pH) (Fig. 3.2) and also documented in the SEM images where increasing changes in the experimental conditions were associated with major impacts on tests exposed to the lowest pH levels (Fig. 3.9).

Despite the low number of 'live' specimens being insufficient to be fully representative and therefore conclusive of the potential impact of OA on *E. williamsoni* size-weight relationships, the comparison of slopes between 'live' and recently dead specimens used in this study may be a useful tool for future comparative studies focused on taphonomic process induced by OA.

3.4.6 SEM observations of the morphological response of 'live' specimens

SEM images of intact tests of 'live' specimens of *E. williamsoni* have provided further details of the different stages of dissolution on foraminiferal tests exposed to high CO_2 concentrations/low pH over a period of 4 weeks (Fig. 3.9). These progressive morphological alterations have been also found on tests of *Ammonia aomoriensis* cultured in similar seawater pCO_2 concentrations for a period of 6 weeks (Haynert et al. 2011). In addition, Khanna et al. (2013) presented evidence of extreme morphological modifications to the functional structures in specimens of *H. germanica* experimentally exposed to similar seawater pH levels over a period of 36 weeks.

Although it is true that the current study and that of Khanna et al. (2013) exhibit a substantial corrosion of apertural teeth and also of test surface, the effect of OA is probably

more clearly observed on specimens of *H. germanica* (Khanna et al. 2013) compared to *E. williamsoni* (this study) as the former were exposed to similar high CO_2 concentrations/low pH for a much longer period.

However, based on the exposure time, our findings may also indicate that *E. williamsoni* show a high vulnerability (low resilience) to changes in carbonate seawater chemistry despite their low-Mg calcite tests that should help resist corrosion and dissolution at early stages when exposed to low pH and calcite undersaturated waters (Table 3.1).

3.4.7 Ecological significance and future work

As benthic foraminifera play a crucial role in biogeochemical cycles in coastal environments (Moodley et al. 2000), the study of short-term effects of OA on benthic foraminifers are important to better understand how ecosystem services of intertidal mudflat environments may change under future high CO_2 scenarios.

In this study, although biological parameters of 'live' *E. williamsoni* such as maximum test diameter, test weight and growth rate were not significantly affected by future pH levels, the main biological impact of OA was observed on their apertural feeding structures. These functionally compromised structures may considerably alter the foraminiferal feeding mechanisms and their feeding efficiency, leading to a loss of ecological competitiveness, long-term fitness and survival (Khanna et al. 2013). This potential negative impact driven by OA may ultimately cause a shift in benthic foraminiferal assemblages composition, giving more benefits to other calcareous foraminiferal species or, in turn, to non-calcifying

species showing a higher resilience to high CO_2/low pH levels (Khanna et al. 2013; Pettit et al. 2015; Dias et al. 2010).

Experimental evidence has shown that two co-occurring species of benthic foraminifera under the same unfavourable environmental conditions exhibit species-specific features that may help one species to prevail over other foraminiferal species in both the short- and long-term (Prazeres et al. 2015). The observations from SEM images of compromised feeding structures suggest that *E. williamsoni* is highly sensitive to high CO_2 concentrations over short periods whereas the negative effect observed by Khanna et al. (2013) on *H. germanica* were observed over a longer experimental period. Differential sensitivity expressed by *E. williamsoni* and *H. germanica* as species-specific responses to low seawater pH should be assessed by conducting further long-term CO_2 experiments where both dominant species are studied together.

Results from post-mortem test dissolution experiments support the suggestion that under future scenarios of high CO_2 concentrations/low pH the dissolution of carbonate tests may strongly affect the foraminiferal test fossilization processes in the sedimentary record, with important implications for long-term storage of carbon in the next decades and centuries. Further test dissolution rates estimations of benthic foraminifera are required to provide new insight into the potential damage (e.g. via changes in weight and morphology) that living and dead assemblages may face under near-future high CO_2 /low pH scenarios.

In general, these results provide relevant insights, highlighting the potential short-term responses of one of the dominant intertidal foraminiferal species of the mid-latitudes to

future scenarios of a high CO_2 world. Further longer-term experiments with a larger number of specimens of both *E. williamsoni* and *H. germanica* are required to assess the significance of OA impacts on their biological parameters such as survival rate, size, weight, size-weight relationship, growth/calcification rate, feeding efficiency and morphology. In addition, further dissolution experiments must be conducted to verify our findings on test dissolution rates and their potential impact on the production and preservation of calcium carbonate in coastal marine environments such as intertidal mudflats.

3.5 Conclusions

This short-term study has shown:

(1) Maximum test diameter, test weight, survival rate, growth rate, SEM imaging and post-mortem dissolution process suggest that a period longer than 4 weeks is required to observe more severe alterations in morphology and biological parameters of *E. williamsoni.*

(2) SEM imaging and post-mortem test dissolution observed suggest that under future scenarios with high CO_2 concentration, 'live' and dead assemblages of *E. williamsoni* may be highly sensitive to OA effects in the short-term, and this may ultimately affect the carbon cycling and total production and preservation of $CaCO_3$ in coastal environments such as intertidal mudflats.

(3) Studies of the potential negative impacts of OA on foraminiferal species from coastal environments are still required to evaluate whether or not their abundance, distribution and diversity, food web-dynamics and the role in the biogeochemical processes (e.g. nutrient fluxes, carbon sink, etc.) are affected by future CO_2/low pH levels.

Chapter 4. Multiple biological responses of *E. williamsoni* and *H. germanica* exposed to short-term high CO_2/ low pH levels and low carbonate ion concentrations [CO_3^{2-}] in an experimental recirculating seawater system

4.1 Introduction

Benthic foraminifera, as one of the dominant groups of unicellular microorganisms inhabiting coastal environments, have shown diverse biological responses to OA in both manipulative experiments and natural CO_2-rich habitats (B B Dias et al. 2010; Hikami et al. 2011; Vogel & Uthicke 2012; McIntyre-Wressnig et al. 2013; Pettit et al. 2013). However, it is predicted that groups belonging to calcareous benthic foraminifera may be more severely affected by future reduced seawater pH associated with increased atmospheric CO_2-induced OA (Fujita et al. 2011; Saraswat et al. 2015).

The observed detrimental effects of OA and concomitant dissolution processes on multiple biological parameters of benthic foraminifera may cause a decline in their abundance, distribution and diversity. This may cause a shift in foraminiferal benthic community structure, increasing the likelihood of greater ecological advantage to non-calcifiers over calcifying species in coastal benthic habitats in the long-term. Furthermore, loss of stress-intolerant species with an active role in food web-dynamics and biogeochemical processes (e.g. nutrient fluxes, carbon sink, etc.) may considerably alter carbon cycling, ecosystem productivity and ecological services usually provided by shallow-water environments (Kroeker et al. 2011).

Many of the studies of OA effects on benthic foraminifera come from coral reef habitats (Kuroyanagi et al. 2009; Fujita et al. 2011; Vogel & Uthicke 2012; Briguglio & Hohenegger 2014; Engel et al. 2015; Prazeres et al. 2015), and this research has improved the ability to accurately predict ecological responses under elevated CO_2 concentrations (Edmunds et al. 2016). However, it is limited to coral reef and not representative of many other coastal sediments habitats whose resident benthic communities may exhibit different vulnerability levels to future changes in the ocean carbonate chemistry. Therefore, further progress on understanding ecological responses by benthic foraminifera from other coastal environments (i.e. intertidal mudflats) is required. To date, studies on the effects of OA on foraminiferal species from intertidal mudflats are limited, and in some cases, this research has been restricted to a single species of benthic foraminifera (Allison et al. 2010; Allison et al. 2011b; Khanna et al. 2013; Khanna 2014), including results presented in Chapter 3 of this thesis.

As more CO_2 experiments are required to build on current information on OA effects on intertidal benthic foraminifera, the presented research highlights some improvements in experimental design used in Chapter 3 to increase the numbers of two calcareous foraminiferal species at the onset of CO_2 experiments and to survival at the end, improving analysis of treatments effects on a larger representative population. The aim of this study is to enhance assessment of, via laboratory-based experiments, the effect of increased CO_2 concentrations and associated decrease in pH levels and $[CO_3^{-2}]$ on multiple biological parameters such as: survival rate; test size and weight; growth/calcification rates and

morphology changes of two dominant intertidal benthic foraminifera *E. williamsoni* and *H. germanica.*

Findings from this experimental study will improve knowledge of the biological responses of multi-species benthic foraminifera assemblages to longer-term OA exposure in comparison with the previous experiment performed in Chapter 3, where results of reduced survivorship and altered morphology were limited to *E. williamsoni.* It is hypothesized that there would be a similarly adverse impact from OA on multiple measures of biological parameters of *E. williamsoni* and *H. germanica*; causing a potential decline in the production of biogenic $CaCO_3$ by benthic calcareous foraminifera as a response to simulating predicted high CO_2/low pH levels.

4.2 Materials and Methods

4.2.1 Field sampling and isolation of target foraminifera

Surface sediment scrapes from the top first centimetre were collected in late July 2015 at a low tide from high intertidal mudflats of the Eden Estuary, N.E. Scotland (56°22'N, 2°50 W) (Chapter 2, Fig. 2.1).

Upon returning to the laboratory, all sediment samples were mixed and sieved over a set of 63 µm and 500 µm screens. The sieved sediment fraction was kept to settle in plastic containers. Observation of a small amount of sediment through a stereoscopic binocular microscope confirmed that living foraminiferal specimens of *E. williamsoni* and *H. germanica* were present in densities of 10-20 live specimens/cm^3 and 1-5 live specimens/cm^3, respectively. Subsequently, the sediment with living foraminiferal assemblages of both species was used for calcein incubation as described below. Detailed information on sampling, foraminiferal identification and isolation is outlined in Chapter 2, Sections 2.1 and 2.2.

4.2.2 Foraminiferal calcein incubation

Unlike the procedure in Chapter 3, Section 3.2.2, in order to ensure a larger number of surviving specimens at the end of this calcein incubation, living foraminiferal assemblages of *E. williamsoni* and *H. germanica* were incubated in the same natural sediment in which they were collected in (Fig. 4.1 A). For this experimental design, approx. 100 cm^3 of mixed sediment was placed in a series of 500 cm^3 filtering flasks. The total number of specimens used for this experiment was approx. 20,000 live specimens. The glass containers were

filled with filtered natural (~33 salinity) seawater containing a final concentration of 10 mg/L of the fluorescent marker calcein (Bernhard et al. 2004; Dissard et al. 2009; Dissard et al. 2010). Each flask was sealed with a rubber stopper with three inlets on top, one for air tubing and two to allow seawater with calcein to be continually recirculated into and out of the flask through a 1 L reservoir glass bottle. Multi-channel peristaltic pumps controlled the flow between the experimental flasks and the calcein bottles (Fig. 4.1 A).

This seawater calcein incubation was kept inside the temperature-controlled room at 13°C for 5 weeks with a light condition of 12:12-hr light: dark cycle (Fig. 4.1 A). Filtered seawater with calcein was changed once a week. Fortnightly sampling observations through a fluorescence microscope provided information on incorporation processes of calcein into the new growth of foraminiferal tests (Fig. 4.1 B).

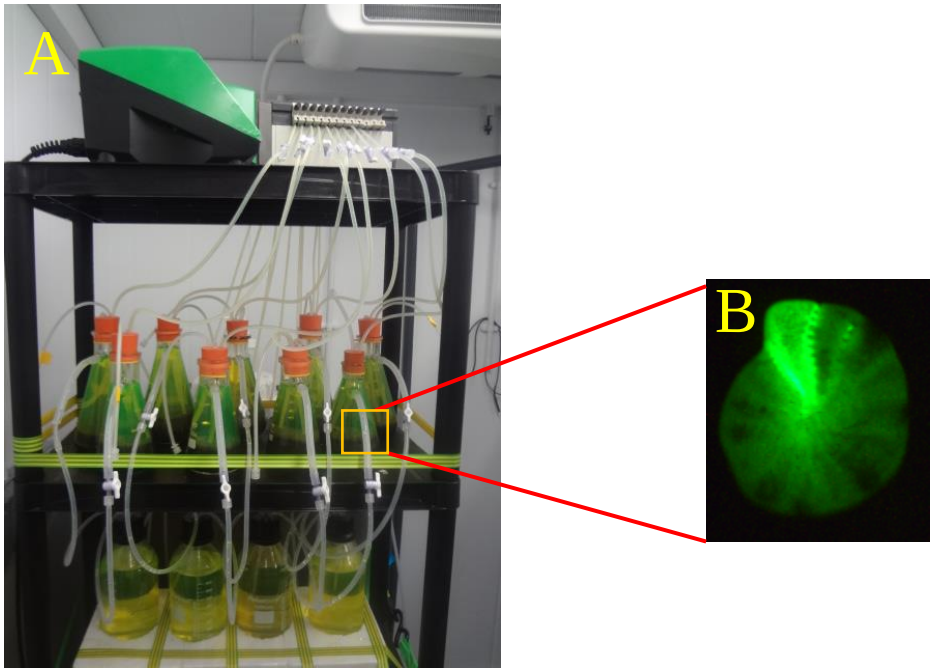

Figure 4. 1 A) Seawater recirculating system used for calcein incubation of *Elphidium williamsoni* and *Haynesina germanica* under controlled conditions of 13°C for 5 weeks with a light condition of 12:12-hr light: dark cycle. B) Specimen of *Elphidium williamsoni* showing the incorporation of calcein into the new growth of foraminiferal test.

4.2.3 Experimental conditions

When the calcein incubation period was concluded (5 weeks), viable ('live') specimens of *E. williamsoni* and *H. germanica* were examined through a fluorescence microscope. The surviving specimens with calcein labelled pre-existing chambers were picked out and cleaned of any detritus attached to their shells (tests) using a fine paintbrush. The total number of live individuals collected after calcein incubation included 4000 specimens of *E. williamsoni* and 800 specimens of *H. germanica*. Thus, 250 specimens of *E. williamsoni* and 50 specimens of *H. germanica* were randomly selected and placed in foraminiferal culturing chambers containing already silica onto the polycarbonate membrane insert, as

detailed in Chapter 2, Section 2.4 (Hintz et al. 2004; Allison et al. 2010; Allison et al. 2011a; Khanna 2014).

Three hundred specimens (corresponding to a mix of both species) were placed into each chamber, with a total of 16 culture chambers set up for this experiment. Four culturing chambers (replicates) with the same number of foraminifera inside were used for each pH treatment, and these were subsequently connected to a manipulative mesocosm, as described in Chapter 2, Section 2.4.

Prior to the start of the CO_2 experiment, a period of 10-day acclimation was allowed in order to gradually reduce the seawater pH until each treatment reached its required pH level/pCO_2. After this time period, foraminiferal culturing chambers were maintained for 6 weeks in a controlled recirculating seawater system within a temperature-controlled room at 13°C with a 12:12-hr light: dark cycle.

Foraminifera were exposed to one of four different pH conditions: pH~8.1 (ambient), pH 7.9, pH 7.7 and pH 7.3 (see more detailed information on culturing chamber and carbonate chemistry manipulation system in Chapter 2, Sections 2.4 and 2.5, respectively).

During the calcein incubation and throughout the entire experiment, the foraminifera were fed weekly with ~10µL/cm^2 of each of the algae *Dunaliella tertiolecta* and *Rhodomonas salina* (typically 1×10^7 cells ml^{-1}).

4.2.4 Biological parameters

After completing the experiment, all chambers were opened; foraminifera were picked out, transferred into clean petri dishes, and washed carefully with distilled water to remove excess silica and food cells.

The relative abundance distributions of 'live' and 'recently dead' individuals of both foraminiferal species and their morphological response to low pH levels were determined. Biological parameters of selected 'live' specimens with intact tests only were recorded for both foraminiferal species: the number of newly formed chambers, maximum test diameter, test weight and morphological changes (i.e. via SEM imaging). Subsequently, 'live' individuals of both species were sorted into groups of different classes of size, weight and chambers added to determine potential OA effects on different growth stages of the foraminifera.

Survival rate, mortality rate, growth rate, test size-normalized weights (SNW) and size-weight relationships across the different pH conditions were calculated as detailed in Chapter 2, Section 2.6.

4.2.6 Statistical Method

All statistical analyses were run in the statistical programme R 3.1.2 (R Development Core Team. 2014) and all of the steps are explained in Chapter 2, Section 2.9.

4.3 Results

4.3.1 Composition of dominant foraminiferal species from field samples

Foraminiferal assemblages of field samples collected in July 2015 showed that both target species were the two dominant (~ 90% abundance) in comparison to other foraminiferal species. However, *E. williamsoni* exhibited a much higher number of living individuals (20-30 living specimens/cm^3 of wet sediment) than *H. germanica* (5-10 living specimens/cm^3 of wet sediment). Living specimens of both foraminiferal species were used for this experimental research.

4.3.2 Seawater carbonate chemistry

Measurements of seawater carbonate parameters taken on a fortnightly basis indicate that these parameters were constant throughout the entire time period of 42 days only for the CO_2 experiment (Table 4.1).

Table 4. 1 Average seawater measurements taken fortnightly from carbonate chemistry manipulation system. Values account for mean ± SD, n = 3.

Measured parameters					**Calculated parameters**					
Treatment	**pH (Total)**	**T (°C)**	**Salinity (ppt)**	**AT (µmol/Kg)**	**DIC (µmol/kg)**	***p*CO2 (µatm)**	**HCO_3^- (µmol/kg)**	**CO_3^{2-} (µmol/kg)**	**$\Omega_{Calcite}$**	**$\Omega_{Aragonite}$**
pH 8.1 (ambient)	8.10 ± 0.02	13.17 ± 0.05	33.02 ± 0.14	2486.19 ± 118.41	2250.90 ± 114.60	380.70 ± 31.43	2063.54 ± 107.99	172.16 ± 8.60	4.15 ± 0.21	2.64 ± 0.13
pH 7.9	7.93 ± 0.04	13.2 ± 0.11	33.10 ± 0.08	2420.86 ± 60.71	2266.41 ± 52.50	578.35 ± 53.92	2122.49 ± 47.96	120.85 ± 10.97	2.91 ± 0.27	1.85 ± 0.17
pH 7.7	7.76 ± 0.03	13.2 ± 0.08	33.20 ± 0.13	2368.49 ± 54.21	2282.17 ± 55.31	885.01 ± 72.32	2164.84 ± 53.15	82.06 ± 4.77	1.97 ± 0.12	1.26 ± 0.07
pH 7.3	7.34 ± 0.02	13.2 ± 0.08	33.19 ± 0.20	2409.40 ± 103.49	2458.78 ± 106.99	2484.36 ± 162.43	2326.09 ± 100.84	33.67 ± 2.00	0.81 ± 0.05	0.51 ± 0.03

4.3.3 Morphological response

The three levels of morphological responses of 'live' and 'recently dead' specimens of *E. williamsoni* and *H. germanica* as a potential response to the experimental pH conditions were observed. Detailed information on potential morphological responses by benthic foraminifera is outlined in Chapter 2, Section 2.6.1.

For *E. williamsoni*, the highest number of intact tests (Level 1) was found at a pH of 8.1 (ambient), followed by a pH of 7.9, pH 7.7 and pH 7.3; whereas the highest number of specimens with minor alterations (Level 2) was found at a pH of 8.1 (ambient), pH 7.7, pH 7.3 and pH 7.9. Contrary to what one may expect, the number of broken tests decreased with declining pH levels. Despite these observations, there are no obvious trends related to the pH effect on foraminiferal morphology (Fig. 4.2).

For *H. germanica*, the highest number of intact tests (Level 1) was observed at a pH of 8.1 (ambient), pH 7.9, pH 7.7 and pH 7.3; whereas the highest number of specimens with minor alterations (Level 2) was found at a pH of 7.3, pH 8.1 (ambient), pH 7.7 and pH 7.9. The highest number of broken tests (Level 3) was found at a pH of 7.7 followed by pH 7.3 pH 7.9 and pH 8.1 (ambient). Despite these observations, there are no obvious trends related to the pH effect on foraminiferal morphology (Fig. 4.2).

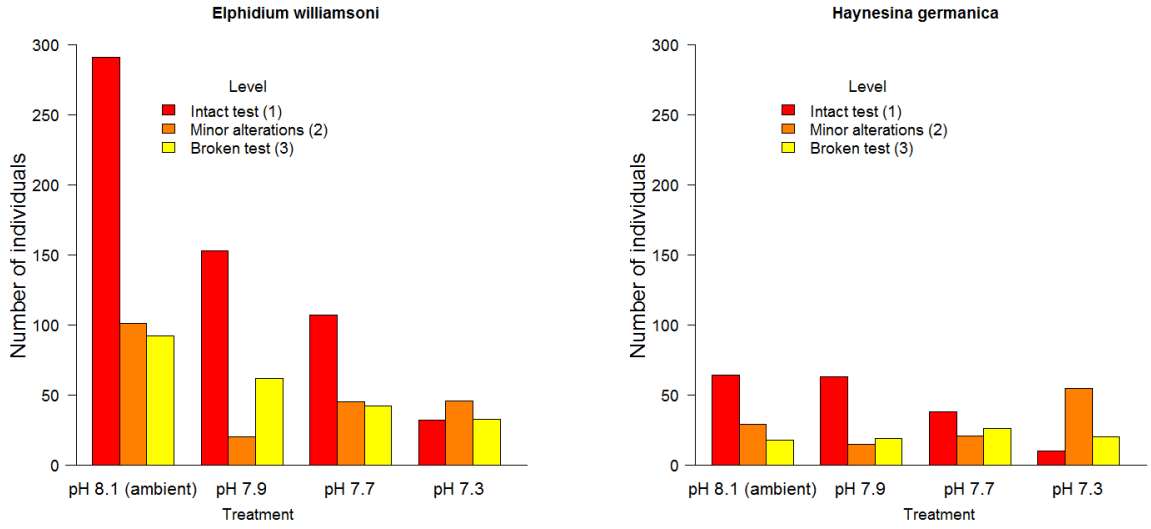

Figure 4. 2 Number of individuals (live and dead) with morphological changes observed in tests of *Elphidium williamsoni* and *Haynesina germanica* as a potential response to experimental pH conditions. Morphological response levels were: **Level 1**= intact test, **Level 2** = minor changes and **Level 3** = broken test.

4.3.4 'Live' and 'dead' foraminiferal individuals and survival rate

At the end of the experiment, specimens from all culturing chambers were collected and only 3528 individuals of *E. williamsoni* and 574 individuals of *H. germanica* were retrieved. This was a loss of 472 and 226 individuals for both species, respectively (Table 4.2).

For *E. williamsoni*, individuals cultured at pH 8.1 (ambient) showed the largest number of surviving specimens (n_{Live} = 373) followed by treatments pH 7.9 and pH 7.7. In contrast, individuals cultured at pH 7.3 showed the lowest number of surviving specimens (n_{Live} = 111). The number of 'live' individuals in each treatment follows a decreasing trend as the pH level decreased. Furthermore, the mortality rate (presumably as a response to OA

effects) increased from 21.9 to 33.2 % when the seawater pH decreased from 7.9 to 7.3 (Table 4.2).

For *Haynesina germanica*, individuals cultured at pH 8.1 (ambient) showed the largest number of surviving specimens (n_{Live} = 111) followed by treatments pH 7.9. In contrast, individuals cultured at pH 7.7 and pH 7.3 showed the lowest number of surviving specimens (n_{Live} = 85). Despite the number of 'live' individuals in some treatments decreased as the pH level falls, the mortality rate (presumably linked to OA effects) shows no obvious trend when the seawater pH decreased from 7.9 to 7.3 (Table 4.2).

Table 4. 2 Survival rates (SR) of *Elphidium williamsoni* and *Haynesina germanica* cultured for 52 days under different pH conditions: pH~8.1 (ambient), pH 7.9, pH 7.7 and pH 7.3.

	Total number of individuals					
Species and treatment	**Start of experiment**	**End of experiment**		**Survival rate (%)**	**Total Mortality rate (%)**	**Mortality rate by OA (%)**
		Retrieved/ Analyzed	**Alive (Chambers added)**			
E. williamsoni						
pH 8.1 (ambient)	1000	801	373	46.6	53.4	0.0
pH 7.9	1000	952	235	24.7	75.3	21.9
pH 7.7	1000	945	194	20.5	79.5	26.0
pH 7.3	1000	830	111	13.4	86.6	33.2
H. germanica						
pH 8.1 (ambient)	200	174	111	63.8	36.2	0.0
pH 7.9	200	134	97	72.4	27.6	-8.6
pH 7.7	200	139	85	61.2	38.8	2.6
pH 7.3	200	127	85	66.9	33.1	-3.1

An improved visualization of the dataset presented in Table 4.2 is shown in Fig. 4.3 A and B for dead and 'live' specimens of *E. williamsoni* and *Haynesina germanica.*

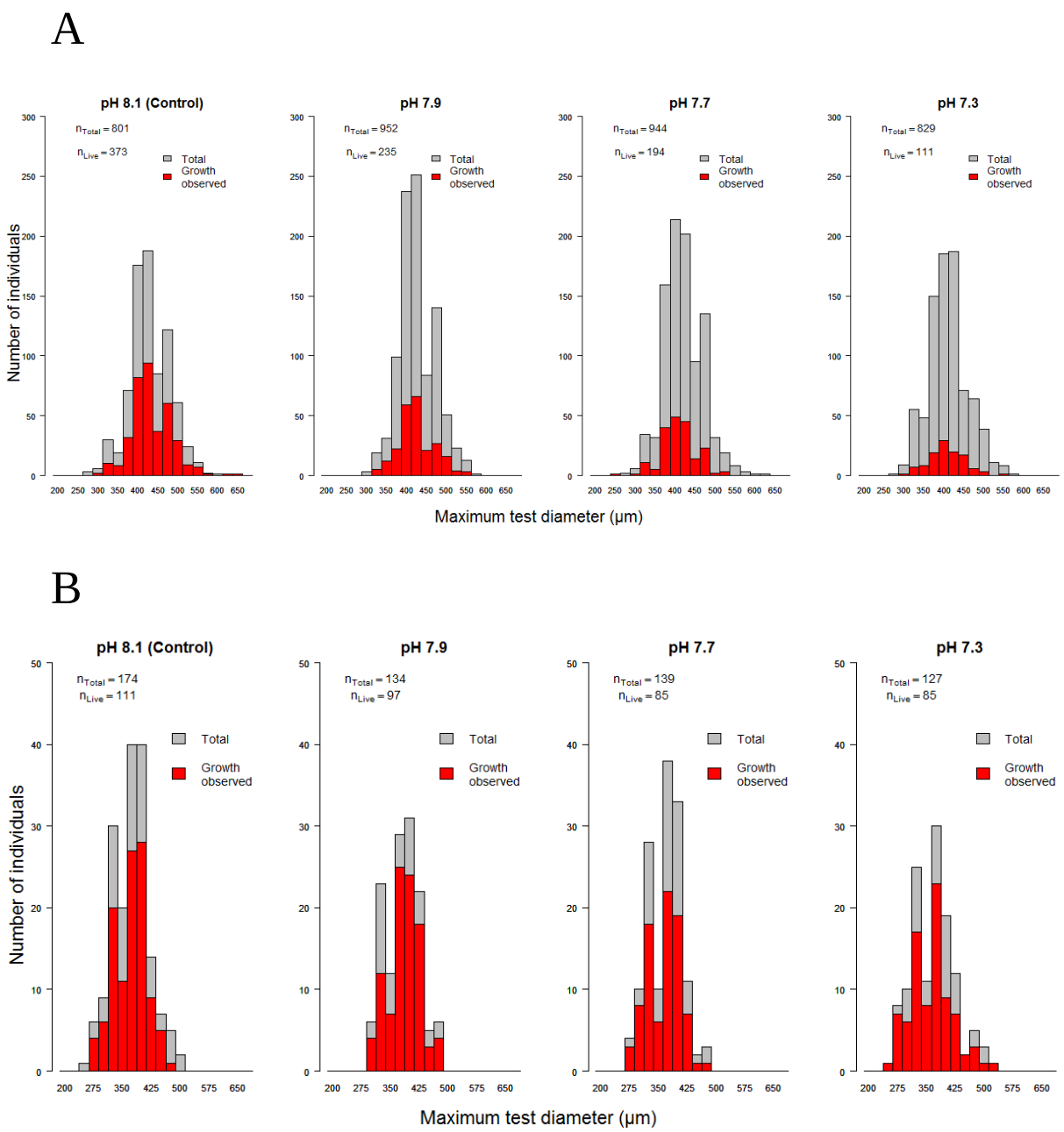

Figure 4. 3 The total number of individuals of (A) *Elphidium williamsoni* and (B) *Haynesina germanica* sorted into size classes after being collected at the end of the experimental period from each culture condition (pH~8.1 (ambient), pH 7.9, pH 7.7 and pH 7.3). The individuals analysed (n_{Total}) and live specimens (n_{Live}) observed are shown in grey and red, respectively. Bandwidth for each size class was 25 µm. Note the relatively high mortality rates in *E. williamsoni* compared to *H. germanica*.

4.3.5 Biological parameters

Table 4.3 illustrates maximum test diameter, weight and the number of new chambers added to live specimens with intact tests for both foraminiferal species.

Table 4. 3 Maximum test diameter, weight and new chambers added to live *Elphidium williamsoni* and *Haynesina germanica* cultured for 52 days under different experimental pH conditions: pH~8.1 (ambient), pH 7.9, pH 7.7 and pH 7.3.

Measured variables	pH conditions	Min.	Max.	Mean	Standard deviation (1σ)	Standard error of mean	n
E. williamsoni							
Maximum test diameter (µm)	8.1 (ambient)	335.80	657.50	454.80	45.39	3.01	227
	7.9	349.80	559.60	444.20	42.41	3.43	153
	7.7	335.80	531.60	427.40	39.30	3.80	107
	7.3	363.70	559.60	439.80	45.50	8.04	32
Test weight (µg)	8.1 (ambient)	6.30	48.00	16.07	5.29	0.35	227
	7.9	7.30	32.70	15.86	4.85	0.39	153
	7.7	4.70	27.30	13.39	4.12	0.40	107
	7.3	6.30	20.30	12.21	4.09	0.72	32
Number of chambers added	8.1 (ambient)	1.00	11.00	4.04	2.28	0.15	227
	7.9	1.00	12.00	5.12	2.57	0.21	153
	7.7	1.00	9.00	3.82	2.21	0.21	107
	7.3	1.00	8.00	4.03	1.93	0.34	32
H. germanica							
Maximum test diameter (µm)	8.1 (ambient)	293.80	461.70	385.60	38.53	4.82	64
	7.9	321.80	489.60	400.40	36.97	4.66	63
	7.7	321.80	475.70	393.90	34.66	5.62	38
	7.3	279.80	475.70	363.70	53.58	16.94	10
Test weight (µg)	8.1 (ambient)	4.00	16.30	8.82	2.48	0.31	64
	7.9	5.30	18.30	10.75	3.14	0.40	63
	7.7	5.00	17.00	8.49	2.80	0.45	38
	7.3	3.00	10.00	5.19	2.03	0.36	10
Number of chambers added	8.1 (ambient)	1.00	7.00	3.13	1.28	0.16	64
	7.9	1.00	7.00	3.64	1.29	0.16	63
	7.7	1.00	6.00	3.90	1.06	0.17	38
	7.3	2.00	6.00	3.90	1.05	0.33	10

4.3.6 Foraminiferal size, weight and chamber addition

In general, for 'live' *E. williamsoni* and *H. germanica*, the highest number of individuals distributed across the greatest number of size classes was found in the treatment pH 8.1 (ambient) followed by treatments pH 7.9 and pH 7.7. In contrast, individuals cultured at pH 7.3 were distributed across the lowest number of size classes and number of individuals (Fig. 4.4 A and 4.5 A). Both, the number of individuals per size class and the number of size classes declined as the pH decreased.

In terms of foraminiferal weight for both species, more individuals were distributed in more weight classes in the treatment pH 8.1 (ambient) followed by treatments pH 7.9 and pH 7.7. In contrast, individuals cultured at pH 7.3 showed the lowest the number of weight classes and the lowest number of individuals (Fig. 4.4 B and 4.5 B). Both the number of individuals per weight class and the number of weight classes declined as the pH decreased.

The highest number of individuals with new chambers added and distributed in more chamber addition classes was found in the treatment pH 8.1 (ambient) followed by treatments pH 7.9 and pH 7.7. In contrast, individuals cultured at pH 7.3 showed the lowest number of individuals with new chambers added (Fig. 4.4 C and 4.5 C). Both the number of individuals per class and the number of new chambers classes declined as the pH was reduced.

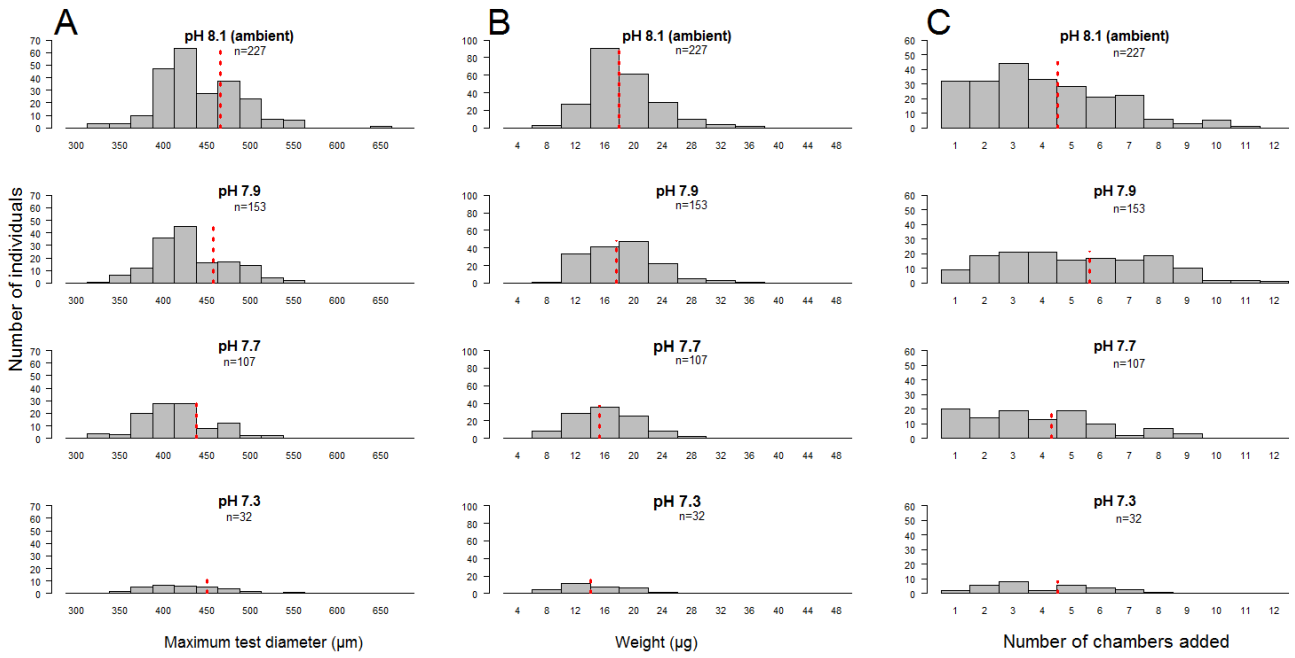

Figure 4. 4 Distribution of 'live' *Elphidium williamsoni* in relation to A) maximum test diameter, B) test weight, and C) number of chambers added for each culture condition (pH 8.1 (ambient), pH 7.9, pH 7.7 and pH 7.3). Individuals were sorted into groups of different bandwidths for each parameter. The bandwidth was equal to 25 µm for each size class, 4 µg for each weight class and 1 for each new chamber added class. The dashed red line represents the mean value of measurements within each pH treatment.

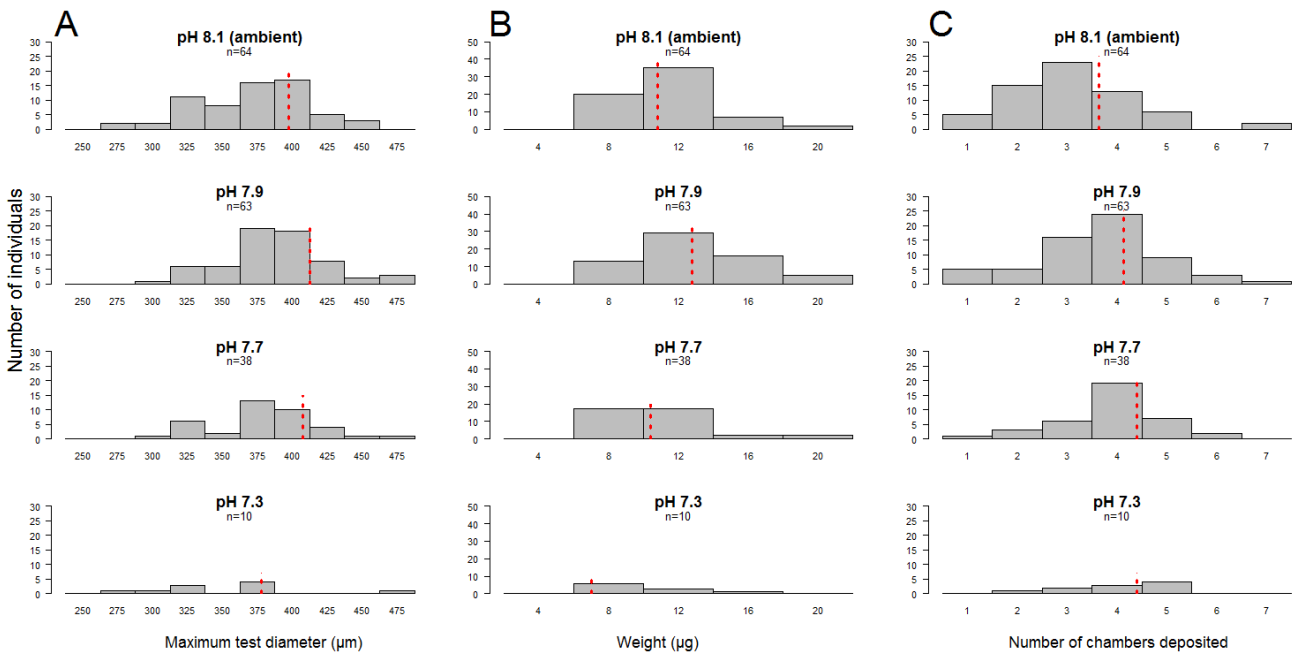

Figure 4. 5 Distribution of 'live' *Haynesina germanica* in relation to A) maximum test diameter, B) test weight, and C) number of chambers added for each culture condition (pH 8.1 (ambient), pH 7.9, pH 7.7 and pH 7.3). Individuals were sorted into groups of different bandwidths for each parameter. The bandwidth was equal to 25 µm for each size class, 4 µg for each weight class and 1 for each new chamber added class. The dashed red line represents the mean value of measurements within each pH treatment.

4.3.7 Foraminiferal mean maximum test diameter (size) of 'live' specimens

For 'live' *E. williamsoni*, individuals cultured at a pH of 8.1 (ambient) showed the largest mean maximum test diameter followed by the treatments at a pH of 7.9 and pH 7.3. In contrast, individuals cultured at a pH of 7.7 showed the lowest mean maximum test diameter (Fig. 4.6 and Table 4.3). Shapiro-Wilk and Levene tests verified the normality (p = 1.45 x 10^{-7}) and homogeneity (p = 0.7227) of the maximum test diameter dataset. A Kruskal-Wallis test indicates there is a statistically significant effect of pH treatments on foraminiferal size (p = 3.07 x 10^{-6}). A Dunn's-test (pairwise comparison) also indicates a

significant difference between the treatment at a pH of 7.7 (group “b”) and the remaining pH treatments (groups “a” and “ab”) (Fig. 4.6 and Table 4.3).

For ‘live’ *H. germanica*, individuals cultured at a pH of 7.9 showed the largest mean maximum test diameter followed by the treatments at a pH of 7.7 and pH 8.1 (ambient). In contrast, individuals cultured at a pH of 7.3 showed the smallest mean size (Fig. 4.6 and Table 4.3). Shapiro-Wilk and Levene tests verified the normality (p = 0.07258) and homogeneity (p = 0.4325) of the maximum test diameter dataset. One-way ANOVA indicates that there is a statistically significant effect of pH treatments on foraminiferal size (p = 0.0175). The Tukey’s test (pairwise comparison) also indicates a significant difference between the treatment at a pH of 7.3 and the remaining pH treatments (groups “a” and “ab”) (Fig. 4.6 and Table 4.3).

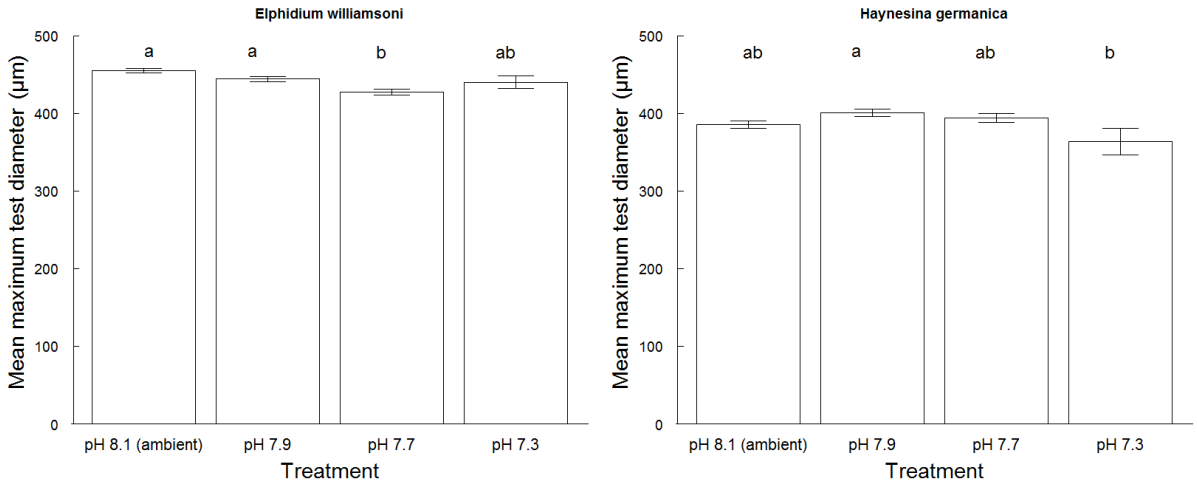

Figure 4. 6 Mean values (± standard error) of maximum test diameter (µm) for live *Elphidium williamsoni* and *Haynesina germanica* cultured at different pH conditions. Treatments with significant differences are indicated by different letters (i.e. **a** and **b**) above bars at $p < 0.05$. Treatments with shared letters (i.e. **ab**) above bars indicate no significant differences ($p > 0.05$) observed between groups according to the Dunn's-test or Tukey's test.

4.3.8 Foraminiferal weight of 'live' specimens

For *E. williamsoni*, individuals cultured at a pH of 8.1 (ambient) showed the heaviest mean weight followed by the treatments at a pH of 7.9 and pH 7.7. In contrast, individuals cultured at a pH of 7.3 showed the lowest weight (Fig. 4.7 and Table 4.3). Shapiro-Wilk and Levene tests verified the normality ($p = 6.5x10^{-14}$) and homogeneity ($p = 0.471$) of the test weight dataset. The Kruskal-Wallis test indicates there is a statistically significant effect of pH treatments on foraminiferal weight ($p = 1.05x\ 10^{-7}$). A Dunn's-test (pairwise comparison) also indicates that the treatments at a pH of 7.3 and 7.7 (group "b") differ significantly from the pH 8.1 (ambient) and pH 7.9 treatments (group "a") (Fig. 4.7). *E. williamsoni* showed a trend of declining weight as pH decreased.

For *H. germanica*, individuals cultured at a pH of 7.9 showed the heaviest mean weight followed by treatments at a pH of 8.1 (ambient) and pH 7.7. In contrast, individuals cultured at a pH of 7.3 showed the lowest weight (Fig. 4.7 and Table 4.2). Shapiro-Wilk and Levene tests verified the normality (p = 2.8 x10^{-6}) and homogeneity (p = 0.2517) of the test weight dataset. The Kruskal-Wallis test indicates there is a statistically significant effect of pH treatments on foraminiferal weight (p = 8.47 x 10^{-6}). A Dunn's-test (pairwise comparison) also indicates that the treatments at a pH of 8.1 (ambient), 7.7 and 7.3 (group "b") differ significantly from the pH 7.9 treatment (group "a") (Fig. 4.7).

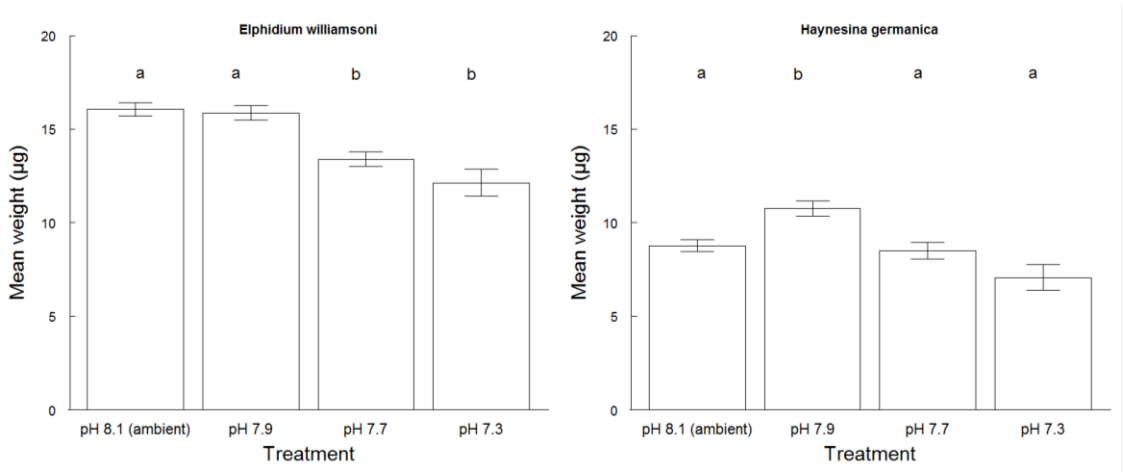

Figure 4. 7 Mean values (± standard error) of weight (µg) for live *Elphidium williamsoni* and *Haynesina germanica* cultured at different pH conditions. Treatments with significant differences are indicated by different letters (i.e. **a** and **b**) above bars at $p < 0.05$. Treatments with shared letters (i.e. **ab**) above bars indicate no significant differences ($p > 0.05$) observed between groups according to the Dunn's-test.

4.3.9 Newly deposited chambers

Slight differences in the number of new chambers added by 'live' specimens of both species were observed within and between treatments (Table 4.3).

For *E. williamsoni*, individuals cultured at a pH of 7.9 showed the largest number of new chambers deposited followed by the treatments at a pH of 8.1 (ambient) and pH 7.3. In contrast, individuals cultured at a pH of 7.7 showed the lowest number of new chambers added (Fig. 4.8 and Table 4.3). Shapiro-Wilk and Levene tests verified the normality (p = 6.14 x10^{-13}) and homogeneity (p = 0.04543) of the test weight dataset. The Kruskal-Wallis test indicates there is a statistically significant effect of pH treatments on foraminiferal chamber addition (p = 5.3 x10^{-5}). A Dunn's-test (pairwise comparison) also indicates that the pH 7.9 treatment (group "b") differ significantly from the pH 8.1 (ambient), pH 7.7 and pH 7.3 treatments (groups "a" and "ab") (Fig. 4.8).

For *H. germanica*, individuals cultured at a pH of 7.7 and pH 7.3 showed the largest number of new chambers deposited followed by the treatment at a pH of 7.9. In contrast, individuals cultured at a pH of 8.1 (ambient) showed the lowest number of new chambers deposited (Fig. 4.8 and Table 4.3). Shapiro-Wilk and Levene tests verified the normality (p = 6.18 x10^{-7}) and homogeneity (p = 0.539) of the chamber addition dataset.

A Kruskal-Wallis test indicates there is a statistically significant effect of pH treatments on foraminiferal weight (p = 1.2 x10^{-3}). A Dunn's-test (pairwise comparison) also indicates that the treatment at a pH of 8.1 (ambient) (group "a") differ significantly from the pH 7.9, pH 7.7 and pH 7.3 treatments (group "b" and "ab") (Fig. 4.8). The number of new chambers deposited by *H. germanica* showed a trend to increase as pH decreased.

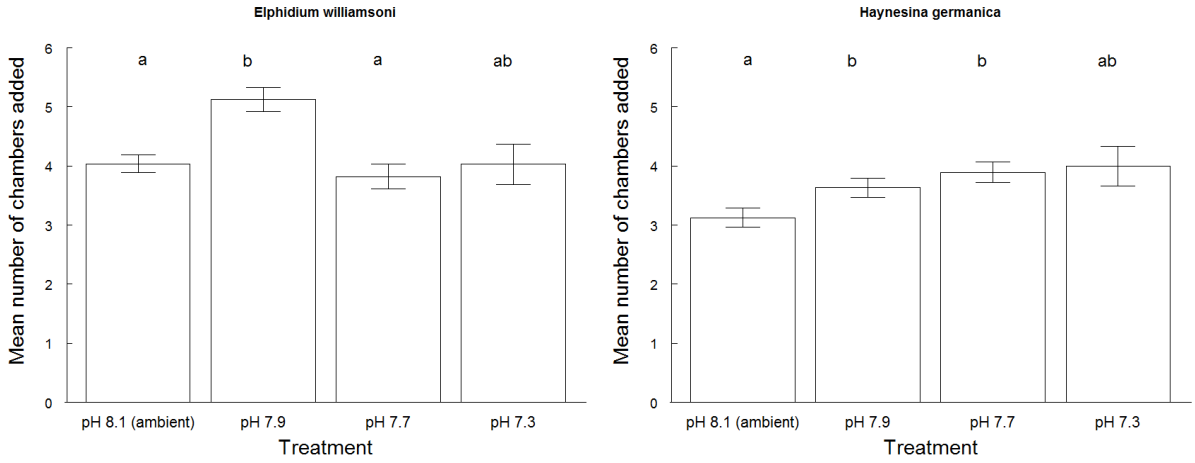

Figure 4. 8 Mean values (± standard error) of newly formed chambers for *Elphidium williamsoni* and *Haynesina germanica* cultured at different pH conditions. Treatments with significant differences are indicated by different letters (i.e. **a** and **b**) above bars at $p < 0.05$. Treatments with shared letters (i.e. **ab**) above bars indicate no significant differences ($p > 0.05$) observed between groups according to the Dunn's-test.

4.3.10 Growth rate

Table 4.4 illustrates the mean growth rates (chambers day^{-1}) of benthic foraminifera observed across different experimental pH conditions.

Table 4. 4 Mean growth rates based on the number of chambers added by *Elphidium williamsoni* and *Haynesina germanica* cultured for 52 days under different experimental pH conditions: pH~8.1 (ambient), pH 7.9, pH 7.7 and pH 7.3.

Measured variables	pH conditions	Min.	Max.	Mean	Standard deviation (1σ)	Standard error of mean	n
E. williamsoni							
Growth rate (chambers day^{-1})	pH 8.1 (ambient)	0.02	0.21	0.08	0.04	0.003	227
	pH 7.9	0.02	0.23	0.10	0.05	0.004	153
	pH 7.7	0.02	0.17	0.07	0.04	0.004	107
	pH 7.3	0.02	0.15	0.08	0.04	0.007	32
H. germanica							
Growth rate (chambers day^{-1})	pH 8.1 (ambient)	0.02	0.13	0.06	0.02	0.003	64
	pH 7.9	0.02	0.13	0.07	0.02	0.003	63
	pH 7.7	0.02	0.12	0.07	0.02	0.003	38
	pH 7.3	0.04	0.12	0.07	0.02	0.006	10

For *E. williamsoni*, individuals cultured at a pH of 7.9 showed the highest mean growth rate followed by the treatment at a pH of 8.1 (ambient) and pH 7.3. In contrast, individuals cultured at a pH of 7.7 showed the lowest mean growth rate (Fig. 4.9 and Table 4.4). Shapiro-Wilk and Levene tests verified the normality (p = 6.14 x10^{-13}) and homogeneity (p = 0.04543) of the mean growth rate dataset. A Kruskal-Wallis test indicates there is a statistically significant effect of pH treatments on foraminiferal growth (p = 5.3 x10^{-5}). The Dunn's-test (pairwise comparison) indicates that the pH 7.9 treatment (group "b") differ significantly from the pH 8.1 (ambient), pH 7.7 and pH 7.3 treatments (groups "a" and "ab") (Fig. 4.9).

For *H. germanica*, individuals cultured at a pH of 7.7 and pH 7.3 showed the highest mean growth rate followed by treatment at a pH of 7.9. In contrast, individuals cultured at a pH of 8.1 (ambient) showed the lowest mean growth rate (Fig. 4.9 and Table 4.4). Shapiro-Wilk and Levene tests verified the normality (p = 6.18 x10^{-7}) and homogeneity (p = 0.539) of the mean growth rate dataset. A Kruskal-Wallis test indicates there is a statistically significant effect of pH treatments on foraminiferal growth (p = 1.2 x10^{-3}). A Dunn's-test (pairwise comparison) also indicates that the pH 8.1 (ambient) (group "a") differ significantly from the pH 7.9, pH 7.7 and pH 7.3 treatments (groups "b" and "ab") (Fig. 4.9). Mean growth of *H. germanica* showed a trend to increase as pH decreased.

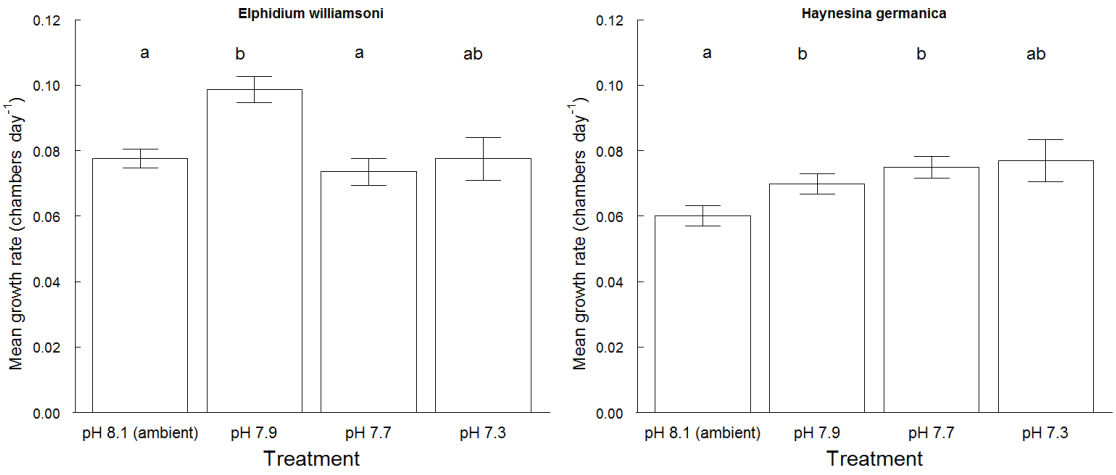

Figure 4. 9 Mean values (± standard error) of mean growth rates for *Elphidium williamsoni* and *Haynesina germanica* cultured for 52 days at different pH conditions. Treatments with significant differences are indicated by different letters (i.e. **a** and **b**) above bars at $p < 0.05$. Treatments with shared letters (i.e. **ab**) above bars indicate no significant differences ($p > 0.05$) observed between groups according to the Dunn's-test.

4.3.11 Foraminiferal size-normalized test weight (SNW)

For *E. williamsoni*, individuals cultured at a pH of 8.1 (ambient) showed the highest mean SNW followed by treatments at a pH of 7.9 and pH 7.7. In contrast, individuals cultured at a pH of 7.3 showed the lowest SNW (Fig. 4.10).

Shapiro-Wilk and Levene tests verified the normality ($p = 4.6x10^{-6}$) and homogeneity ($p = 0.633$) of the SNW dataset. A Kruskal-Wallis test indicates there is a statistically significant effect of pH treatments on foraminiferal SNW ($p = 1.9x10^{-8}$). The Dunn's-test (pairwise comparison) also indicates that the pH 7.3 and 7.7 (group "b") differ significantly from the pH 8.1 (ambient) and pH 7.9 treatments (group "a") (Fig. 4.10).

For *H. germanica*, individuals cultured at a pH of 7.9 showed the highest mean SNW followed by treatments at a pH of 8.1 (ambient) and pH 7.7. In contrast, individuals cultured at a pH of 7.3 showed the lowest SNW (Fig. 4.10). Shapiro-Wilk and Levene tests verified the normality ($p = 2.6 x10^{-5}$) and homogeneity ($p = 0.2099$) of the SNW dataset. The Kruskal-Wallis test indicates there is a statistically significant effect of pH treatments on foraminiferal SNW ($p = 7.06 x 10^{-7}$). A Dunn's-test (pairwise comparison) indicates that the pH 7.9 (group "b") differ significantly from the pH 8.1 (ambient), pH 7.7 and pH 7.3 treatments (group "a") (Fig. 4.10).

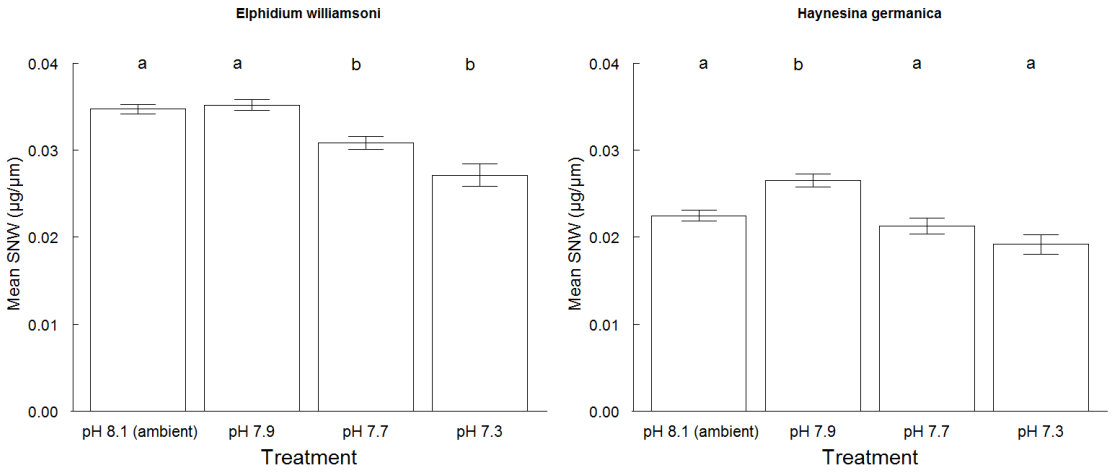

Figure 4. 10 Mean values (± standard error) of size-normalized test weight (SNW) for *Elphidium williamsoni* and *Haynesina germanica* cultured for 52 days at different pH conditions. Treatments with significant differences are indicated by different letters (i.e. **a** and **b**) above bars at $p < 0.05$. Treatments with shared letters (i.e. **ab**) above bars indicate no significant differences ($p > 0.05$) observed between groups according to the Dunn's-test.

4.3.12 SEM observations of the morphological response of 'live' *E. williamsoni*

SEM images of intact tests of 'live' *E. williamsoni* showed remarkable morphological differences between pH treatments. These observations indicated a progressive alteration of the foraminiferal morphology (test) when individuals were exposed to high CO_2 concentrations/low pH for 52 days. The most notable features observed on the test surface are the presence of cracks and signs of dissolution on individuals exposed to the low pH levels (Fig. 4.11, D-H). Specimens cultured at pH 7.7 and 7.3 clearly displayed dissolution around the apertural region, notably on apertural teeth (Fig. 4.11, E & G). In addition, chambers of foraminiferal specimens cultured at pH 7.7 and pH 7.3 (Fig. 4.11, F & H) displayed large and irregular septal bridges and sutures with clear signs of corrosion in

comparison to those cultured at pH 8.1 and pH 7.9, which exhibited smooth surfaces and regular shapes of these structures (Fig 4.11, A & C).

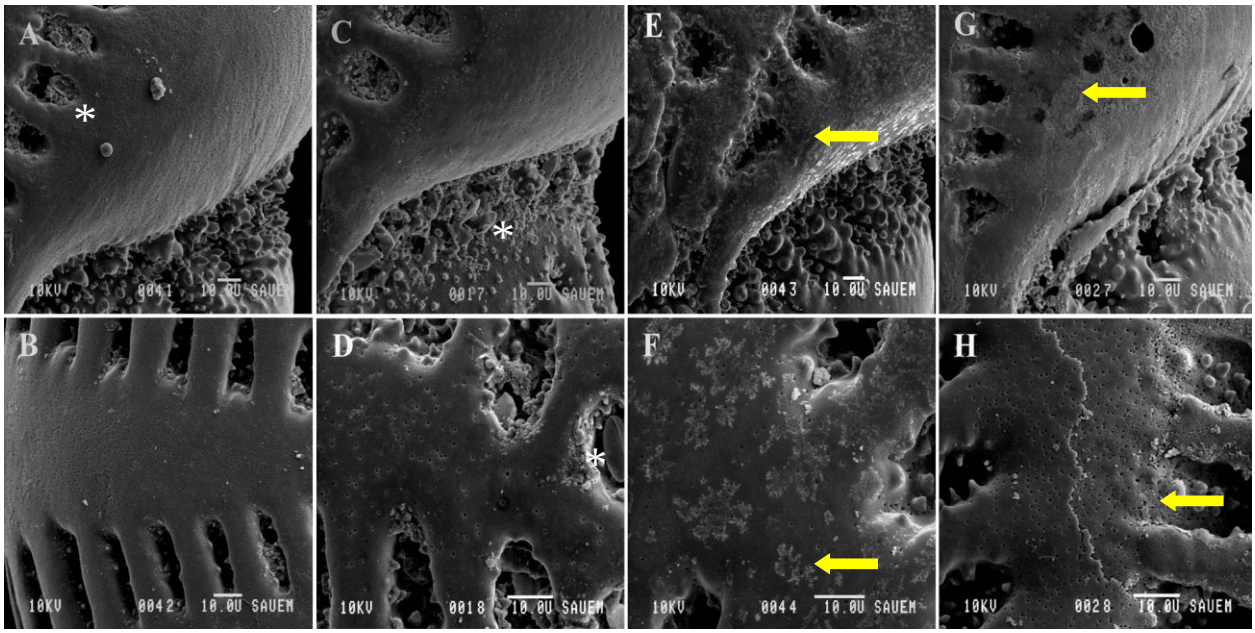

Figure 4. 11 Scanning electron micrographs (SEM) images of live specimens of *Elphidium williamsoni* cultured at pH 8.1 (A & B), pH 7.9, (C & D), pH 7.7 (E & F) and pH 7.3 (G & H). (A) SEM image of side view of the apertural region showing numerous teeth and tubercles. A frustule of the diatom *Navicula sp*. (*) and organic detritus are visible by a septal bridge. (B) A side view of the test surface of specimen A. (C) SEM image of a side view of the apertural region, showing numerous teeth and tubercles with some impaled frustules of the diatom *Navicula sp*. (*). (D) A side view of the smooth test surface of specimen C. (E) SEM image of a side view of the apertural region, where signs of dissolution and cracking are clearly observed. Teeth and tubercles are less sharp with rounded shape. No frustules of diatom species are observed. (F) A side view of the test surface of specimen E affected by dissolution and cracking processes. (G) SEM of a side view of the apertural region showing a reduction in the number of teeth and tubercles. Dissolution and cracking processes are clearly observed in multiple structures with a severe effect on septal bridges and sutures. No frustules of diatom species are observed. (H) A side view of the test surface of specimen G, showing several

test wall layers on septal bridges and sutures affected by dissolution and cracking processes. (←) Yellow arrows highlight areas affected by dissolution. White scale bar represents 10 µm.

4.3.13 SEM observations of the morphological response of 'live' *H. germanica*

SEM images of intact tests of live *H. germanica* showed remarkable morphological differences between pH treatments. These observations indicated a progressive alteration of the foraminiferal morphology (test) when individuals were exposed to high CO_2 concentrations/low pH for 52 days. The most notable features observed on the test surface are the presence of cracks, asymmetric and larger test pores with clear signs of dissolution on individuals exposed to all pH levels except for pH 8.1 (ambient) (Fig. 4.12, C-H). Specimens cultured at pH 7.7 and 7.3 clearly displayed dissolution and cracks around the apertural region, notably on apertural teeth (Fig. 4.12, E & G). In addition, test surfaces and sutures of foraminiferal specimens cultured at pH 7.9, pH 7.7 and pH 7.3 (Fig. 4.12, D, F & H) showed clear signs of corrosion in comparison to those cultured at pH 8.1 (ambient), which exhibited smooth surfaces and regular shapes of these structures (Fig 4.12, A & B).

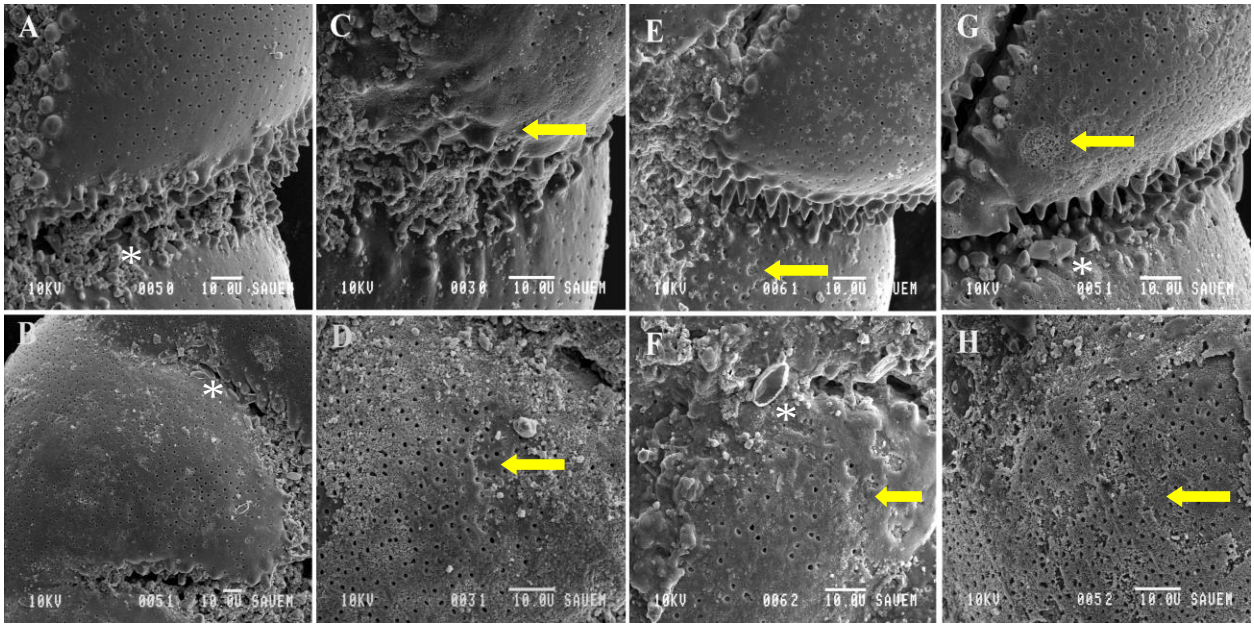

Figure 4. 12 Scanning electron micrographs (SEM) images of live specimens of *Haynesina germanica* cultured at pH 8.1 (A & B), pH 7.9, (C & D), pH 7.7 (E & F) and pH 7.3 (G & H). (A) SEM image of a side view of the apertural region showing numerous teeth and tubercles. A frustule of the diatom *Navicula sp*. (*) and organic detritus are visible. (B) A side view of the test surface of specimen A. (C) SEM image of a side view of the apertural region, showing organic detritus and rounded and slightly corroded teeth and tubercles (D) A side view of dissolution effect on test surface of specimen C. (E) SEM image of a side view of the apertural region, where signs of dissolution and cracking are clearly observed. Teeth and tubercles are less sharp with round shape. (F) A side view of the test surface of specimen E affected by dissolution and cracking processes. A frustule of an unidentified diatom species is observed (*). (G) SEM of a side view of the apertural region showing a reduction in the number of teeth and tubercles. Dissolution and cracking processes are clearly observed in multiple structures with a severe effect on teeth and sutures. A frustule of an unidentified diatom species is observed (*). (H) A side view of the test surface of specimen G, showing several test wall layers severely affected by dissolution and cracking processes. (←) Yellow arrows show areas affected by dissolution. White scale bar represents 10 µm.

4.4 Discussion

4.4.1 Survival rate

In this study, *E. williamsoni* showed a steady decrease in the number of surviving individuals as the pH decreased over the 52 day-experiment, suggesting an important impact of OA on survival rate of *E. williamsoni*. The mortality rate of *E. williamsoni* rose to 33 % when the seawater pH decreases from 7.9 to 7.3 (Fig. 4.3 A and Table 4.2). A similar decreasing trend of survival with lowering pH was observed for *E. williamsoni* and is described in Chapter 3, Section 3.3.4. These findings resemble those reported for other calcareous benthic foraminifera which reported negatively impacted survivorship from high pCO_2/low pH levels under a laboratory and natural acidified setting (Bernhard et al. 2009; Dias et al. 2010).

However, *H. germanica* showed only a slight decrease in the number of surviving individuals as the pH decreased, and no additional mortality driven by OA was clearly observed as a potential impact on survival rate (Fig. 4.3 B and Table 4.2). The biological response of *H. germanica* to OA is more consistent with previous works on benthic foraminifera (i.e. calcareous species), where most individuals incubated under conditions of high pCO_2 and in some cases in undersaturated seawater ($\Omega < 1$), were unaffected (survived) (Bernhard et al. 2009; Dissard et al. 2010; Haynert et al. 2011; McIntyre-Wressnig et al. 2013; Haynert et al. 2014; McIntyre-Wressnig et al. 2014).

In the present research, as benthic foraminifera *E. williamsoni* and *H. germanica* were isolated from both the natural sediment and predators which are themselves known factors

playing a key role in foraminiferal mortality/survival and test destruction (Haynert et al. 2012; Haynert et al. 2014), our results suggest that high CO_2/low pH levels may be the most likely cause for the high rate of mortality (low survival rate) observed. However, this sensitivity (vulnerability) of foraminifera (i.e. via survival /mortality rates) to future OA levels may vary when experiments incorporate a natural sediment habitat because the pore water alkalinity and consequently the calcite saturation state (Ω_{cal}) may be higher in the sediment than the overlying water column, reducing the potential effects of OA on foraminifera (Haynert et al. 2014). Therefore, despite the apparent species-specific responses of *E. williamsoni* and *H. germanica* to multiple future OA scenarios (different pH conditions and [CO_3^{2-}]), these differential responses of both species may also vary under a longer exposure time.

4.4.2 Foraminiferal maximum test diameter and test weight

Despite the longer duration of the experimental period of this study compared to the previous experiment (see Chapter 3), juvenile specimens (<100 µm) of *E. williamsoni* and *H. germanica* were again not observed at the end of the experiment. This indicates that reproduction events were unlikely to have occurred during the experimental period across all pH treatments. These observations may be similar to those reported for natural assemblages, where the capacity of postponing the reproductive phase due to unfavourable environmental conditions have been described (Murray 1983). However, other experimental studies have shown that foraminiferal species reproduce as a stress response to elevated pCO_2 levels during the first weeks of an experimental period (Bernhard et al. 2009; McIntyre-Wressnig et al. 2013).

With regards to the mean maximum test diameter measured for both foraminiferal species, this parameter was significantly affected at pH 7.7 for *E. williamsoni* and at pH 7.3 for *H. germanica* (Fig.4.6 and Table 4.3). There is, therefore, an inconsistent trend between decreased test size and decreased pH levels. These results resemble those reported for *E. williamsoni* in Chapter 3, Section 3.3.6, and also for *Ammonia* sp. (Keul et al. 2013), *Amphistegina lessonii* and *Marginopora vertebralis* (Prazeres et al. 2015), where foraminiferal size was not significantly affected by lowering pH and concomitant low carbonate concentration [CO_3^{2-}]. In general, these findings may indicate that *E. williamsoni* and *H. germanica* grew at approximately the same rate regardless of CO_2/pH level. In contrast, Kuroyanagi et al. (2009) found a significant decrease in maximum test diameter of *Marginopora kudakajimensis* when specimens were exposed for 10 weeks to similar pH conditions to those used in this study.

The mean test weight of *E. williamsoni* and *H. germanica* were affected to different degrees by high CO_2/low pH levels. For *E. williamsoni*, a progressive decrease in test weight was observed with decreasing pH, mainly at pH 7.7 (Ω_{cal} >1) and pH 7.3 (Ω_{cal} <1) (Fig. 4.7 and Tables 4.1, 4.3). These results are consistent with those reported for *M. kudakajimensis* (Kuroyanagi et al. 2009) and *A. lessonii* (Prazeres et al. 2015), where even in saturated seawater (Ω >1) and pH of 7.7 or slightly lower, foraminiferal specimens were much lighter than those cultured at pH from 7.9 to 8.3. Furthermore, a similar decline in test weight was also reported for *Ammonia* sp. but in undersaturated seawater (Ω = 0.5) and pH 7.95 (Keul et al. 2013). The latter finding highlights the importance of measuring the [CO_3^{2-}] and concomitant seawater carbonate saturate state (Ω) because they are the main factors

affecting biological parameters (e.g. weight) of benthic foraminifera rather than pH levels (Keul et al. 2013).

For *H. germanica,* despite there being no statistically significant OA effect on the mean test weight across all pH treatments, specimens exposed to pH 7.9 were heavier compared to those specimens cultured at the remaining pH treatments including ambient conditions (Fig. 4.7 and Table 4.3). This non-linear response to pH treatments was observed for *M. kudakajimensis* also cultured across a similar pH levels range (Kuroyanagi et al. 2009).

In this study, although the effects of pH treatment on both mean size and weight were opposed to what one would expect or hypothesize (i.e. decline in size and weight with decreased pH), the reduction in number of individuals belonging to a specific class of size or weight (Fig .4.4 and 4.5) suggest that both *E. williamsoni* and *H. germanica* may be smaller and lighter, and found in very low abundance when exposed to lowering pH. Again, similar results were observed for *M. kudakajimensis* cultured under similar pH conditions to those used in this research (Kuroyanagi et al. 2009).

Our results suggest a species-specific response of *E. williamsoni* to future OA scenarios, with a greater impact of OA on mean test weight rather than other parameters such as mean size and mean number of new chambers. However, a much longer exposure time to elevated pCO_2 may show more outstanding changes in size and weight such as smaller tests, thinner chambers and lighter tests as those observed in other studies (Kuroyanagi et al. 2009; Allison et al. 2010; Prazeres et al. 2015).

4.4.3 Growth rate

For *E. williamsoni* and *H. germanica*, the growth rate expressed as the number of new chambers added over a period of 52 days, was significantly affected by low pH conditions.

Thus, *E. williamsoni* cultured at pH 7.9 showed a significantly higher mean growth rate (0.10 chambers day^{-1}) than the remaining pH conditions (~ 0.08 chambers day^{-1}) including the ambient conditions (Fig. 4.9 and Table 4.4). This non-linear response to pH treatments resembles that reported for *M. kudakajimensis* when comparing the number of new chambers added/growth rates between pH 7.9 and pH 8.2 (control) (Kuroyanagi et al. 2009). However, Khanna (2013), using a similar experimental set-up design to this study, reported a considerable and steady increase in the mean growth rate of *E. williamsoni* as the pH was reduced: at pH values of 7.7 the mean growth rate was 0.042 chambers day^{-1}, whereas at a pH of 7.3 the mean growth increased up to 0.099 chambers day^{-1}.

Results of mean growth rate for *E. williamsoni* obtained in this study and by Khanna (2013) contrast to those reported by Allison et al. (2010) and the findings of Chapter 3 (this study), where no significant difference in the growth rate was observed across different pH treatments. Furthermore, Austin (2013) experimentally determined that *E. williamsoni* was able to grow up to 0.10 chambers day^{-1} at a pH ~8.1 (ambient) and 10°C, which is slightly higher than that found in this study for a pH ~8.1 (0.08 chambers day^{-1}) at 13°C. It is likely that these differences in growth rate of *E. williamsoni* between are related to a multifactorial effect such as different length of each culture experiment, type of food

source, a potential temperature effect and ontogenetic stage (i.e. juveniles grow faster than adults).

H. germanica cultured at a pH~8.1 (ambient) showed the lowest mean growth rate (0.06 chambers day^{-1}) compared to the mean growth rate observed (~ 0.08 chambers day^{-1}) in the remaining pH conditions (Fig. 4.9 and Table 4.4). This progressive trend of the mean growth rate to increase (enhanced calcification) with lowering pH was also observed by Khanna (2013) for *H. germanica* culture at 15°C for 6 weeks at pH values of 7.7 (0.013 chambers day^{-1}) and 7.3 (0.017 chambers day^{-1}). However, the mean growth rate for *H. germanica* in the current study is ~4 times higher than values reported by Khanna (2013). It is more likely that this difference in growth rate of *H. germanica* between both studies is related to the different food sources that may have resulted in a differential feeding efficiency. The effect of temperature could also be considered as a potential factor influencing the mean growth rate.

In this study, these contrasting findings observed in the mean growth between *E. williamsoni* and *H. germanica* apparently indicate species-specific growth/calcification responses to OA. As these findings are opposed to what one would expect or hypothesize (i.e. decline in number of new chambers/growth rate with decreased pH as reported by Kuroyanagi et al. (2009).

4.4.4 Size-weight relationship of 'live' specimens

Live *E. williamsoni* and *H. germanica* with intact tests exhibited a minimal response to OA based on size-weight relationships across pH treatments (Fig. 4.13). These size-weight changes (slopes) are not statistically significant in both species ($p > 0.05$).

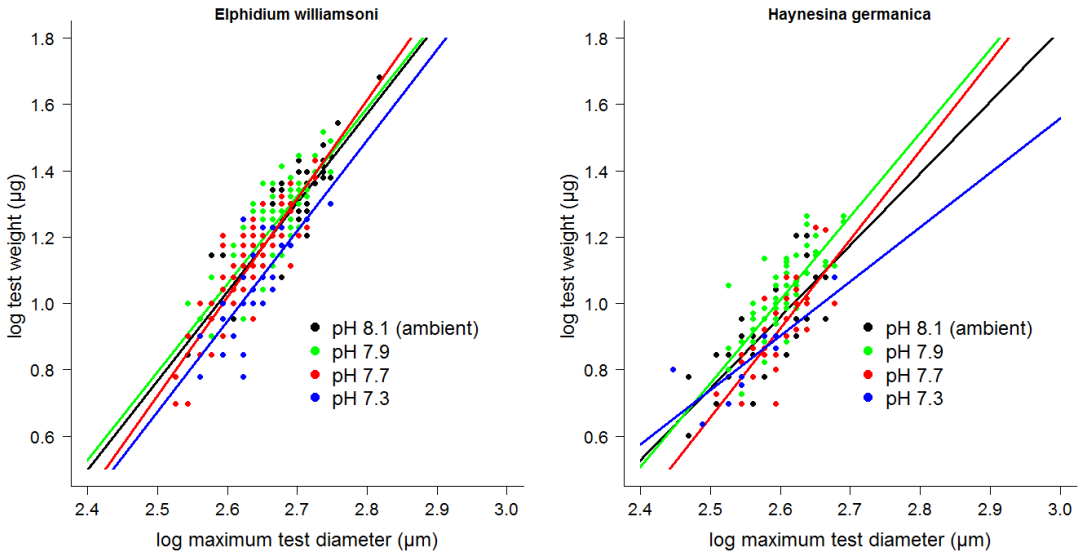

Figure 4. 13 Regression lines of the relationship between maximum log test diameter and log test weight of *Elphidium williamsoni* and *Haynesina germanica* cultured under different experimental pH conditions: black (ambient: pH 8.1/ ~400 µatm CO_2); green (pH 7.9/ ~600 µatm CO_2); red (pH 7.7/ ~950 µatm CO_2) and blue (pH 7.3/ ~2600 µatm CO_2).

This insensitivity of size-weight relationship to change due to OA in *E. williamsoni* and *H. germanica* was previously reported for specimens of *E. williamsoni* (see Chapter 3). In addition, similar findings were also found in other laboratory experiments using similar set-up (pH conditions of 8.1, 7.9, 7.7 and 7.6, and an experimental period of 30 days at 25°C) on specimens of the large benthic foraminifera *Marginopora vertebralis* which have high-

Mg calcite tests and live in coral reef environment (Prazeres et al. 2015). In contrast, a similar experiment carried out *Amphistegina lessonii* tests, a benthic foraminifera with a low-Mg calcite test, resulted in significant alterations in size-weight relationship as a consequence of changing pH levels (8.1, 7.9, 7.7 and 7.6) for an experimental period of 30 days at 25°C (Prazeres et al. 2015).

4.4.5 Foraminiferal calcification via size-normalized test weight (SNW) and seawater carbonate concentration

The parameter SNW has been used as an equivalent to estimating the test thickness or density of planktonic and benthic foraminifera, and consequently to measure the potential change in their calcification rate (efficiency) (Marshall et al. 2013; Mewes et al. 2014). In this study, however, following the suggestions that calcification rates in multiple calcifiers respond to seawater carbonate ions concentration [CO_3^{-2}] rather than pH (Langdon 2002; Kleypas et al. 2006; Keul et al. 2013), the combination of datasets of SNW and seawater [CO_3^{-2}] allowed an assessment of the impact of future OA levels on calcification/growth for *E. williamsoni* and *H. germanica* (Fig. 4.14 and Table 4.1).

The negative relationship between SNW and [CO_3^{-2}] observed at pH levels from 7.9 (calcite saturated seawater) to 7.3 (calcite undersaturated seawater) suggest important differences in calcification efficiency across all pH treatments and between foraminiferal species (Fig. 4.14). This reduced calcification/growth by *E. williamsoni* and *H. germanica* with declining seawater [CO_3^{-2}] support results reported by other studies (Langdon 2002; Kleypas et al. 2006; Keul et al. 2013).

The observed difference in magnitude of test thickness or calcification rate estimated via SNW in combination with [CO_3^{-2}] between both species is because *E. williamsoni* is naturally larger and heavier than *H. germanica* rather than a direct effect of OA between both species.

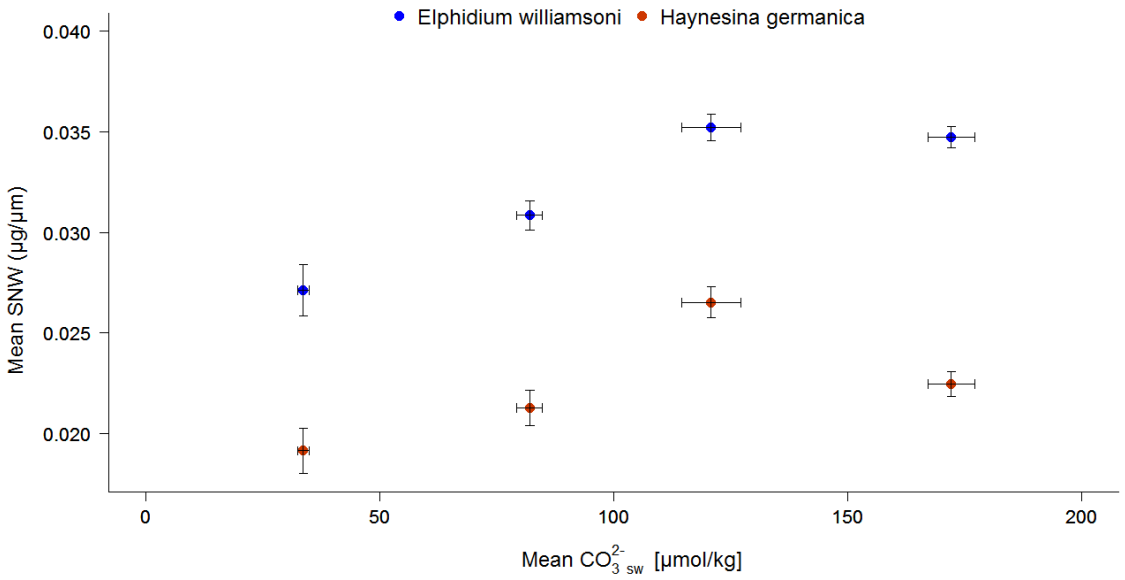

Figure 4. 14 Relationship between mean values (± standard error) of seawater [CO_3^{2-}] and size-normalized weight (SNW) for *Elphidium williamsoni* (in blue) and *Haynesina germanica* (in red) cultured at different pH conditions.

4.4.6 SEM observations of the morphological response of 'live' foraminifera

SEM images showed different stages of external dissolution on foraminiferal tests of live *E. williamsoni* and *H. germanica* exposed to high CO_2 concentrations/low pH over a period of 7 weeks (Fig. 4.11 and 4.12). These observations support the preliminary results illustrated in Chapter 3 (Figure 3.8). Similarly, Khanna et al. (2013), presented evidence of extreme

morphological modifications (i.e. changes in number of apertural teeth and shape) to the functional feeding structures in specimens of *H. germanica* experimentally exposed to similar seawater pH levels over a period of 36 weeks. Nevertheless, the effect of OA is probably more clearly observed on specimens of *H. germanica* studied by Khanna et al. (2013) compared to this study, as the former were exposed to similar high CO_2 concentrations/low pH for a much longer period. In addition, progressive morphological alterations have also been reported for tests of *A. aomoriensis* cultured in similar seawater pCO_2 concentrations for a period of 6 weeks (Haynert et al. 2011).

Although *E. williamsoni* and *H. germanica* are two co-occurring dominant foraminiferal species from intertidal mudflats with similarities in: test composition, feeding/sequestration mechanisms of primary producers (Austin et al., 2005), natural prey consumption (mainly diatoms) and morphological responses (i.e. altered feeding structures around the apertural area) to reduced pH; the findings via SEM imaging are inconclusive to provide a better understanding of species-specific responses to future OA scenarios. Instead, the results appear to indicate that living assemblages of *E. williamsoni* and *H. germanica* are highly vulnerable (low resilience) to changes in carbonate seawater chemistry despite their low-Mg calcite tests. This feature usually helps tests to resist corrosion and dissolution at early stages when exposed to low pH and undersaturated seawater with respect to calcite (Engel et al. 2015).

4.4.7 Future work

Further CO_2 experiments are required to: 1) verify our findings of growth rate expressed as the number of new chambers added. These new results could be combined with measurements of test thickness and weight to quantify the potential decreased/enhanced calcification of *E. williamsoni* and *H. germanica* as responses to OA; 2) assess the combined effect of OA and rising temperature on foraminiferal calcification/growth rates of these two benthic species; 3) evaluate whether the altered feeding structures (i.e. reduction in the number of teeth, modified shape and corroded structures) due to OA could modify their feeding efficiency via alteration in the carbon consumption rates, ultimately affecting the energy transfer to higher trophic levels.

In general, such experiments would provide a better understanding of species-specific responses and highlight the wider ecosystem changes that may be driven by future OA scenarios.

4.5 Conclusions

Based on the results of this short-term study on the biological responses of multispecies assemblages of benthic foraminifera to OA, the following conclusions may be drawn:

(1) Survival rate of *E. williamsoni* was negatively affected by the lowest pH treatments. The estimated OA-induced survival suggests that the observed natural mortality may increase by up to ~30 %. Survival rates of *H. germanica* appear to be less affected by OA, suggesting a species-specific response in survival rates under conditions of OA.

(2) Mean maximum test diameter and growth rate via size-weight relationships for *E. williamsoni* and *H. germanica* were not significantly altered by lowering pH.

(3) Mean growth rate estimated via the chamber addition method indicates that *E. williamsoni* appears to be less affected by lowering pH, except in pH 7.9 where an increase in growth rate was observed. However, mean growth rates of *H. germanica* showed a progressive increase in the mean growth (i.e. enhanced calcification) with reduced pH; again suggesting a species-specific response.

(4) Test weight and SNW (i.e. equivalent to test thickness/calcification efficiency) of *E. williamsoni* and *H. germanica* were strongly affected by reduced pH and low [CO_3^{-2}].

(5) The SNW appears to be a useful parameter to determine biological changes induced by OA on benthic foraminifera. Further, SNW combined with [CO_3^{-2}] datasets allow a better assessment of the impacts of future OA levels on thickness/calcification for *E. williamsoni* and *H. germanica*.

(6) SEM imaging suggest that under future scenarios with high CO_2 concentrations, live assemblages of *E. williamsoni* and *H. germanica* may be highly vulnerable to OA effects in the short-term, and this may ultimately affect the carbon cycling and total production and preservation of $CaCO_3$ in coastal environments such as intertidal mudflats

Chapter 5. Feeding efficiency and carbon uptake by benthic foraminifera under Ocean Acidification conditions

5.1 Introduction

Understanding the key role foraminifera play in benthic biogeochemical cycles (notably in the carbon cycle) from the deep sea to intertidal mudflats has stimulated notable scientific interest in the last decades. Numerous laboratory and *in-situ* based studies have investigated the fate of organic carbon derived from primary producers to benthic foraminifera, demonstrating the importance of microphytobenthos in the nutrition of some benthic foraminifera (Gooday 1988; Gooday 1996; Moodley et al. 2000; Heinz et al. 2001; Heinz et al. 2002; Allison et al. 2005; Nomaki et al. 2005; Wukovits et al. 2018). This foraminiferal heterotrophy may be a considerable loss pathway of primary producers in benthic environments.

These new insights into foraminiferal feeding behaviour have also improved our understanding of the potential link between organic carbon uptake by foraminifera and their contribution to abundance, distribution, biomass and diversity in meiobenthic communities, as well as their key role in processing organic material and nutrient fluxes within benthic food webs (Gooday 1988; Hohenegger et al. 1989; Lambshead & Gooday 1990; Gooday et al. 1992; Gooday 1996; Moodley et al. 2000; Heinz et al. 2001; Heinz et al. 2002; Moodley et al. 2002; Nomaki et al. 2005; Nomaki et al. 2008; Wukovits et al. 2017).

However, the amount of autotrophic organic carbon taken up by foraminifera may differ between species due to selective ingestion of the algal material available such as diatoms,

bacteria, microchlorophytes (Moodley et al. 2000; Mojtahid et al. 2011; Wukovits et al. 2017; Wukovits et al. 2018). This food preference is a common feature among a wide range of benthic foraminiferal species (Moodley et al. 2000; Nomaki et al. 2005; Nomaki et al. 2006), suggesting that selective feeding strategies of benthic foraminifera may be biological responses triggered by a high diversity of available carbon sources mainly found in shallow environments (Moodley et al. 2000). This fact, to some extent, may make it more difficult to select specific microphytobenthos species to be ingested by foraminifera in simulated feeding experiments that may help explain analogous feeding behaviour in natural settings.

An increasing number of complementary studies have also indicated that environmental factors such as pressure (Turley et al. 1993); oxygen (Alve & Bernhard 1995; Heinz et al. 2001; Altenbach et al. 2003; Enge et al. 2014; LeKieffre et al. 2017); temperature (Linke & Lutze 1993; Turley et al. 1993; Sayles et al. 1994; Wukovits et al. 2017; Wukovits et al. 2018); quantity and quality of food available and the periodicity of food inputs (Linke 1992; Sayles et al. 1994; Pfannkuche et al. 1999; Gooday 2002; Suhr et al. 2003; Martins et al. 2015); may control the grazing response by foraminifera to phytodetrital organic carbon supply.

However, despite the progress made to investigate the factors controlling the fate of organic carbon uptake into benthic foraminifera, the role of other environmental factors is still limited, such as increased atmospheric CO_2-induced Ocean Acidification (OA) and their impact on foraminiferal feeding efficiency and phytodetrital-derived organic carbon uptake in coastal environments, including estuaries and intertidal mudflats.

One of the first attempts to provide information on potential changes in the carbon uptake by benthic foraminifera (i.e. *H. germanica*) under projected increases in atmospheric CO_2 concentrations was carried out by Khanna (2014). The laboratory experiment setting for that study consisted of pre-conditioning benthic foraminifera to long-term exposure of high concentrations of CO_2 and low pH levels. After 32 weeks, live specimens of *H. germanica*, with observed damage to feeding structures as a consequence of the experimental conditions (see Khanna et al. 2013), were used for a 3 day-feeding experiment with ^{13}C-labelled native diatoms as the only food source. The results from that study did not conclusively demonstrate the link between observed negative impact of OA on feeding structures of *H. germanica* and its subsequent feeding efficiency. However, Khanna's experiment was extremely useful in sign-posting the next steps for future feeding experiments related to OA impacts on benthic foraminifera.

As studies on the effects of OA on foraminiferal feeding behaviour of intertidal benthic species are still limited (Khanna et al. 2013; Khanna 2014; Wukovits et al. 2017; Wukovits et al. 2018); the present study aims to provide evidence of the response of preconditioned *E. williamsoni* and *H. germanica* to organic carbon supply from fresh diatoms provided as a single food pulse, using stable isotope ^{13}C-labelling.

Although the current experimental design follows a similar methodology used by Khanna (2014) for a long-term laboratory CO_2 experiment and associated feeding study; some laboratory procedures were modified to successfully quantify OA-induced changes in uptake rates of fresh ^{13}C-labelled diatoms by *E. williamsoni* and *H. germanica*. It is assumed that the short-term precondition (e.g. deleterious effects of OA on apertural

feeding structures reported in Chapters 3 and 4) has the potential to modify foraminiferal organic carbon uptake with ecological implications for carbon cycling within the marine food web.

5.2 Materials and Methods

5.2.1 Field sampling and isolation of target foraminifera

Surface sediment scrapes from the top first centimetre were collected in late July 2016 at low tide from high intertidal mudflats of the Eden Estuary, N.E. Scotland (56°22'N, 2°50 W) (Chapter 2, Fig. 2.1). On return to the laboratory, all sediment samples were mixed and sieved over a set of 125 µm and 300 µm screens to remove stones, organic particles and larger meiofauna.

The sieved sediment fraction (bulk sieved sediment) was left to settle in big plastic trays for three hours, and direct observation of a small amount of sieved sediment through a stereoscopic binocular microscope confirmed that living foraminiferal specimens of *E. williamsoni* and *H. germanica* were present in densities of 10-20 live specimens/cm^3 and 1-5 live specimens/cm^3, respectively. Surface sediment scrapes from the bulk sieved sediment were collected and placed uniformly on the bottom of a series of 5-litre plastic containers to form a thin layer of approx. ~ 2-3 mm of sediment in each container. The total number of specimens of both foraminiferal species used for this experiment was approx. 20,000 live specimens (5,000 specimens per pH treatment). These plastic containers were filled with filtered natural (~33 salinity) seawater and sealed with a lid with an inlet on top to allow seawater to be continually recirculated into the containers from the main 400 L seawater reservoirs. A lateral outlet allowed seawater to flow out of plastic containers and return to seawater reservoirs. Multi-channel peristaltic pumps controlled the flow in/out between the experimental containers and the principal seawater reservoir.

5.2.2 Experimental conditions

Once the plastic containers with sediment containing living foraminiferal assemblages of both species were connected to a CO_2 manipulative mesocosm, the seawater pH was gradually reduced over 10 days (acclimation period) until each treatment reached its required pH level/pCO_2, ensuring that the measured foraminiferal responses were due to the treatments as described in Chapter 2.

For this experimental design, during the acclimation period (10 days) and the 6 week-CO_2 experiment itself, living foraminiferal assemblages of *E. williamsoni* and *H. germanica* were maintained in a controlled recirculating seawater system within a temperature-controlled room at 13°C with a 12:12-hr light: dark cycle. Foraminifera were exposed to different pH conditions: pH~8.1 (ambient), pH 7.9, pH 7.7 and pH 7.3 (Fig. 5.1) (information on carbonate chemistry manipulation system in detail in Chapter 2.4 and 2.5, respectively).

In contrast to previous experiments performed as part of this research programme (Chapter 3 and 4), and to ensure a larger number of surviving specimens at the end of the 6-week experiment, calcein incubation was not required.

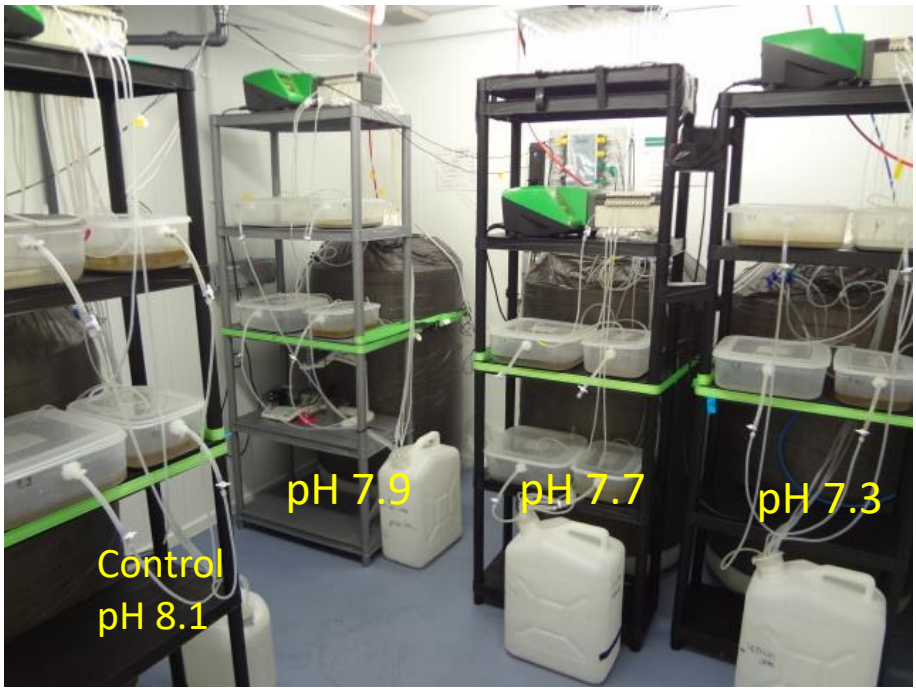

Figure 5. 1 Controlled recirculating seawater system used for CO_2 experiments. *Elphidium williamsoni* and *Haynesina germanica* cultured in natural sediment under controlled conditions of 13°C for 52 days with a light condition of 12:12-h light: dark cycle. From left to right, the reservoir tanks contain with seawater bubbled with equivalent atmospheric CO_2 concentrations of: 380 µatm /pH 8.1; 578 µatm /pH 7.9; 885 µatm /pH 7.7; and > 2484 µatm/pH 7.3

5.2.3 Production of non-labelled diatom *Navicula sp.*

The diatom *Navicula sp.* was incubated in sterilized and prefiltered natural seawater that contained modified f/2 medium (Guillard & Ryther 1962). After 30 days of incubation at 20°C and light: dark cycle = 12:12 h, part of the diatom culture was harvested by centrifugation at 800 G for 10min in 50 mL falcon tubes. These non-labelled diatom samples were stored at -20°C until they were required to feed foraminifera during the CO_2 experiment. The remaining part of the culture was used for the production of ^{13}C-labelled

diatoms for feeding experiments, and also to estimate the background signature of the diatom *Navicula sp.* before the start of the feeding experiment (as detailed below). The average concentration of ^{13}C in the non-labelled diatom sample was of 1.10 atom%.

5.2.4 Foraminiferal feeding process prior to feeding experiments

Foraminifera assemblages were fed weekly with ~10μL/cm^2 of each algae *Dunaliella tertiolecta*, *Rhodomonas salina* and *Navicula sp.* (typically 1×10^7 cells ml^{-1}). Concentrated algae were defrosted prior to use for foraminiferal feeding. Algae species were axenic clones provided by the Culture Collection of Algae and Protozoa (CCAP) at SAMS.

In the manipulative mesocosm, peristaltic pumps were switched off during this feeding procedure for 2 hours, and the level of seawater within the plastic container was reduced to the minimum level before the addition of food supply to allow algae to settle faster and also to avoid loss of this food material by resuspension when system back on again. The feeding procedure involves the use of a Pasteur pipette to add the algae into the plastic container with sediment. All foraminifera in all containers were fed at approximately the same time.

Navicula sp. was prepared as a representative of common natural phytoplankton in the Eden Estuary where sediments were collected for this research. Moreover, *Navicula sp.* was chosen for the experiment because it has been previously observed attached to test surfaces and feeding structures of both target foraminiferal species used in this study (Khanna, 2014, Chapter 3 and 4 of this research). *Dunaliella tertiolecta* and *Rhodomonas salina* were used again as the other main carbon sources because it is confirmed (see Chapter 3 and 4) that the supply of both microalgae in previous CO_2 experiments promoted the growth of *E.*

williamsoni and *H. germanica*. Furthermore, *Dunaliella tertiolecta* is well known to be assimilated by benthic foraminifera when it is provided as the main food source in the laboratory and *in-situ* ^{13}C experiments (Heinz et al. 2001; Heinz et al. 2002; Nomaki et al. 2005; Nomaki et al. 2006).

In this study, throughout the CO_2 experimental period, benthic foraminifera assemblages potentially utilized the three different carbon sources, and this may have ensured a larger number of surviving specimens. Preferences for any particular algae are not discussed further here.

5.2.5 Production of ^{13}C-labelled diatom for feeding experiments

Navicula sp. was selected to be the only ^{13}C-labelled food source for foraminiferal specimens for the feeding experiments. This diatom was incubated in sterilized and prefiltered natural seawater that contained modified f/2 medium (Guillard & Ryther 1962) and approx. 0.1 mol L^{-1} of additional $NaHCO_3$ enriched with 99.9% ^{13}C (Cambridge Isotope Laboratories, Inc., USA) (Nomaki et al. 2005; Nomaki et al. 2006; Hunter et al. 2012). After 28 days of incubation at 13°C and a light: dark cycle = 12:12 hr., the diatom culture (non-aerated) was harvested by centrifugation at 800 G for 10min in 50 mL falcon tubes.

The supernatant was removed, and the algal pellets were collected and homogenized. This retained algal slurry was centrifuged again and rinsed three times in sterilized natural seawater (Nomaki et al. 2005; Nomaki et al. 2006). For the last centrifugation step, the algal pellet was transferred to 5 mL cryotubes. The algal pellet of only 1 cryotube was used

for feeding experiments in order to provide a fresh food source for foraminifera as described below. The remaining cryotubes were shock frozen in liquid nitrogen and lyophilized at -55°C and 0.180 mbar for 3 days and stored at -20°C for further ^{13}C analysis. The final concentration of ^{13}C in labelled diatom sample was of 5.5 atom%.

5.2.6 Feeding experiments

Once the CO_2 experiment was concluded (after 52 days), viable ('live') specimens of *E. williamsoni* and *H. germanica* were examined through a stereoscopic binocular microscope. These surviving specimens were selected based on their colourful protoplasm which was extensively distributed across the test, except in the last two or three chambers which in some cases were empty. Selected specimens were carefully wet-picked and cleaned of any detritus and sediment particles attached to their tests using a fine paintbrush (Enge et al. 2014), and finally, foraminifera were sorted into species to manage them separately throughout the feeding experiments.

Due to the high mortality observed (~80%) throughout the CO_2 experiment, total foraminiferal abundance was low. 'Live' individuals collected from all pH treatments included approx. 1,800 specimens of *E. williamsoni* and 960 specimens of *H. germanica*. This total number of living specimens for each species allowed setting the maximum number of replicates for each treatment and the duration of the feeding experiment. For instance, the experiment had a maximum time span of 72 hours of incubation; mainly for *E. williamsoni* which had a much larger number of surviving specimens compared to *H. germanica*.

At the start of the feeding experiment, 90 specimens of *E. williamsoni* and 80 specimens of *H. germanica* from each pH treatment were separately transferred to clean Petri dishes and carefully rinsed three times in sterile natural seawater with their specific pH level. Paintbrushes used here were previously cleaned in a solution of CH_2Cl_2 and CH_4O (1:1, v:v) (Enge et al. 2014). Each Petri dish with a specific number of living specimens was immediately filled with their respective reduced pH seawater. Specimens were kept in the dark for 5 days within a temperature-controlled room at 13°C and no food source was provided (starvation period) up to the start of the ^{13}C incubation.

Once the starvation period was concluded and the feeding experiment started, the first batch of selected Petri dishes corresponding to $\mathbf{T_0}$ for both species was removed to estimate the isotopic composition ($\delta^{13}C$) of these samples. These represented the laboratory background signature or control used for further calculations. Hence, the changes of $\delta^{13}C$ observed in the other incubation periods (i.e. after 3 and 72 hours) were compared to $\delta^{13}C$ values determined at $\mathbf{T_0}$ for each foraminiferal species.

The remaining Petri dishes with foraminifera of each species were incubated with 100 µL of concentrated ^{13}C-labelled diatom *Navicula sp.* (~72 µg C*)*. As the experimental design included only two incubation periods, 3 and 72 hours after the start of feeding, every batch of selected Petri dishes corresponded to $\mathbf{T_1}$ and $\mathbf{T_2}$, respectively. These were removed (subsampled) from the experiment and processed prior to estimation of uptake of ^{13}C by foraminifera (Fig. 5.2).

In order to keep the same experimental physicochemical conditions, the entire feeding experiment was conducted in the dark in a temperature-controlled room at 13°C. In the case of pH in the experimental Petri dishes, incubated seawater from each petri dish with foraminifera and labelled diatom was carefully replaced every day for fresh seawater at the respective pH level.

Experimental species	Number of replicates	CO_2 concentrations/pH conditions											
		400 ppm/ pH 8.1			750 ppm/ pH 7.9			1000 ppm/ pH 7.7			2400 ppm /pH 7.3		
		T_0	T_1	T_2	T_0	T_1	T_2	T_0	T_1	T_2	T_0	T_1	T_2
Elphidium williamsoni + *Navicula sp.* (+)	Replicate #1	●	●	●	●	●	●	●	●	●	●	●	●
	Replicate #2	-	●	●	-	●	●	-	●	●	-	●	●
Haynesina germanica + *Navicula sp.* (+)	Replicate #1	●	●	-	●	●	-	●	●	-	●	●	-
	Replicate #2	-	●	-	-	●	-	-	●	-	-	●	-

Figure 5. 2 Schematic diagram of the feeding experiment design with 4 pH treatments. Specimens of *E. williamsoni* and *H. germanica* were fed once with ^{13}C enriched diatom *Navicula sp.* **(+). T_0** represents Petri dishes with foraminifera subsampled after the 5-day starvation period (laboratory background); T_1 and T_2 represent Petri dishes with foraminifera subsampled after 3 and 72 hours of the start of feeding experiment, respectively. **(●)** Circles represent each replicate incubated and removed from the experiment at specific incubation period. (-) represents the absence of live specimens for that replicate at that specific incubation period.

After each incubation period (from T_0 to T_2), and based on the same criterion of selection for viable (live) specimens described above, selected specimens were wet-picked and transferred from experimental Petri dishes to new Petri dishes to be carefully cleaned and

rinsed three times in sterile natural seawater, and subsequently placed into a watch glass. Here, foraminifera were again counted and transferred into 1.5 mL Eppendorf tubes using pipettes where the excess water was removed, and each foraminiferal sample was frozen via immersion in liquid nitrogen.

The number of live specimens of each foraminiferal species used for the analysis of isotopic composition in each sample was lower than the initial number of specimens used for the feeding experiments (Table 5.2). These samples were stored at -20°C until further processes were carried out (Enge et al. 2014; Wukovits et al. 2017).

5.2.7 Sample preparation for ^{13}C analysis

Each sample with foraminiferal specimens was naturally defrosted and transferred from the Eppendorf tube to a watch glass filled with Deionized water (DI H_2O). Specimens of each species were rinsed three times and finally recounted and transferred into tin (Sn) capsules using Pasteur pipettes. Excess water was carefully removed with a microsyringe. Each Sn capsule was previously cleaned in CH_2Cl_2:CH_4O (Wukovits et al. 2017), and weighed using a micro scale and placed in a 96 well plate.

All samples placed in the sample rack (96 well plate) were dried at 50°C. Samples were continually monitored until they were completely dried and subsequently weighed. Samples were kept in a desiccator until the decalcification process started. Foraminiferal specimens were decalcified (dissolution of all calcium carbonate) by adding gradually 5 µL of 4% HCl in every capsule (Enge et al. 2011; Enge et al. 2014). Formation of carbon dioxide bubbles was observed as part of the decalcification reaction. Samples were placed for 3 days in an

oven at 50°C to complete drying (Enge et al. 2011; Enge et al. 2014). Capsules were closed, weighted and crushed into a small ball with no edges by using clean tweezers and a petri dish as an underlay. Finally, acidified samples were wrapped in a silver capsule for further analysis as described below.

Samples of foraminiferal cytoplasm were analysed at the Large-Instrument Facility for Advanced Isotope Research at the University of Vienna 145 (SILVER). Isotopic composition and Total Organic Carbon (TOC) content were analysed using an elemental analyser (EA 1110, CE Instruments) connected to an Isotope Ratio Mass Spectrometer (IRMS; DeltaPLUS, Thermo Finnigan).

For calculations of carbon uptake, only samples showing a higher $\delta^{13}C$ value or sufficient organic carbon with respect to the $\delta^{13}C$ values of background were used (Nomaki et al. 2005; Enge et al. 2014). In this study, only one sample was excluded from the original dataset for further calculations.

The carbon isotope composition of the samples ($atom\%_{sample}$) was obtained from isotope ratio data ($^{13}C/^{12}C$) and calculated against the international Vienna Pee Dee Belemnite (for carbon, RVPDB=0.0112372) as the reference standard (see Equation 5.1) (Enge et al. 2016; Wukovits et al. 2017; Wukovits et al. 2018) as follows:

$$atom\%\ C_{sample} = \frac{100 \times R_{standard} \times ((\delta^{13}C_{sample}/1000) + 1))}{1 + ((R_{standard}) \times ((\delta^{13}C_{sample}/1000) + 1))} \quad \text{(Eq. 5.1)}$$

The excess (E_{sample}) of isotopic content within the samples was calculated as the difference between data of enriched samples and background samples (bkgd) data at different time

periods as detailed below (see Equation 5.2) (Hunter et al. 2012; Enge et al. 2016; Wukovits et al. 2017; Wukovits et al. 2018)

$$\boldsymbol{E}_{\mathbf{sample}} = (\text{atom\% } {}^{13}C_{sample} - \text{atom\% } {}^{13}C_{bkgd})/100 \quad \text{(Eq. 5.2)}$$

The amount of incorporated isotopes derived from food source I_{iso} [µg ind^{-1}] was calculated as the product of isotope excess (see Equation 5.2) and Total Carbon Content (TOC) in the sample and divided by the number of analysed specimens (see Equation 5.3) (Hunter et al. 2012; Enge et al. 2016; Wukovits et al. 2017; Wukovits et al. 2018).

$$I_{iso} = E \text{ x } TOC_{sample} \text{ /number of specimens} \quad \text{(Eq. 5.3)}$$

Subsequently, the amount of phytodetrital carbon (pC [µg ind^{-1}]) within the foraminiferal cytoplasm was calculated using I_{iso} divided by atom% ^{13}C of enriched diatom sample as follows (see Equation 5.4) (Hunter et al. 2012; Enge et al. 2016; Wukovits et al. 2017; Wukovits et al. 2018)

$$I_{phyto} = I_{iso} / (\text{atom\% } {}^{13}C_{diatom}/100) \quad \text{(Eq. 5.4)}$$

5.2.8 Natural isotope signatures for foraminifera

Following the steps mentioned in this Chapter 5.2.1, specimens of *E. williamsoni* and *H. germanica* were collected in late April 2016 and isolated to be processed for the ^{13}C analysis as mentioned in Chapter 5.2. These $\delta^{13}C$ values from foraminiferal cytoplasm represented the natural signature of δ ^{13}C for each foraminiferal species before CO_2 and feeding experiments. Despite the fact that these values were not used for the calculations of species uptake per individual, $\delta^{13}C$ values obtained from natural signatures allowed

estimation of the optimum amount needed for isotope and elemental analysis when the feeding experiments concluded as recommended by Wukovits et al. (2017).

5.2.9 Statistical Method

The present statistical tests applied to datasets have been selected to keep robustness with the present study design and address potential problems due to small samples sizes as recommended by Wukovits et al. (2017). Homogeneity of variances was tested using Fligner–Killeen's (Conover et al. 1981; Wasserstein & Boyer Jr 1991; Wukovits et al. 2017). Additionally, Welch's *t*-test (for sample sizes<10) was used to observe significant differences of phytodetrital carbon (pC) and uptake rates between species (Moser & Stevens 1992; Ruxton 2006; Wukovits et al. 2017). A one-way ANOVA was carried out to observe significant differences between pH treatments at different incubation periods for each foraminiferal species dataset. Where possible, a two-way ANOVA was carried out to observe significant differences within each foraminiferal species dataset, with the incubation periods and pH treatments as independent factors and pC and uptake rates as dependent variables.

All statistical analyses were run in the statistical programme R 3.1.2 (R Development Core Team. 2014).

5.3 Results

5.3.1 Seawater carbonate chemistry

Fortnightly measurements of parameters of seawater carbonate were constant throughout the entire time period of 42 days for the CO_2 experiment (Table 5.1), where foraminifera were preconditioned to different CO_2 concentrations and pH levels before feeding experiments.

Table 5. 1 Average seawater measurements taken fortnightly from carbonate chemistry manipulation system. Values account for mean ± SD, n = 3.

Measured parameters					**Calculated parameters**					
Treatment	**pH (Total)**	**T (°C)**	**Salinity (ppt)**	**AT (µmol/Kg)**	**DIC (µmol/kg)**	***p*CO2 (µatm)**	**HCO_3^- (µmol/kg)**	**CO_3^{2-} (µmol/kg)**	**$\Omega_{Calcite}$**	**$\Omega_{Aragonite}$**
pH 8.1 (ambient)	8.12 ± 0.01	13.10 ± 0.05	32.77 ± 0.09	2535.70 ± 67.65	2294.17 ± 63.12	378.41 ± 11.59	2101.24 ± 57.85	177.76 ± 5.19	4.29 ± 0.12	2.73 ± 0.08
pH 7.9	7.91 ± 0.01	13.2 ± 0.04	32.80 ± 0.08	2522.74 ± 65.80	2376.07 ± 64.39	641.42 ± 30.40	2231.47 ± 61.16	118.97 ± 4.15	2.87 ± 0.10	1.83 ± 0.06
pH 7.7	7.72 ± 0.01	13.2 ± 0.04	32.72 ± 0.09	2492.24 ± 50.38	2418.49 ± 49.33	1024.67 ± 23.69	2298.69 ± 46.84	78.85 ± 2.07	1.90 ± 0.05	1.21 ± 0.03
pH 7.3	7.36 ± 0.01	13.2 ± 0.05	32.72 ± 0.09	2518.95 ± 50.46	2564.12 ± 52.72	2461.90 ± 102.61	2429.08 ± 49.49	36.67 ± 1.30	0.88 ± 0.03	0.56 ± 0.02

5.3.2 Measurement of $\delta^{13}C$

In general, due to the high mortality rate throughout the experimental period (i.e. CO_2 experiment, starvation period and feeding experiment), the number of live specimens used for the analysis of isotopic composition in each sample was lower than expected. This sample limitation, in most the cases, resulted in a maximum of two replicates for each treatment. These were mainly for *E. williamsoni* as it had a much larger number of

surviving specimens (Table 5.2). $\delta^{13}C$ values measured in enriched and non-enriched samples of both diatom and cytoplasm of preconditioned foraminifera across different pH treatments and at different incubation periods are shown in Table 5.2

Table 5. 2 Values of $\delta^{13}C$ in samples of the diatom *Navicula sp*. and protoplasm of live *E. williamsoni* and *H. germanica* previously exposed (preconditioned) for 52 days to different CO_2/pH levels prior to feeding experiment. Natural abundance signature is referred as to the measurement of $\delta^{13}C$ in the protoplasm of specimens collected from the Eden Estuary in April 2016. For the feeding experiment, T_0 represents the laboratory abundance signature (background) subsampled after the 5-day starvation period; T_1 represents foraminifera subsampled after 3 hours of the start of feeding experiment, and T_2 represents to foraminifera subsampled 72 hours after the start of feeding experiment. D^+ represents labelled diatom added at different incubation periods (T_1 and T_2). n represents the number of analysed samples (replicates) used for calculated $\delta^{13}C$.

Sample	**Background**		**T_0**		**T_1 (D^+)**		**T_2 (D^+)**	
	$\delta^{13}C$	**n**	**$\delta^{13}C$**	**n**	**$\delta^{13}C$**	**n**	**$\delta^{13}C$**	**n**
***Navicula sp*.**								
Non-labelled	-10.40 to -10.39	2	-	-	-	-	-	-
Labelled	3776.93 to 4579.15	2	-	-	-	-	-	-
Elphidium williamsoni								
Nature signature	-13.77	1	-	-	-	-	-	-
pH 8.1 (ambient)	-		-21.713	1	13.82 to 28.55	2	316.89 to 370.50	2
pH 7.9	-		-20.768	1	17.98 to 24.07	2	118.74 to 474.28	2
pH 7.7	-		-21.584	1	0.06 to 7.10	2	296.42 to 386.10	2
pH 7.3	-		-20.697	1	-4.34 to 18.70	2	-11.14 to 421.47	2
Haynesina germanica								
Nature signature	-13.41 to -12.47	2	-	-	-	-	-	-
pH 8.1 (ambient)	-		-18.068	1	-13.02	1	-	-
pH 7.9	-		-15.902	1	-10.01 to -8.03	2	-	-
pH 7.7	-		-16.77	1	-13.64 to -12.31	2	-	-
pH 7.3	-		-17.081	1	-11.94 to -7.74	2	-	-

An improved visualization of the dataset presented in Table 5.2 is shown in Fig. 5.3 and 5.4 for specimens of *E. williamsoni* and *H. germanica,* respectively.

5.3.3 Food uptake by foraminifera from a single feeding pulse

In general, isotopic composition analysed in foraminiferal cytoplasm samples differed strongly between species and incubation periods. These $\delta^{13}C$ values demonstrate highly active ingestion of labelled diatom by *E. williamsoni* in comparison to *H. germanica* across all pH treatments over time.

For *E. williamsoni,* prior to the feeding experiment, measured $\delta^{13}C$ of preconditioned foraminifera showed a value of approx. -21 ‰ (n = 1). After 3 days of incubation with labelled *Navicula sp.*, individuals cultured at a pH of 8.1 (ambient) showed the highest incorporation of the labelled ^{13}C followed by the treatments at a pH of 7.9 and pH 7.3. In contrast, individuals cultured at a pH of 7.7 showed the lowest value of incorporated $\delta^{13}C$. Across all pH treatments, $\delta^{13}C$ values consistently increased over time until 72 hours after the start of incubation, maintaining a similar decreasing trend of uptake with pH as observed at T_1 (3 hours) where the values of incorporated ^{13}C at a pH of 8.1 differed notably from the treatment at a pH of 7.3 (Fig. 5.3 and Table 5.2).

Large error bars represent standard deviations (SD) calculated for foraminiferal cytoplasm samples incubated for 72 hours. These reflect the wide variation between the two $\delta^{13}C$ values analysed for *E. williamsoni* mainly at pH 7.9 and pH 7.3.

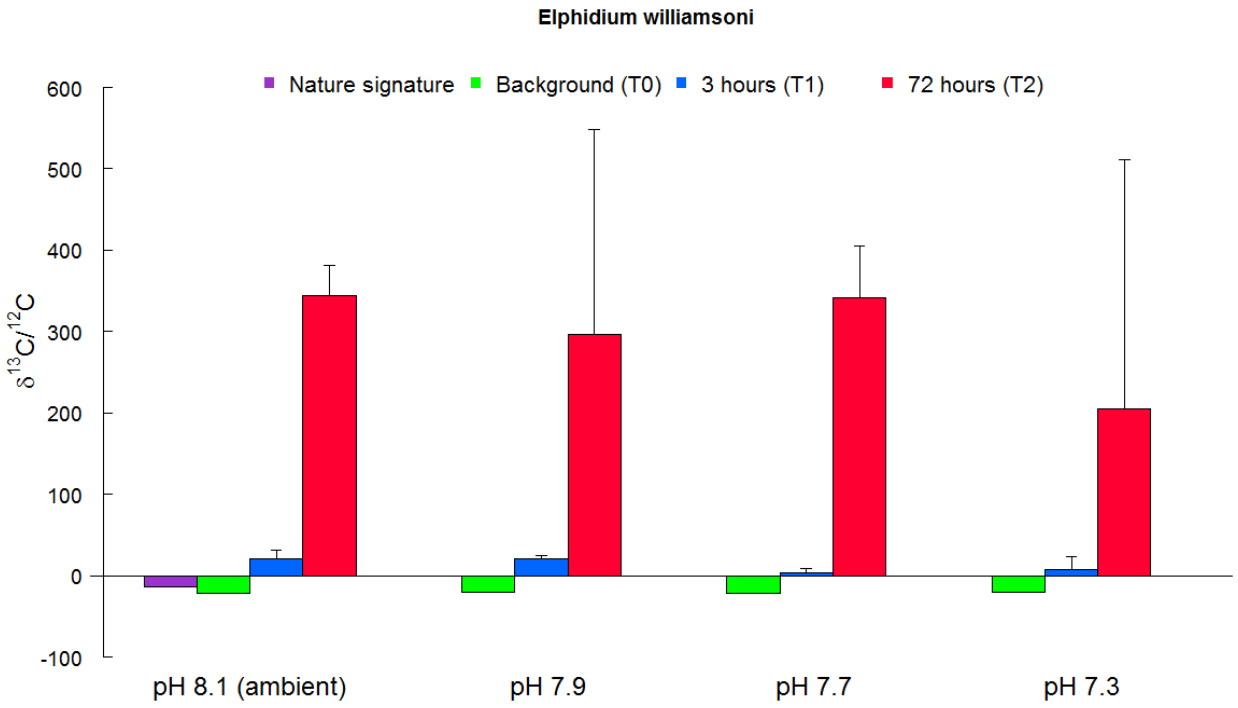

Figure 5. 3 Comparison of carbon isotope ratios ($\delta^{13}C$) of cytoplasm samples of preconditioned *Elphidium williamsoni* after feeding experiments. Natural signature (purple), experimental background at the start of the incubation period (T_0) (green), labelled treatments after 3 hours (T_1) (blue) and 72 hours (T_2) (red) of the start of feeding experiment. Mean + 1SD are based on two points measurements (n = 2), whereas other $\delta^{13}C$ values are just based on one-point measurement.

H. germanica showed a limited amount of excess ^{13}C after three hours of incubation. Individuals cultured at a pH of 7.9 showed the highest enrichment of ^{13}C followed by the treatment at a pH of 7.3. In contrast, individuals cultured at a pH of 8.1 (ambient) and pH 7.7 showed the lowest enrichments of incorporated ^{13}C. Despite the evidence for a slight enrichment of ^{13}C within the foraminiferal cytoplasm of *H. germanica* across all pH treatments, there are no obvious trends related to pH effect on the incorporated ^{13}C food

label by *H. germanica.* There were no live specimens for 72-hour incubation (Fig. 5.4 and Table 5.2).

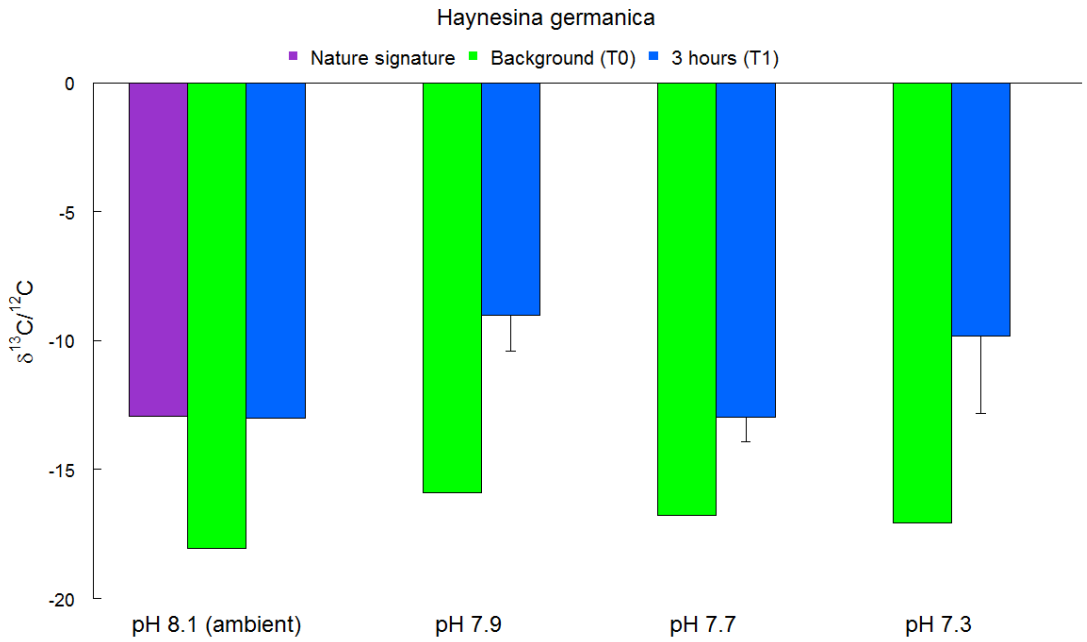

Figure 5. 4 Comparison of carbon isotope ratios ($\delta^{13}C$) of cytoplasm samples of preconditioned *Haynesina germanica*. Natural signature (purple), experimental background at the start of the incubation period (T_0) (green), labelled treatments at 3 hours (T_1) (blue) after the start of feeding experiment. Mean + 1SD observed are based on two points measurements (n = 2), whereas other $\delta^{13}C$ values are just based on one-point measurement.

5.3.4 Content of phytodetrital carbon (pC)

The content of phytodetrital carbon (pC) was calculated based on the number of individuals per sample, total organic carbon (TOC) and values of atom %. The latter were calculated for *E. williamsoni*, *H. germanica* and labelled *Navicula sp.* (Table 5.3).

Table 5. 3 Number of individuals per sample and total organic carbon (TOC) for *E. williamsoni* and *H. germanica*. Calculated values of atom % (based on values of $\delta^{13}C$) for both foraminiferal species and labelled *Navicula sp.* are shown. Calculated mean values (± standard deviation) of pC (based on values of TOC, atom % and number of individuals) for both foraminiferal species are also shown.

T_0 represents the laboratory abundance signature (background) subsampled after the 5-day starvation period; T_1 represents foraminifera subsampled after 3 hours of the start of feeding experiment, and T_2 represents to foraminifera subsampled 72 hours after the start of feeding experiment.

Sample	T_0			T_1 (3 hours)				T_2 (72 hours)			
	Individuals per sample	TOC (mg)	atom %	Individuals per sample	TOC (mg)	atom %	pC (µg ind^{-1})	Individuals per sample	TOC (mg)	atom %	pC (µg ind^{-1})
Diatom										-	
Non-labelled	-	-	1.100	-	-	-		-	-	-	
Labelled (+)	-	-	5.499	-	-	-		-	-	-	
Elphidium williamsoni											
Natural signature	50	0.068	1.096	-	-	-		-	-	-	
pH 8.1 (ambient)	76	0.030	1.087	84	0.036	1.135	3.6 ± 0.90	76	0.028	1.487	26.93 ± 5.18
pH 7.9	75	0.031	1.088	74	0.027	1.134	3.07 ± 0.25	79	0.023	1.436	20.20 ± 18.67
pH 7.7	80	0.038	1.088	80	0.031	1.115	1.92 ± 0.40	79	0.028	1.485	25.60 ± 0.77
pH 7.3	66	0.012	1.088	81	0.037	1.119	2.6 ± 1.67	78	0.036	1.336	21.73 ± 29.53
Haynesina germanica											
Natural signature	50	0.064	1.104	-	-	-		-	-	-	
pH 8.1 (ambient)	58	0.021	1.091	63	0.021	1.090	0.34	-	-	-	
pH 7.9	60	0.020	1.094	54	0.018	1.101	0.46 ± 0.08	-	-	-	
pH 7.7	60	0.017	1.093	65	0.020	1.097	0.23 ± 0.04	-	-	-	
pH 7.3	58	0.021	1.092	75	0.024	1.100	0.46 ± 0.11	-	-	-	

For *E. williamsoni,* after 3 hours of incubation with labelled diatom, individuals cultured at a pH of 8.1 (ambient) showed the highest pC values followed by the treatments at a pH of 7.9 and pH 7.3. In contrast, individuals cultured at a pH of 7.7 showed the lowest value of carbon uptake. Across all pH treatments, pC value significantly increased over time until 72 hours after the start of incubation, keeping a similar decreasing trend observed at T_1 (3 hours) where the amount of pC decreased as the pH decreased (Fig. 5.5 and Table 5.4).

Table 5. 4 Two-way ANOVA to compare the effects of the time period and pH treatments on dependent variable pC (content of phytodetrital carbon) mainly in *Elphidium williamsoni* cultured under different experimental pH conditions: pH~8.1 (ambient), pH 7.9, pH 7.7 and pH 7.3. Numbers in bold indicate statistically significant *p*-values.

Species	**Variable**	**Factor**	**Df**	***F* value**	***p* value**
E. williamsoni	pC	time period	1	11.067	**0.010**
		treatment	3	0.070	0.974
		time period x treatment	3	0.065	0.977
		residuals	8		

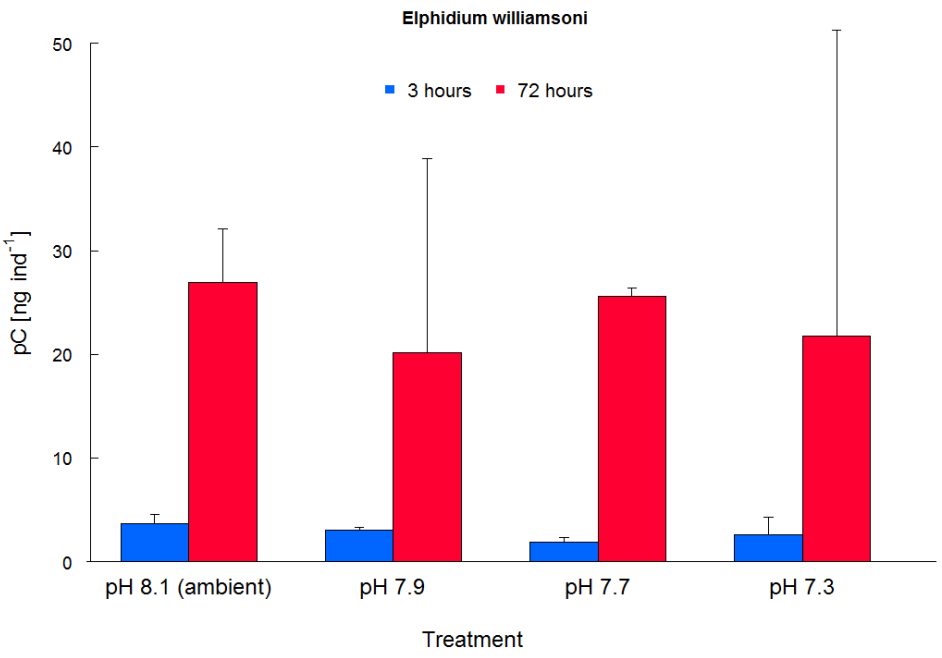

Figure 5. 5 Comparison of individual uptake of phytodetrital carbon (pC) by preconditioned *Elphidium williamsoni* for 3 hours (T_1) (blue) and 72 hours (T_2) (red) after the start of feeding experiment. Mean + 1SD observed are based on two points measurements (n = 2).

For *H. germanica*, after 3 hours of incubation with labelled diatom, individuals cultured at a pH of 7.9 showed the highest pC values followed by the treatments at a pH 7.3 and 8.1 (ambient). In contrast, individuals cultured at a pH of 7.7 showed the lowest value of carbon uptake. Calculated pC values are approximately 10-fold lower than pC values displayed by *E. williamsoni* within the first 3 hours of incubation with labelled diatom. Despite the values of pC show a slight food uptake by *H. germanica* across all pH treatments, there are no obvious trends related to the pH effect on carbon uptake by *H. germanica* (Fig. 5.6).

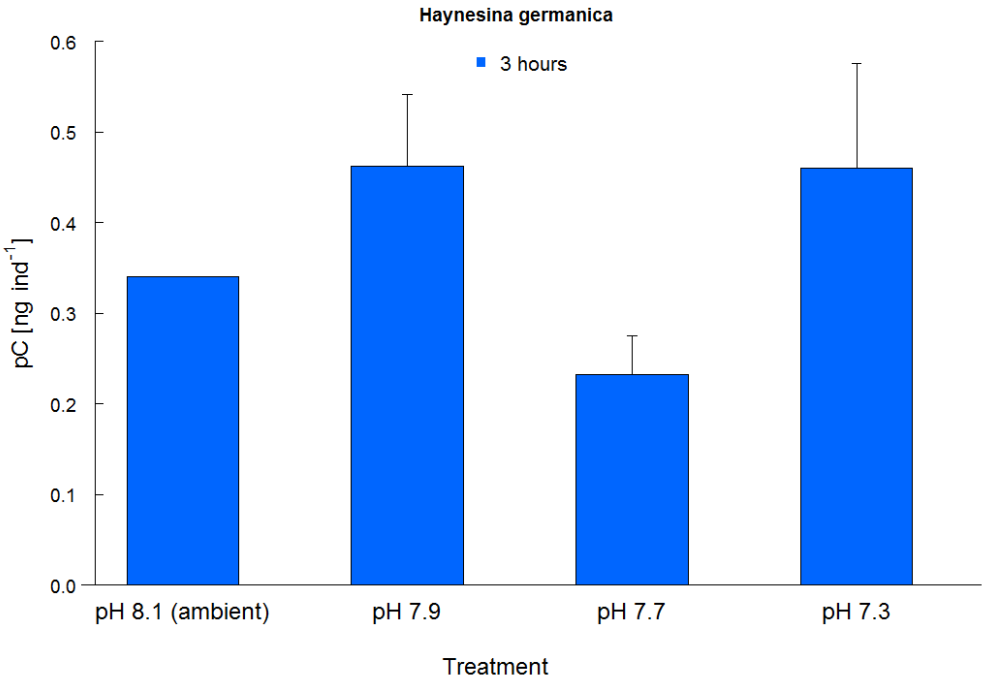

Figure 5. 6 Comparison of individual uptake of phytodetrital carbon (pC) by preconditioned *Haynesina germanica* for 3 hours (T_1) (blue) after the start of the feeding experiment. Mean + 1SD observed are based on two points measurements (n = 2).

In summary, the calculated content of pC within foraminiferal cytoplasm differed significantly among species (Welch's test, $p < 0.05$) and incubation periods (Two-way ANOVA, $p = 0.010$, Table 5.4). However, pC did not differ significantly between pH treatments within each incubation period (One-way ANOVA, $p > 0.05$ (Fig. 5.5 and 5.6).

5.3.5 Foraminiferal carbon uptake rate at a single food pulse

Carbon uptake rates (ngC) for each foraminiferal species were calculated based on the content of phytodetrital carbon (pC) accumulated in the cytoplasm within a specific time period (Fig. 5.7 and 5.8).

E. williamsoni showed a rapid and active response to a single pulse of labelled food within the first three hours of the incubation experiment. The carbon uptake rates differed between pH treatments. For instance, individuals cultured at a pH of 8.1 (ambient) showed the highest carbon uptake rate (1.22 ngC ind^{-1} h^{-1}) followed by the treatments at a pH of 7.9 (1.02 ngC ind^{-1} h^{-1}) and pH 7.3 (0.85 ngC ind^{-1} h^{-1}). In contrast, individuals cultured at pH 7.7 showed the lowest carbon uptake rate of 0.64 ngC ind^{-1} h^{-1}. However, after 72 hours of incubation, carbon uptake rates substantially decreased and were 4-fold lower than those rates observed within the first three hours of the feeding experiment in each pH treatment; the decreasing trend in uptake rates is also apparent, except for individuals exposed to a pH of 7.7 (Fig. 5.7).

Table 5. 5 Two-way ANOVA to compare the effects of the time period and pH treatments on dependent variable carbon uptake rate mainly by *Elphidium williamsoni* cultured under different experimental pH conditions: pH~8.1 (ambient), pH 7.9, pH 7.7 and pH 7.3. Numbers in bold indicate statistically significant *p*-values.

Species	**Variable**	**Factor**	**Df**	***F* value**	***p* value**
E. williamsoni	uptake rate	time period	1	17.833	**0.003**
		treatment	3	0.763	0.546
		time period x treatment	3	0.722	0.566
		residuals	8		

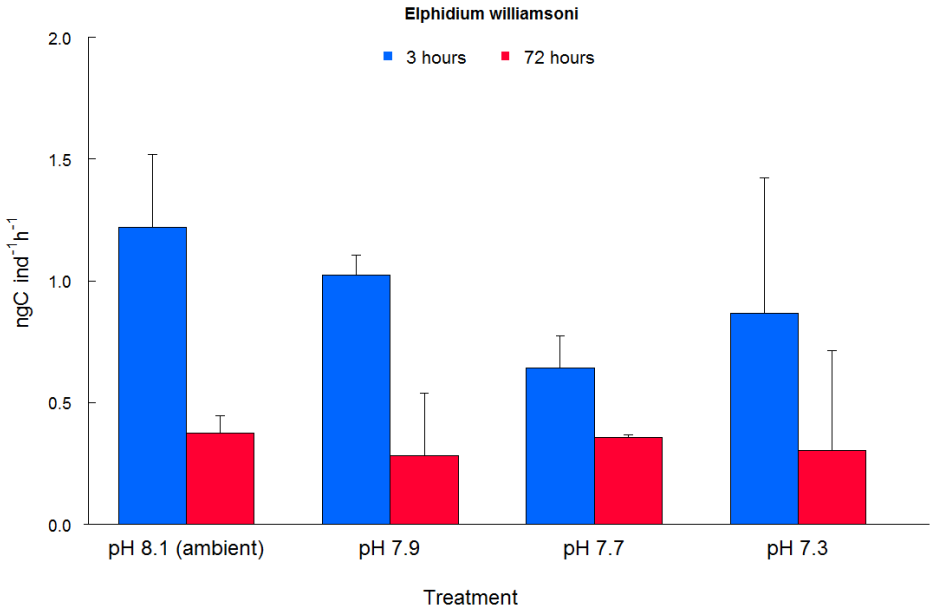

Figure 5. 7 Comparison of individual uptake rates of phytodetrital carbon (pC) by preconditioned *Elphidium williamsoni* for 3 hours (T_1) (blue) and 72 hours (T_2) (red) after the start of feeding experiment. Mean + 1SD observed are based on two points measurements (n = 2).

H. germanica displayed a slow and limited response to a single pulse of labelled *Navicula sp.* within the first hours of the incubation experiment. For instance, individuals cultured at pH 7.3 showed the highest uptake rate (0.16 ngC ind^{-1} h^{-1}) followed by treatments at pH 7.9 (0.15 ngC ind^{-1} h^{-1}) and pH 8.1 (ambient) (0.11 ngC ind^{-1} h^{-1}). In contrast, individuals cultured at pH 7.7 showed the lowest carbon uptake rate of 0.08 ngC ind^{-1} h^{-1}. Despite remarkable differences between treatments, there are no obvious trends related to the pH effect on uptake rates of incorporated ^{13}C by *H. germanica* (Fig. 5.8).

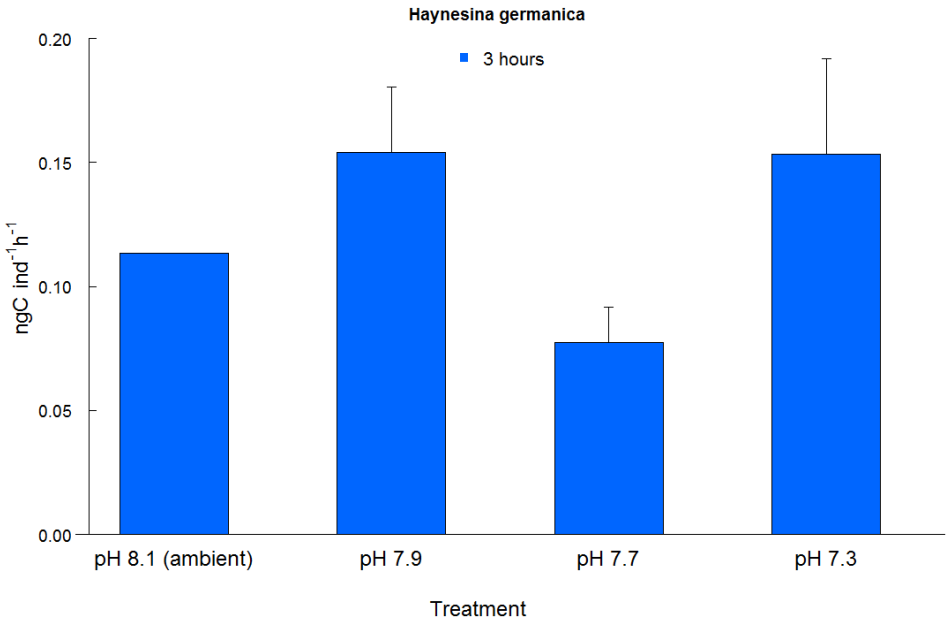

Figure 5. 8 Comparison of individual uptake rates of phytodetrital carbon (pC) by preconditioned *Haynesina germanica* for 3 hours (T_1) (blue) after the start of the feeding experiment. Mean + 1SD observed are based on two points measurements (n = 2).

In summary, the calculated carbon uptake rate by foraminifera differed significantly among species (Welch's test, $p < 0.05$) and incubation periods (Two-way ANOVA, $p = 0.003$, Table 5.5). However, pC did not differ significantly between pH treatments within each incubation period (One-way ANOVA, $p > 0.05$ (Fig. 5.7 and 5.8).

5.4 Discussion

Quantitative evidence of the potential impact of climate change (i.e. increased temperature and ocean acidification) on the important role played by foraminifera in the carbon cycling of coastal environments is fundamental to understanding how the dynamics of organic material processing and nutrients fluxes in benthic habitats may change in the next decades and centuries due to environmental perturbations.

In this study, laboratory findings suggest potential changes in the fate of organic carbon derived from fresh phytodetritus consumed by benthic foraminifera under future OA scenarios as detailed below.

5.4.1 The response of benthic foraminifera to a single pulse of labelled *Navicula sp.*

In general, the ingestion of ^{13}C-labelled diatoms by *E. williamsoni* and *H. germanica* was demonstrated by observing that δ^{13}C values from labelled samples exceeded respective laboratory background isotopic values for each pH treatment over the different incubation periods (Table 5.2). Furthermore, analysis of δ^{13}C data of foraminiferal cytoplasm indicates that *E. williamsoni* showed a remarkably positive and quick response to the single pulse of ^{13}C-labelled *Navicula sp.* in comparison to *H. germanica* which showed a much lower preference (i.e. one order of magnitude difference) for the same food source provided during the feeding experiment.

Usually, in highly populated habitats, feeding preferences may be beneficial for some species due to the high competitiveness for organic material and space (Enge et al. 2014). However, in this study, as *E. williamsoni* and *H. germanica* were independently managed

during the experimental period, the differential ingestion of labelled *Navicula sp.* observed between both foraminiferal species mainly within the first three hours strongly support the idea of food selectivity rather than a consequence of a potential foraminiferal interspecific competition for the same carbon sources.

The selective ingestion of organic carbon derived from phytodetritus by benthic foraminifera have been observed in species from the open sea (Heinz et al. 2001; Heinz et al. 2002; Moodley et al. 2002; Enge et al. 2014; Enge et al. 2016) to intertidal mudflats (Moodley et al. 2000; Wukovits et al. 2017; Wukovits et al. 2018). Thus, this feeding behaviour was expected to occur in our feeding experiment with the two dominant and co-occurring benthic species displaying similar feeding/sequestration mechanisms of primary production and also as a consequence of feeding structures compromised by the pre-exposure to high CO_2 and low pH levels.

5.4.2 Amount of organic ^{13}C and uptake rates by target benthic foraminifera

As the number of surviving specimens of *H. germanica* was lower than *E. williamsoni,* the amount of incorporated organic carbon and uptake rates for both species can only be reliably compared within the first 3 hours of incubation (Fig. 5.5 and 5.6). Thus, *E. williamsoni* showed carbon uptake rates of ^{13}C derived from *Navicula sp*. up to 10-fold higher compared to those observed in *H. germanica* across all pH treatments (Fig. 5.7 and 5.8). Similar differential feeding behaviour between *E. williamsoni* and *H. germanica* has been observed in a labelling experiment where lyophilized ^{13}C-labelled *Chlorella* was used as the main food supply (Moodley et al. 2000).

Although the incorporated amount of fresh diatom-derived ^{13}C increased significantly across all pH treatments mainly for *E. williamsoni* after 72 hours of the incubation period ($p < 0.05$) (Fig. 5.5), organic carbon uptake rates for *E. williamsoni* showed a significant decrease (up to 4-fold lower) in comparison with uptake rates recorded within the first 3 hours ($p < 0.05$) (Fig. 5.7). These results are consistent with earlier studies on *Ammonia tepida* which also displayed an opportunistic strategy by accumulating a large amount of organic carbon derived from a phytodetritus pulse within the first hours or days of incubation (Wukovits et al. 2017; Wukovits et al. 2018), with a subsequent decrease of uptake rate presumably due to either a limited storage capability or satiation.

These results clearly support previous findings on the feeding preference by co-occurring foraminiferal species (i.e. *A. tepida*, *H. germanica* and *E. williamsoni*) present in intertidal estuarine sediments (Moodley et al. 2000). The type, quantity and condition-derived quality of food source provided (i.e. fresh or aged material) (Sayles et al. 1994), and experimental conditions under which feeding experiments were performed (i.e. temperature, use of sediment, observations timing, etc) may also be factors influencing the observations of feeding preferences by benthic biota such as foraminifera (Pfannkuche et al. 1999; Rudnick 1989; Moodley et al. 2000; Moodley et al. 2002). In the present study, because of our research aim of providing evidence of alteration of benthic foraminiferal feeding efficiency caused by OA, foraminifera were fed with freshly collected labelled-diatoms in contrast to the use of lyophilized labelled-green algae by Moodley et al. (2000). These factors may have also partially contributed to the notable difference in magnitude of organic carbon incorporated by *E. williamsoni* over time between both studies.

In general, in this study, the observation of the high preference and fast processing of the fresh diatom *Navicula sp.* provided to *E. williamsoni* was limited to a maximum of 72 hours of incubation. Due to the limited number of live specimens available, it was not possible to incorporate more measurement points over time to observe when the amount of incorporated carbon and uptake rates by *E. williamsoni* start to level off as observed for intertidal foraminifer *Ammonia tepida* (Moodley et al. 2000; Wukovits et al. 2017; Wukovits et al. 2018).

The observations from the present work and other earlier studies (Moodley et al. 2000; Wukovits et al. 2017) on the low amount of labelled carbon ingested and uptake rates by *H. germanica* indicate this foraminifer definitely prefer another type of carbon sources (selective feeding) rather than *Navicula sp*.(this study) and *Chlorella* (Moodley et al., 2000). Additionally, Wukovits et al. (2017) also pointed out that in laboratory conditions, *H. germanica* has shown a limited preference for *D. tertiolecta,* suggesting a particular preference for secondary products of microphytobenthic biofilms or other diatoms. For instance, some studies of the diet of *H. germanica* showed a preferential consumption of phytodetritus due to the capacity for kleptoplasty (retention of functional chloroplasts derived mainly from diatoms within the foraminiferal cytoplasm). However, these earlier studies did not provide specific information on what type of diatom *H. germanica* consume (Knight & Mantoura 1985; Pillet et al. 2011; Jauffrais et al. 2016; Cesbron et al. 2017). In general, only a few studies have provided detailed information on the diatom-based diet of *H. germanica* that may help future feeding experiments; thus, pennate diatoms such as Nitzschia sp. (Hohenegger et al. 1989), *Pleurosigma angulatum* (Austin et al. 2005) and

Amphora coffeaeformis (Cevasco et al. 2015) have been identified as carbon sources for *H. germanica*.

Furthermore, low labelled carbon concentrations measured in the cytoplasm of *H. germanica* may also reflect a natural mechanism to reduce to the minimum the energy demand and metabolic activity under unfavourable food supply conditions as observed in *A. tepida* (LeKieffre et al. 2017). This singular capacity may render *H. germanica* more resilient to undertake longer-term starvation periods; giving to this foraminiferal species a potential ecological advantage over other benthic species such as *E. williamsoni.*

5.4.3 Ocean acidification and phytodetritus uptake by benthic foraminifera

Clearly, the results of differential ingestion of fresh ^{13}C-labelled *Navicula sp.* observed between *E. williamsoni* and *H. germanica* to a single food pulse in laboratory conditions suggest only an indication of food preference (selective feeding) by both foraminiferal species (Fig. 5.3 and 5.4). However, the observed differences in magnitude of organic carbon incorporated mainly by *E. williamsoni* over time (from 3 to 72 hours) across different pH treatments provide substantial evidence of the influence of short-term exposure to high CO_2 concentration and low pH in seawater upon the feeding experiments (Fig. 5.3). This experimental precondition, where feeding structures were compromised after the 6-week CO_2 experiment, may have broadly contributed to the alteration of feeding efficiency and subsequent differential organic carbon uptake by *E. williamsoni* as pH levels decreased (Fig. 5.5).

Understanding and quantifying the future impact of OA on the feeding ecology of two dominant benthic foraminiferal species such as E. *williamsoni* and *H. germanica* that represent up to 90% of the total foraminiferal assemblages from intertidal mudflats in the Eden Estuary, Scotland is undoubtedly challenging. Both foraminiferal species have displayed similar levels of damages in feeding structures after a short-term exposure to high CO_2/low pH levels (see Chapter 4 of this study), and it appears that under future OA scenarios, *E. williamsoni* is more likely to be affected by reducing its organic carbon uptake in comparison to *H. germanica;* the latter displays a higher resilience to environmental changes.

The reduction in the phytodetritus-derived carbon uptake of *E. williamsoni* at low pH may also be closely related to the altered growth/calcification rates experimentally observed in Chapter 4 of this study. These combined findings may provide relevant evidence of the potential ecological changes that occur in the population dynamics of benthic foraminifera and their key role in nutrient fluxes in natural settings, as previously described for intertidal mudflat habitats (Wukovits et al. 2018).

5.4.4 Limitations and future work

Despite the low number of surviving specimens at the end of the CO_2 experiment, the current ^{13}C-labelling feeding experiment carried out under laboratory conditions helped determine the differential response of two dominant foraminiferal benthic species from intertidal mudflats preconditioned to future scenarios of high CO_2 concentrations and low pH levels.

Although a time lag between food pulses and the ^{13}C incorporated by marine meiobenthos from a soft-bottom community is commonly observed (Rudnick 1989); in the present study, as the transfer of ^{13}C from labelled diatom *Navicula sp.* to foraminifera occurred within the first hours of the incubation, no considerable time lag was observed during the experiment. This observation supports the idea that our first measurement point of 3 hours after the incubation was enough to determine the initial organic carbon uptake by opportunistic foraminifera. However, future work could focus on increasing measurement points, and the length of the incubation period could be extended up to 7 days to observe when uptake rates by *E. williamsoni* and *H. germanica* start to level off. Furthermore, the number of replicates for each pH treatment for both species should also be increased. A larger number of replicates per pH treatment may help reduce the error due to the wide variation of δ13C values observed in the analysed samples. By collecting more sediment samples containing a larger number of specimens of both target benthic foraminifers, increased numbers of potential surviving specimens after the CO_2 experiments would be available.

As species of *Elphidium* and *Haynesina* apparently prefer to retain chloroplasts from specific diatom clades (Pillet et al. 2011), future work should be focused on culturing different types of local diatoms and determining individually which diatom is preferred by both *E. williamsoni* and *H. germanica.* In this study, despite the use the techniques of collection and culture of diatoms previously described for other feeding experiments with foraminifera (Austin, 2003, Khanna 2014), months before the start of the CO_2 experiment and subsequent feeding experiment, several attempts to culture a locally native species of

diatoms were not successful. Thus, the diatom species to be cultured should be carefully selected.

Finally, as heterotrophy (grazing) by benthic biota may constitute a notable loss pathway of primary producers in coastal environments (Middleburg et al. 2000), future feeding experiments should be continued at foraminiferal species level to observe species-specific responses (feeding preferences) to multiple carbon sources. This information is fundamental to understand the importance of some foraminiferal species and their role in a specific environment (Enge et al. 2014).

5.5 Conclusions

Based on the results of this short-term study on biological responses of multispecies assemblages of benthic foraminifera to OA, the following conclusions may be drawn:

(1) The species-specific responses observed between *E. williamsoni* and *H. germanica* to a single pulse of ^{13}C-labelled diatom *Navicula sp.* is mainly linked to the type of food source provided, suggesting that *H. germanica* may selectively prefer other carbon sources rather than *Navicula sp.*

(2) The observed differential amount of ^{13}C taken up mainly by *E. williamsoni* over time across pH treatments suggests a link between a reduced feeding efficiency and feeding structures compromised by OA effects. The alteration of the common foraminiferal feeding/sequestration mechanisms of primary production observed in short-term experiments may lead to changes in long-term ecological competitiveness between *E. williamsoni* and *H. germanica*

(3) Reduced carbon uptake as a consequence of an impact from OA may cause a potential shift in benthic community structures with negative impacts on the energy transferred within the benthic food web, carbon cycling and total $CaCO_3$ production within mid-latitude intertidal environments.

Chapter 6. The combined effect of increased temperature and elevated CO_2 concentration on benthic foraminifera growth and calcification. A case study of two independent laboratory experiments.

6.1 Introduction

Modern climate change, accelerated by the rising of the concentration of anthropogenic carbon dioxide (CO_2) in the atmosphere, has altered the global average temperature and pH of the surface ocean over the past two centuries (IPCC 2014). As these two major climatic factors of the Anthropocene interact simultaneously in the global ocean, a wide variety of biological responses with a synergistic, antagonistic or cumulative effect are expected to affect thresholds for an optimized physiological performance of marine biota (Doney 2010; Melatunan et al. 2011; Padilla-Gamiño et al. 2013). These changes could ultimately produce cascading effects on community composition, biomass, trophic dynamics and dominance of species across different biological levels of organization in marine habitats (Caldeira & Wickett 2003; Reynaud et al. 2003; Guinotte & Fabry 2008; Miller et al. 2009; Thomsen et al. 2010; Godbold & Solan 2013; Russell et al. 2013; Navarro et al. 2016; Celis-Plá et al. 2017; Keys et al. 2018).

Although the detection of a combined effect of multiple climatic factors may be more complex than those displayed by a single factor (Harley et al. 2006), the current challenge is focused on observations of more complex interactions between multiple climatic drivers and ecosystems to predict ecological consequences of future alterations in ocean productivity and ecosystem services (Boyd & Doney 2002; Guinotte & Fabry 2008; Dunne

2015; Keys et al. 2018), especially in coastal oceans where more severe changes in seawater pCO_2 and temperature are expected to occur in comparison to those projected for the open ocean (Miller et al. 2009; Thomsen et al. 2010; Ceballos-Osuna et al. 2013).

Despite the fact that coastal habitats continuously experience drastic fluctuations in environmental variables such as pCO_2 and temperature (Cai & Wang 1998; Wootton et al. 2008; Miller et al. 2009), it is believed that future OA and ocean warming may strongly influence the adaptive capacity (tolerance limits) of some coastal species (Harley et al. 2006; Wootton et al. 2008; Russell et al. 2013; Queirós et al. 2015), including calcifying organism such as benthic foraminifera.

To date, with exceptions (Dissard et al. 2010; Haynert et al. 2014; Engel et al. 2015; Meadows et al. 2015), most of the ecological laboratory and *in-situ* studies conducted on coastal benthic foraminifera have only focused on their biological responses to a single climatic factor; -either OA (Kuroyanagi et al. 2009; Allison et al. 2010; Allison et al. 2011; Keul et al. 2013; Khanna et al. 2013; Haynert et al. 2014; Khanna 2014; Prazeres et al. 2015; Pettit et al. 2015) or temperature (Wukovits et al. 2017; Wukovits et al. 2018). Little is known about how benthic foraminifera from coastal environments will respond to the interactive effects of OA and elevated temperatures projected by the end of the century. This is particularly true for foraminifera from intertidal environments, where this ecologically relevant information is still lacking or inconclusive (Meadows et al. 2015).

In the present study, to test the effect of combined (synergistic) effects of OA and slight temperature variation on foraminifera, a comparative framework was set-up to assess two

similar CO_2 laboratory experiments performed at different times using assemblages of the dominant benthic foraminifera, *E. williamsoni* and *H. germanica*. In both experiments, foraminiferal species were exposed to similar pH conditions but incubated at two different temperatures.

Throughout this chapter, datasets of several biological parameters of benthic foraminifera obtained from the present experiment under decreasing pH levels and a constant temperature of 15°C are described in detail. However, in the discussion section, only selected parameters of this chapter are compared and statistically analysed against similar datasets from another CO_2 experiment previously performed at 13°C (Chapter 4). The outcomes of this comparison between studies will help establish whether or not a small increase in temperature and declining pH influence growth/calcification rates of intertidal benthic foraminifera. It was hypothesized that a small increase in temperature of 2°C between studies in interaction with elevated CO_2 levels projected by the end of this century will cause a negative impact on the growth/calcification rates of benthic foraminifera

6.2 Materials and Methods

6.2.1 Field sampling and isolation of target foraminifera for an experiment at 15°C

Surface sediment scrapes from the top first centimetre were collected in late July 2017 at low tide from high intertidal mudflats of the Eden Estuary, N.E. Scotland (56°22'N, 2°50 W) (Chapter 2, Fig. 2.1). On return to the laboratory, all sediment samples were mixed and sieved over a set of 75 µm and 425 µm screens. The sieved sediment fraction (bulk sieved sediment) was left to settle in big plastic trays for three hours, and direct observation of a small amount of sieved sediment through a stereoscopic binocular microscope confirmed that living foraminiferal specimens of *E. williamsoni* and *H. germanica* were present in densities of 10-20 'live' specimens/cm^3 and 1-5 'live' specimens/cm^3, respectively.

Subsequently, the sieved sediment with living foraminiferal assemblages of both species was used for calcein incubation as described below. Detailed information on sampling, foraminiferal identification and isolation is outlined in Chapter 2.1 and 2.2.

6.2.2 Foraminiferal calcein incubation

In this study, calcein incubation was carried out following most of the procedures detailed in Chapter 4, Section 4.2.2. In order to ensure a larger number of surviving specimens at the end of this calcein incubation, living foraminiferal assemblages of *E. williamsoni* and *H. germanica* were incubated in the same natural sediment in which they were collected (Fig. 6.1). For this experimental design, approx. 100 cm^3 of mixed sediment was placed in a series of 5-litre plastic containers. The total number of specimens used for this experiment

was approx. 30,000 ‘live’ specimens. These plastic containers were filled with filtered natural ~33 salinity seawater containing a final concentration of 20 mg/L of the fluorescent marker calcein (Bernhard et al. 2004; Dissard et al. 2009; Dissard et al. 2010). Each container was sealed with a lid with two inlets on top, one for air tubing and other to allow seawater with calcein to be continually recirculated into and out of the container through a 1 L reservoir glass bottle containing seawater. Multi-channel peristaltic pumps controlled the flow between the experimental flasks and the calcein bottles.

Unlike the previous calcein incubation carried out in Chapters 3 and 4, the present seawater calcein incubation was kept inside the temperature-controlled room at 15°C for 4 weeks with a light condition of 12:12-hr light: dark cycle (Fig. 6.1 A). This temperature of 15°C (+2°C higher than previous experiments of this study) allowed an opportunity to determinate how small increases in temperature of up to 2°C also projected to occur by the end of this century in Scottish seawaters (Hiscock et al. 2001) may affect several biological parameters of foraminiferal assemblages. Furthermore, this slight increase in temperature of 2°C was also selected because it is part of the range of variation of temperature observed in the sampling area in July.

Finally, filtered seawater with calcein was changed once a week. Fortnightly sampling observations through a fluorescence microscope provided information on incorporation processes of calcein into the new growth of foraminiferal tests (Fig. 6.1 B).

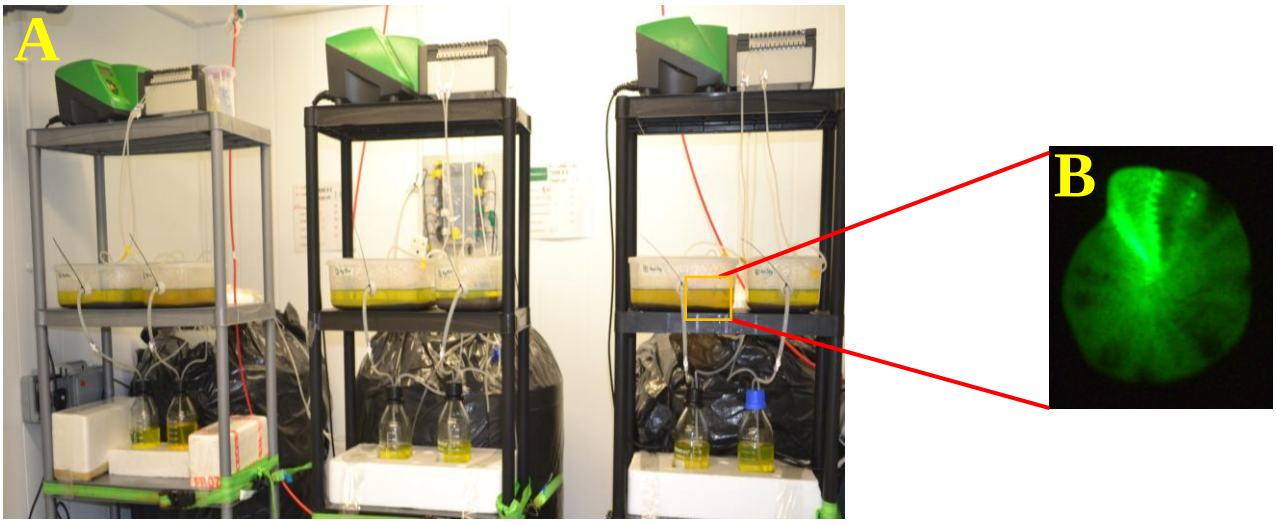

Figure 6. 1 A) Seawater recirculating system used for calcein incubation of *Elphidium williamsoni* and *Haynesina germanica* under controlled conditions of 15°C for 4 weeks with a light condition of 12:12-hr light: dark cycle.

6.2.3 Experimental conditions

When the calcein incubation period was concluded (4 weeks), viable ('live') specimens of *E. williamsoni* and *H. germanica* with calcein labelled pre-existing chambers were collected, including ~3,100 specimens of *E. williamsoni* and 2,000 specimens of *H. germanica*. Subsequently, mixed assemblages containing ~260 specimens of *E. williamsoni* and ~50 specimens of *H. germanica* were randomly selected and placed in each foraminiferal culturing chamber, as detailed in Chapter 2, Section 2.4 (Hintz et al. 2004; Allison et al. 2010; Allison et al. 2011; Khanna 2014).

A total of 310 specimens (corresponding to a mix of both species) were placed into each chamber, with a total of 12 culture chambers set up for this experiment. Four culturing chambers (replicates) with the same number of foraminifera inside were used for each pH

treatment (pH~8.1 (ambient), pH 7.7 and pH 7.3), and these were subsequently connected to a manipulative mesocosm, as described in Chapter 2, Section 2.4.

Prior to the CO_2 experiment, a 10 days acclimation period for foraminifera over which time the seawater was pH gradually reduced until each treatment reached its required pH level/pCO_2, ensuring that the measured foraminiferal responses were due to the pH treatments and not a shock response. After this time period, foraminiferal culturing chambers were maintained for 6 weeks in a controlled recirculating seawater system within a temperature-controlled room at 15°C with a 12:12-hr light: dark cycle.

Unlike the previous experimental conditions performed in Chapters 3-5, due to some changes in the space available within the experimental facilities (remodelling work), meant that foraminifera were exposed to a short-term experiment involving only 3 different pH conditions: pH~8.1 (ambient), pH 7.7 and pH 7.3.

During the calcein incubation and throughout the entire experiment, the foraminifera were fed weekly with ~10µL/cm^2 of each of the algae *Dunaliella tertiolecta* and *Rhodomonas salina* (typically 1×10^7 cells ml^{-1}).

6.2.4. Biological parameters

After completing the experiment at 15°C, all chambers were opened; foraminifera were picked out, transferred into clean petri dishes, and all biological parameters were determined following the procedures detailed in Chapter 4, Section 4.2.4

All specimens were randomly sub-sampled from the natural population in the field and there was no systematic size different at the start of both the present study at 15°C and the earlier experiment conducted at 13°C (Chapter 4). This facilitated the comparison of biological parameters (i.e. size, weight and chambers addition) measured on foraminifera in both experiments which were potentially affected by the combined effects of OA and elevated temperature (see Discussion section).

6.2.6 Statistical Method

The assumptions of homogeneity of variance and normality of datasets for both studies were independently assessed by Shapiro-Wilk and Levene tests. Subsequently, either a one-way ANOVA (parametric analysis) or Kruskal-Wallis rank sum (non-parametric) test was performed when the assumptions of normality and homogeneity for datasets were matched with values of $p > 0.05$ or $p < 0.05$, respectively. A Tukey test and Dunn's-test for multiple comparisons were applied following the one-way ANOVA and Kruskal-Wallis results, respectively. These additional tests helped determine any significant differences between the pH treatments

To detect the combined effects of pH (three levels) and temperature (two levels) on biological parameters for each foraminiferal species, datasets of both experimental studies for each biological parameter were combined and homogeneity of variance and normality were assessed by Shapiro-Wilk and Levene tests. When datasets matched values of $p < 0.05$, data was transformed following the steps for Aligned Rank Transform (ART) for nonparametric analyses of variance on factorial models (Wobbrock et al. 2011; Kay &

Wobbrock 2018), and a two-way ANOVA was performed on the transformed datasets. The statistical significance level of 95% ($p < 0.05$) was set for all tests. Temperature and pH were considered as independent factors; however, size, weight and number of chambers and growth rate were considered as dependent variables.

6.3 Results

6.3.1 Composition of dominant foraminiferal species from field samples

Foraminiferal assemblages of field samples collected in July 2017 for an experiment at 15°C showed that both target species were again the two dominant (~ 90%) in comparison to other foraminiferal species. However, *E. williamsoni* exhibited a much higher number of living individuals (20-30 living specimens/cm^3 of wet sediment) than *H. germanica* (5-10 living specimens/cm^3 of wet sediment). Living assemblages of both foraminiferal species were used for this experimental study.

6.3.2 Seawater carbonate chemistry at 15°C

Measurements of seawater carbonate parameters taken on a fortnightly basis indicate that these parameters were constant throughout the entire 52 day-time period of the CO_2 experiment (Table 6.1).

Table 6. 1 Seawater measurements taken fortnightly from the experimental carbonate chemistry manipulation system. Values account for mean ± SD, n = 3.

Measured parameters					**Calculated parameters**					
Treatment	**pH (Total)**	**T (°C)**	**Salinity (ppt)**	**AT (µmol/Kg)**	**DIC (µmol/kg)**	***p*CO2 (µatm)**	**HCO_3^- (µmol/kg)**	**CO_3^{2-} (µmol/kg)**	**$\Omega_{Calcite}$**	**$\Omega_{Aragonite}$**
pH 8.1 (ambient)	8.13 ± 0.02	15.20	33.57 ± 0.08	2609.07 ± 7.55	2327.91 ± 14.27	367.01 ± 16.08	2107.63 ± 18.91	206.55 ± 5.81	4.97 ± 0.14	3.19 ± 0.09
pH 7.7	7.73 ± 0.02	15.12 ± 0.03	33.46 ± 0.05	2591.34 ± 31.67	2499.03 ± 28.24	1038.44 ± 37.60	2368.41 ± 26.12	91.67 ± 3.83	2.21 ± 0.09	1.41 ± 0.06
pH 7.3	7.33 ± 0.01	15.05 ± 0.06	33.46 ± 0.05	2585.37 ± 19.40	2635.11 ± 21.46	2770.13 ± 95.45	2493.05 ± 19.57	37.94 ± 0.98	0.91 ± 0.02	0.59 ± 0.02

6.3.3 Reproduction events at 15°C

At the end of the CO_2 experiment at 15°C, specimens from all culturing chambers were collected and only 3566 individuals of *E. williamsoni* and 1901 individuals of *H. germanica* were retrieved. There was an increase from the initial number of specimens of *E. williamsoni* used in each pH treatment, presumably due to a reproduction event during the experimental period. For *H. germanica,* however, there was a loss of 41 individuals at a pH of 8.1 and 22 individuals at a pH of 7.3. The increased number of specimens observed at a pH of 7.7 was also presumably due to a reproduction event (Table 6.2). As the new specimens did not display calcein labelling, they were not considered for further analysis and were labelled as "dead" specimens.

6.3.4 'Live' and 'dead' foraminiferal individuals and survival rate at 15°C

For *E. williamsoni*, individuals cultured at a pH of 8.1 (ambient) showed the largest number of surviving specimens (n_{Live} = 246) followed by the treatment at a pH of 7.7 (n_{Live} = 189). In contrast, individuals cultured at a pH of 7.3 showed the lowest number of surviving specimens (n_{Live} = 50). The number of 'live' individuals in each treatment follows a decreasing trend as the pH level decreased. Furthermore, the mortality rate, presumably as a response to OA and temperature effects, increased from 7.8 to 19.5 % when the seawater pH decreased from 7.7 to 7.3 (Table 6.2).

For *H. germanica*, individuals cultured at a pH of 7.7 (n_{Live} = 222) showed the largest number of surviving specimens followed by the treatment at a pH of 8.1 (ambient) (n_{Live} = 200). In contrast, individuals cultured at a pH of 7.3 showed the lowest number of surviving

specimens (n_{Live} = 115). The survival rate shows a decreasing trend in response to a lowering pH level; consequently, the mortality rate presumably linked to OA effects increased from 2.4 to 16.3% when the seawater pH decreased from 7.7 to 7.3 (Table 6.2).

Table 6. 2 Survival rates (SR) of *Elphidium williamsoni* and *Haynesina germanica* cultured for 52 days under different pH conditions: pH~8.1 (ambient), pH 7.7 and pH 7.3.

	Total number of individuals					
Species and treatment	**Start of experiment**	**End of experiment**		**Survival rate (%)**	**Total Mortality rate (%)**	**Mortality rate by OA (%)**
		Retrieved/ Analyzed	**Alive (Chambers added)**			
E. williamsoni						
pH 8.1 (ambient)	1012	1053	246	23.4	76.6	0.0
pH 7.7	1070	1211	189	15.6	84.4	7.8
pH 7.3	1041	1303	50	3.8	96.2	19.5
H. germanica						
pH 8.1 (ambient)	620	579	200	34.5	65.5	0.0
pH 7.7	660	691	222	32.1	67.9	2.4
pH 7.3	653	631	115	18.2	81.8	16.3

An improved visualization of the dataset presented in Table 6.2 is shown in Fig. 6.2 for individuals analysed (n_{Total}) and 'live' specimens (n_{Live}) of *E. williamsoni (*A) and *H. germanica* (B).

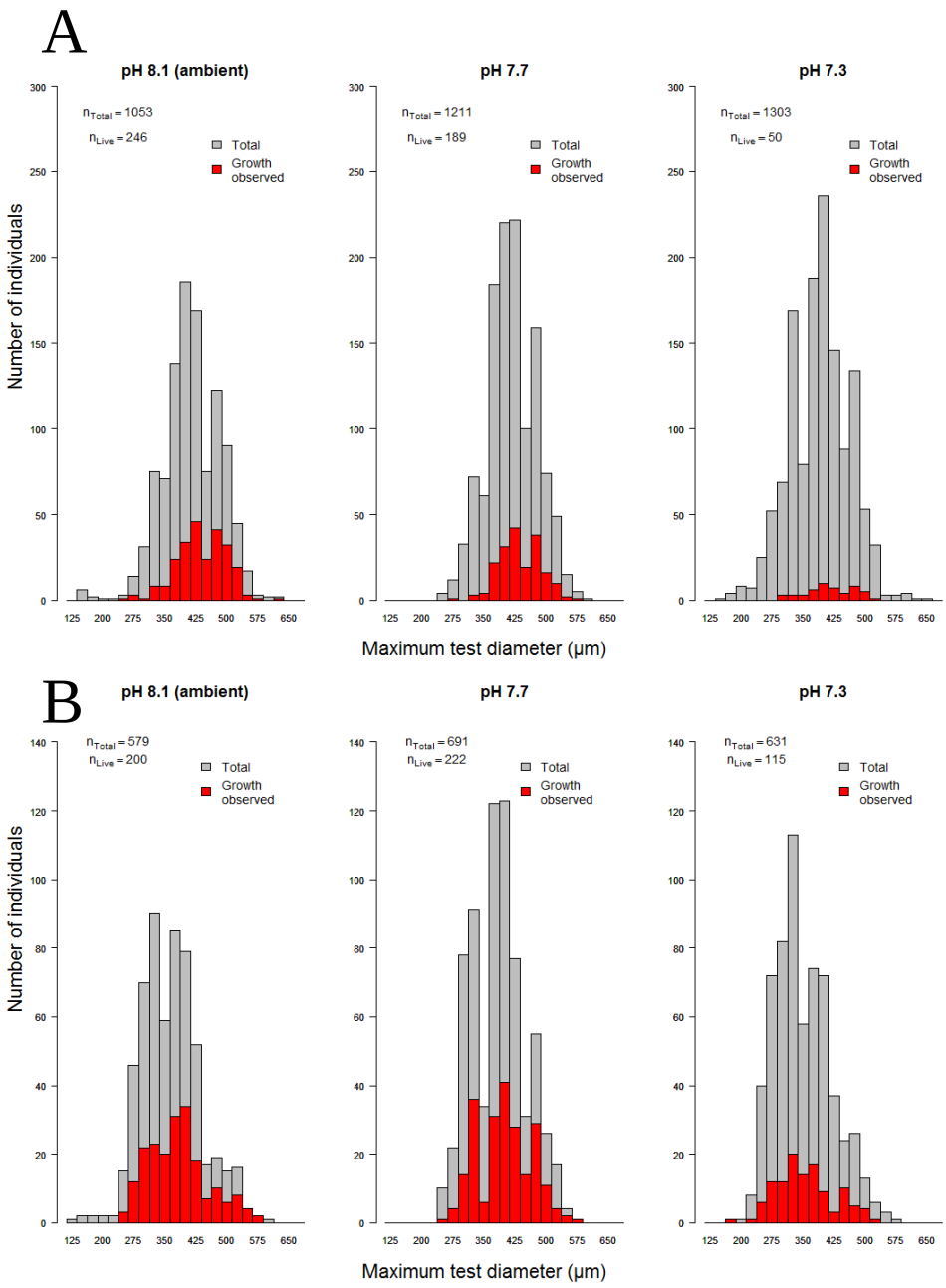

Figure 6. 2 The total number of individuals of (A) *Elphidium williamsoni* and (B) *Haynesina germanica* sorted into size classes after being collected at the end of the experimental period from each culture condition (pH~8.1 (ambient), pH 7.7 and pH 7.3). The individuals analysed (n_{Total}) and 'live' specimens (n_{Live}) observed are shown in grey and red, respectively. Bandwidth for each size class was 25 µm.

6.3.5 Biological parameters at 15°C

Table 6.3 illustrates maximum test diameter, weight and the number of new chambers added to 'live' specimens with intact tests for both foraminiferal species.

Table 6. 3 Maximum test diameter, weight and new chambers added of 'live' *Elphidium williamsoni* and *Haynesina germanica* cultured under different experimental pH conditions: pH~8.1 (ambient), pH 7.7 and pH 7.3.

Measured variables	pH conditions	Min	Max.	Mean	Standard deviation (1σ)	Standard error of mean	n
E. williamsoni							
Maximum test diameter (µm)	8.1 (ambient)	279.80	587.60	453.03	56.15	3.93	204
	7.7	335.80	587.60	449.58	46.50	3.58	169
	7.3	321.80	531.60	434.47	58.78	9.80	36
Test weight (µg)	8.1 (ambient)	3.00	28.67	12.84	4.78	0.34	204
	7.7	7.00	30.00	14.47	4.65	0.36	169
	7.3	4.50	22.50	11.72	4.46	0.74	36
Number of chambers added	8.1 (ambient)	1.00	11.00	4.03	1.99	0.14	204
	7.7	1.00	7.00	2.86	1.64	0.13	169
	7.3	1.00	6.00	2.42	1.44	0.24	36
H. germanica							
Maximum test diameter (µm)	8.1 (ambient)	265.80	587.60	393.40	70.54	5.65	227
	7.7	279.80	587.60	415.00	61.58	4.65	107
	7.3	195.90	531.60	373.90	73.60	8.61	32
Test weight (µg)	8.1 (ambient)	2.50	21.67	8.77	3.85	0.31	227
	7.7	3.00	27.00	11.64	4.21	0.32	107
	7.3	3.00	21.00	7.66	3.39	0.57	32
Number of chambers added	8.1 (ambient)	1.00	8.00	3.74	1.50	0.12	227
	7.7	1.00	7.00	3.31	1.25	0.09	107
	7.3	1.00	7.00	2.85	1.31	0.15	32

6.3.6 Foraminiferal size, weight and chamber addition

For 'live' *E. williamsoni* and *H. germanica*, the highest numbers of individuals distributed in larger size, weight and chambers addition classes were found in the treatment at a pH of 8.1 (ambient) followed by the treatment at a pH of 7.7. In contrast, individuals cultured at a pH of 7.3 showed the lowest number of size, weight and chamber addition classes (Fig. 6.3 and 6.4). In general, these results suggest that biological parameters sorted into classes were affected by declining pH at 15°C.

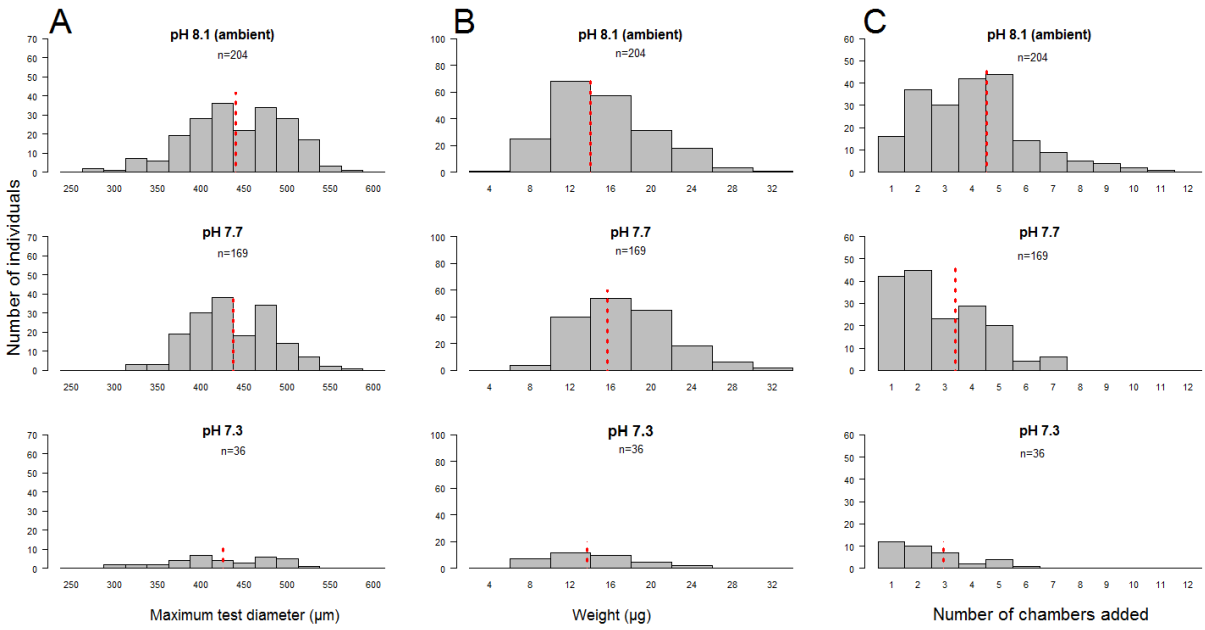

Figure 6. 3 Distribution of 'live' *Elphidium williamsoni* in relation to A) maximum test diameter, B) test weight and C) number of chambers added for each culture condition (pH 8.1 (ambient), pH 7.7 and pH 7.3). Individuals were sorted into groups of different bandwidths for each parameter. The bandwidth was equal to 25 µm for each size class, 4 µg for each weight class and 1 for each new chamber added class. The dashed red line represents the mean value of measurements within each pH treatment.

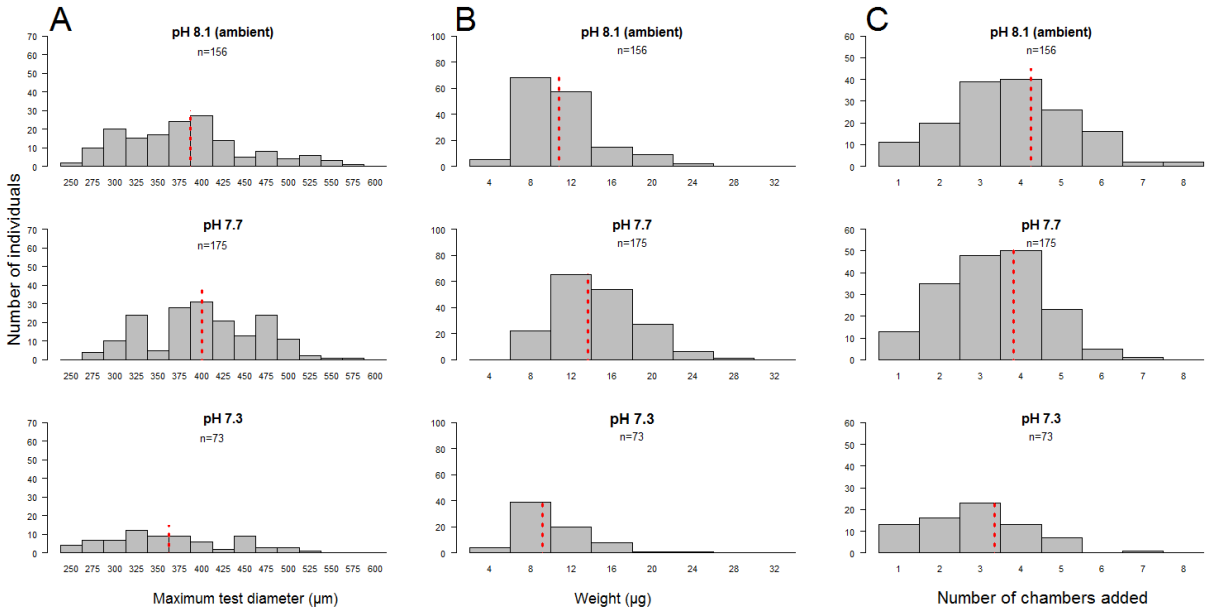

Figure 6. 4 Distribution of 'live' *Haynesina germanica* in relation to A) maximum test diameter, B) test weight and C) number of chambers added for each culture condition (pH 8.1 (ambient), pH 7.7 and pH 7.3). Individuals were sorted into groups of different bandwidths for each parameter. The bandwidth was equal to 25 µm for each size class, 4 µg for each weight class and 1 for each new chamber added class. The dashed red line represents the mean value of measurements within each pH treatment.

6.3.7 Foraminiferal mean maximum test diameter (size) of 'live' specimens

For 'live' *E. williamsoni*, individuals cultured at a pH of 8.1 (ambient) showed the largest mean maximum test diameter followed by a pH of 7.7. In contrast, individuals cultured at a pH 7.7 showed the lowest mean maximum test diameter (Fig. 6.5 and Table 6.3). Shapiro-Wilk and Levene tests verified the normality (p = 0.009485) and homogeneity (p = 0.01938) of the maximum test diameter dataset. A Kruskal-Wallis test indicates there is no statistically significant effect of pH treatments at 15°C on mean foraminiferal size (p =

0.2058). The Dunn's-test (pairwise comparison) also indicates no significant difference between the pH 8.1 treatment (group “a”) and the remaining pH treatments (Fig. 6.5).

For ‘live’ *H. germanica*, individuals cultured at a pH of 7.7 showed the largest mean maximum test diameter followed by the treatment at a pH of 8.1 (ambient). In contrast, individuals cultured at a pH of 7.3 showed the smallest mean size (Fig. 6.5 and Table 6.3). Shapiro-Wilk and Levene tests verified the normality (p = 0.01072) and homogeneity (p = 0.3295) of the maximum test diameter dataset. A Kruskal-Wallis test indicates there is a statistically significant difference in foraminiferal size between pH treatments (p = 3.029×10^{-5}). The Dunn's-test (pairwise comparison) also indicates a significant difference between the treatment at a pH of 7.7 (group “b”) and the remaining pH treatments (group “a”) (Fig. 6.5).

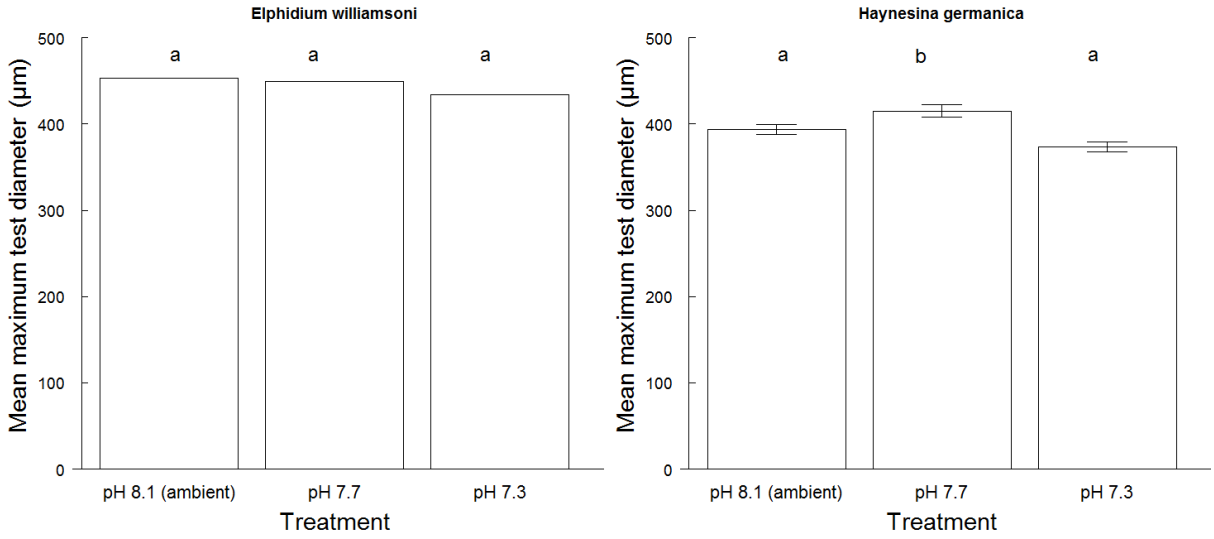

Figure 6. 5 Mean values (± standard error) of maximum test diameter (µm) for 'live' *Elphidium williamsoni* and *Haynesina germanica* cultured at different pH conditions. Treatments with significant differences to each other are indicated by different letters above bars (i.e. **a** and **b**) at $p < 0.05$ observed between groups according to the Dunn's-test.

6.3.8 Foraminiferal weight

For *E. williamsoni*, individuals cultured at a pH of 7.7 showed the heaviest mean weight followed by the treatments at a pH of 8.1 (ambient). In contrast, individuals cultured at pH 7.3 showed the lowest weight (Figure 6.6 and Table 6.3). Shapiro-Wilk and Levene tests verified the normality ($p = 1.38 \times 10^{-5}$) and homogeneity ($p = 0.91$) of the test weight dataset. A Kruskal-Wallis test indicates there is a statistically significant difference in foraminiferal weight between pH treatments at 15°C ($p = 3.457 \times 10^{-6}$). The Dunn's-test (pairwise comparison) also indicates that the pH 7.7 (group "b") differ significantly from the pH 8.1 (ambient) and pH 7.3 treatments (group "a") (Fig. 6.6).

For *H. germanica*, individuals cultured at a pH of 7.7 showed the heaviest mean weight followed by the treatments at a pH of 8.1 (ambient). In contrast, individuals cultured at a pH of 7.3 showed the lowest weight (Fig. 6.6 and Table 6.3). Shapiro-Wilk and Levene tests verified the normality (p = 7.569x^{-10}) and homogeneity (p = 0.03493) of the test weight dataset. A Kruskal-Wallis test indicates there is a statistically significant difference in foraminiferal weight between pH treatments at 15°C (p = 2.2x10^{-16}). The Dunn's-test (pairwise comparison) also indicates that the pH 7.7 (group "b") differ significantly from the pH 8.1 (ambient) and pH 7.3 treatments (group "a") (Fig. 6.6).

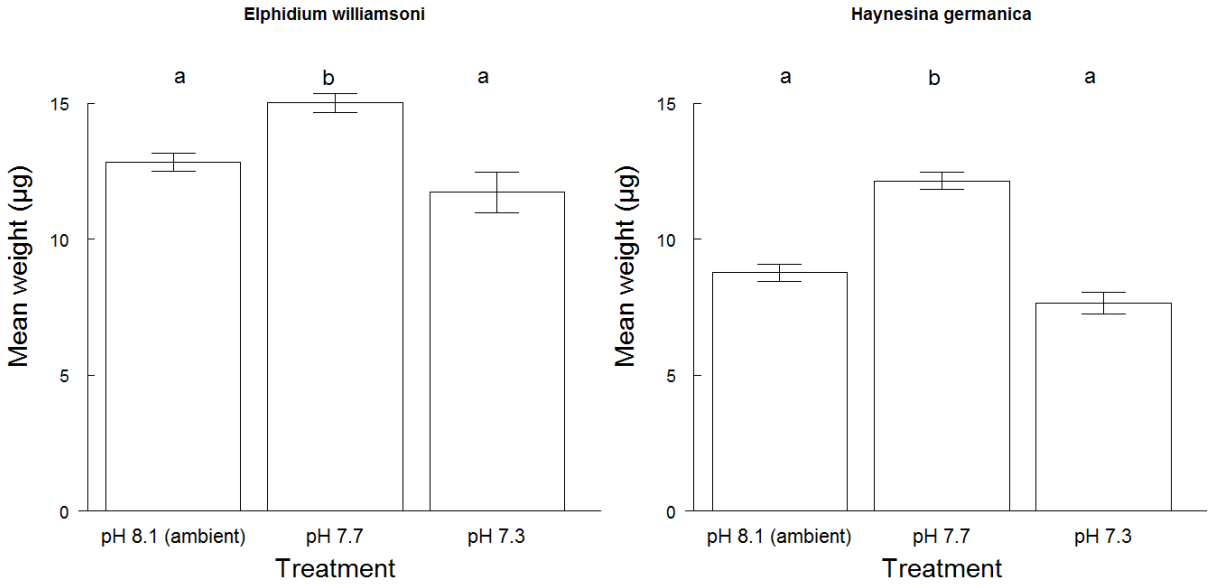

Figure 6. 6 Mean values (± standard error) of weight (µg) for *Elphidium williamsoni* and *Haynesina germanica* cultured at different pH conditions. Treatments with significant differences are indicated by different letters above bars (i.e. **a** and **b**) at $p < 0.05$ observed between groups according to the Dunn's-test.

6.3.9 Newly deposited chambers

For *E. williamsoni*, individuals cultured at a pH of 8.1 (ambient) showed the largest number of new chambers deposited (n = 4.03) followed by the treatment at a pH of 7.7 (n = 2.86). In contrast, individuals cultured at a pH of 7.3 showed the lowest number of new chambers added (n = 2.42) (Fig. 6.7 and Table 6.3). Shapiro-Wilk and Levene tests verified the normality (p = 2.813x10^{-14}) and homogeneity (p = 0.1149) of the test weight dataset. A Kruskal-Wallis test indicates there is a statistically significant effect of pH treatments at 15°C on foraminiferal chamber addition (p = 1.271x10^{-10}). The Dunn's-test (pairwise comparison) also indicates that the pH 8.1 (groups "a") (ambient) differ significantly from the pH 7.7 and pH 7.3 treatments (group "b") (Fig. 6.7).

For *H. germanica*, individuals cultured at a pH of 8.1 (ambient) showed the largest number of new chambers deposited (n = 3.74) followed by the treatment at a pH of 7.7 (n = 3.31). In contrast, individuals cultured at a pH of 7.3 showed the lowest weight (n = 2.85) (Fig. 6.7 and Table 6.3). Shapiro-Wilk and Levene tests verified the normality (p = 4.618x10^{-11}) and homogeneity (p = 0.1461) of the test weight dataset. A Kruskal-Wallis test indicates there is a statistically significant effect of pH treatments at 15°C on foraminiferal chamber addition (p = 4.533e-05). The Dunn's-test (pairwise comparison) also indicates that the pH 8.1 (group "a") differ significantly from the treatments at a pH of 7.7 (group "b") and pH 7.3 (group "c") (Fig. 6.7).

In general, the number of new chambers deposited by *E. williamsoni* and *H. germanica* showed a decline with decreasing pH at 15°C.

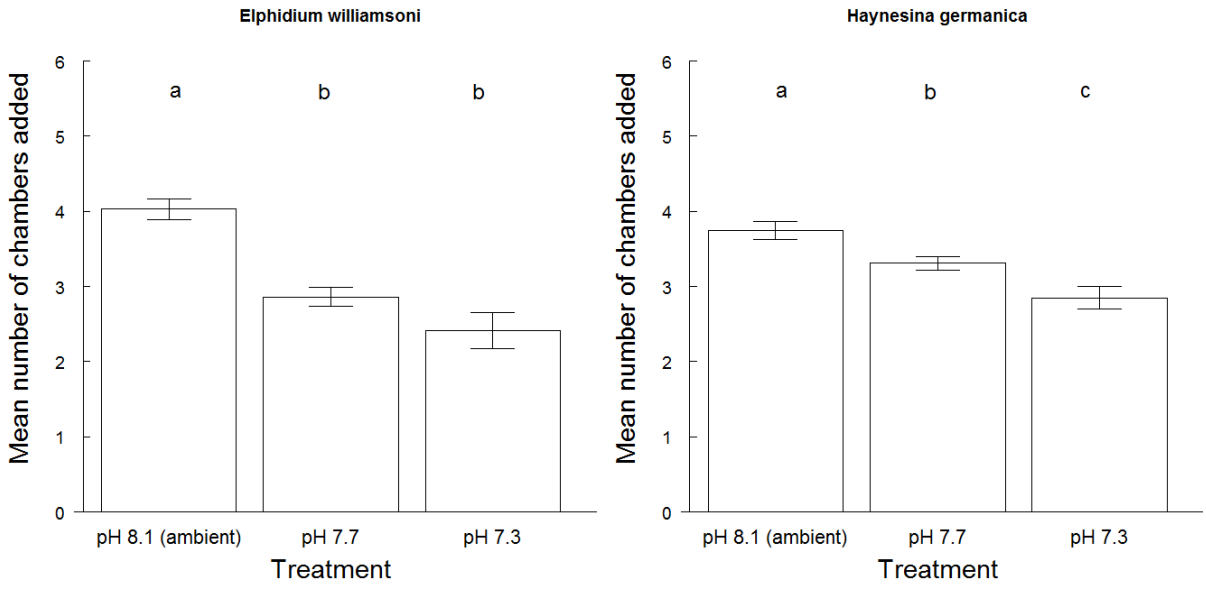

Figure 6. 7 Mean values (± standard error) of newly formed chambers for *Elphidium williamsoni* and *Haynesina germanica* cultured at different pH conditions. Treatments with significant differences are indicated by different letters above bars (i.e. "a", "b" and "c") at $p < 0.05$ observed between groups according to the Dunn's-test.

6.3.10 Growth rate

Mean growth rates (chambers day^{-1}) were calculated based on the average number of new chambers added during the experimental period of 52 days; in general, mean growth rates of *E. williamsoni* and *H. germanica* declined with decreasing pH at 15°C. A Kruskal-Wallis test and the Dunn's-test (pairwise comparison) indicate significant differences between pH treatments for each foraminiferal species (Fig. 6.8).

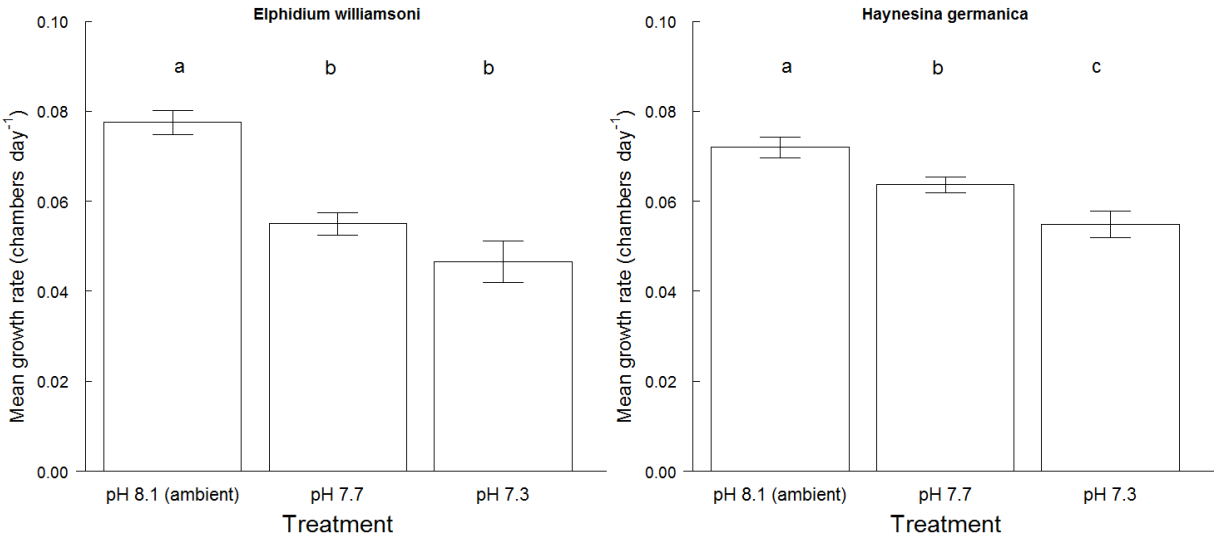

Figure 6. 8 Mean values (± standard error) of mean growth rates (chambers day^{-1}) for *Elphidium williamsoni* and *Haynesina germanica* cultured at different pH conditions. Treatments with significant differences are indicated by different letters above bars (i.e. "a", "b" and "c") at $p < 0.05$ observed between groups according to the Dunn's-test.

6.3.11 Foraminiferal Size-Normalized test Weight (SNW)

For *E. williamsoni* and *H. germanica*, individuals cultured at a pH of 7.7 showed the highest mean Size-Normalized test Weight (SNW) followed by the treatment at a pH of 8.1 (ambient). In contrast, individuals cultured at a pH of 7.3 showed the lowest SNW (Fig. 4.10). A Dunn's-test (pairwise comparison) indicates that the pH 7.7 (group "b") differ significantly from the pH 8.1 (ambient) and pH 7.3 treatments (group "a") (Fig. 6.9)**.**

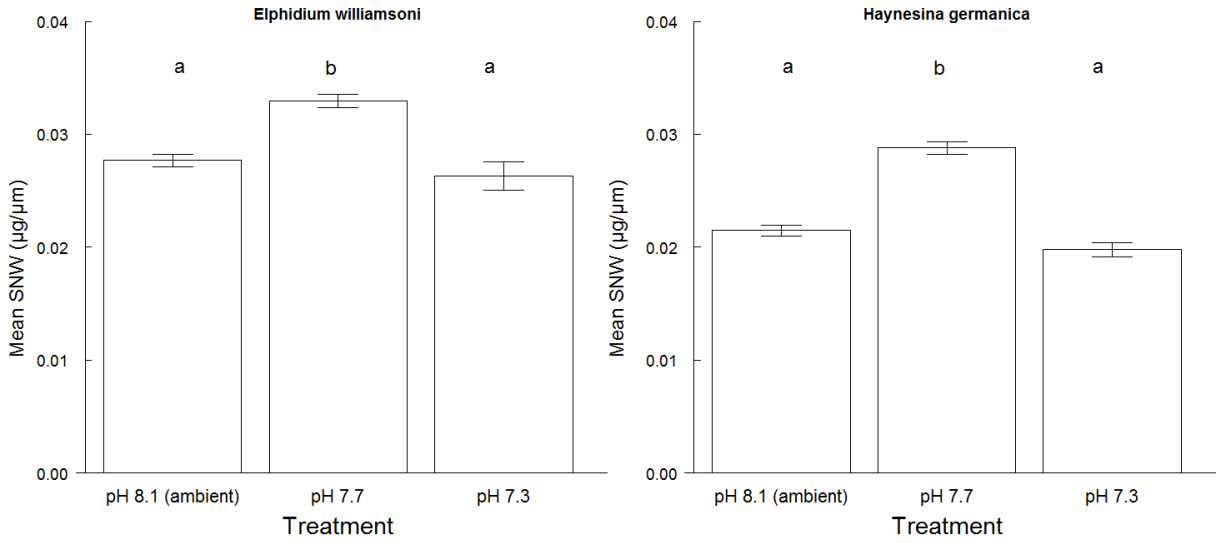

Figure 6. 9 Mean values (± standard error) of Size-Normalized test Weight (SNW) for *Elphidium williamsoni* and *Haynesina germanica* cultured at different pH conditions. Treatments with significant differences are indicated by different letters above bars (i.e. a and b) at $p < 0.05$ according to the Dunn's-test.

6.3.12 SEM observations of the morphological response of 'live' *E. williamsoni*

SEM images of intact tests of 'live' *E. williamsoni* showed a progressive alteration of the foraminiferal morphology (test) when individuals were exposed to high CO_2 concentrations/low pH for 52 days at 15°C. The most remarkable features observed on the test surface are the presence of cracks and signs of dissolution on individuals exposed to the low pH levels (Fig. 6.10, C-F). Specimens cultured at a pH of 7.7 and 7.3 clearly displayed dissolution around the apertural region, notably on apertural teeth where these structures are extremely rounded and with clear signs of corrosion in comparison to those cultured at pH 8.1, which exhibited conical, sharp apertural teeth and smooth surfaces (Fig 6.10 A).

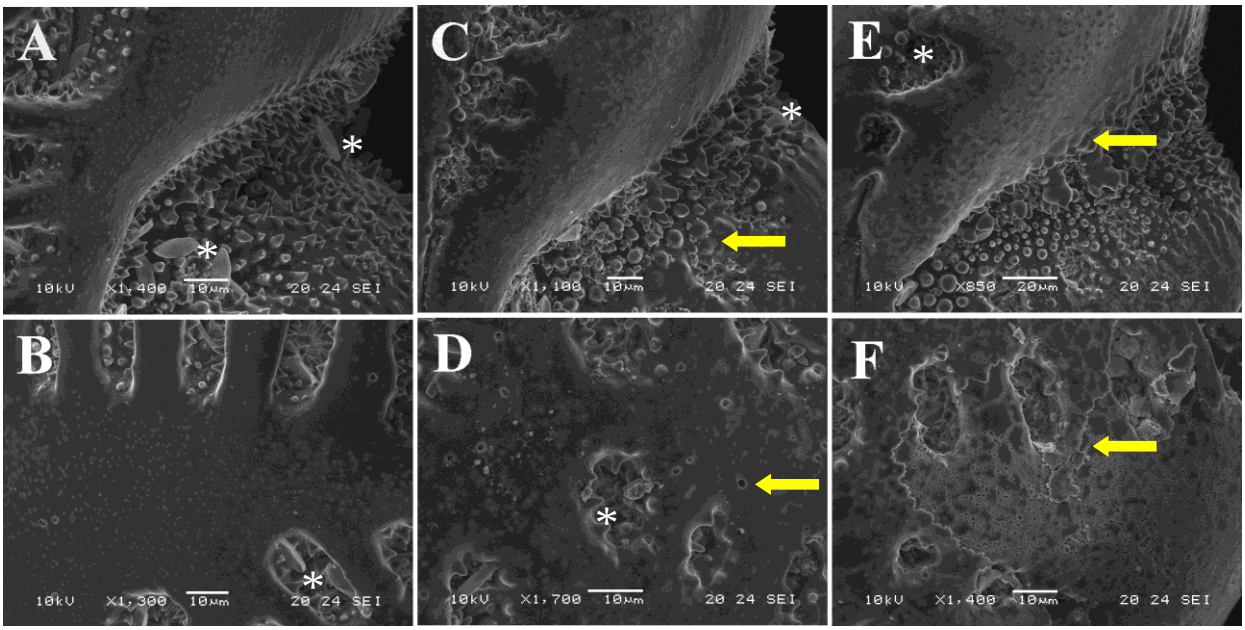

Figure 6. 10 Scanning electron micrographs (SEM) images of 'live' specimens of *Elphidium williamsoni* cultured at pH 8.1 (A & B), pH 7.7 (C & D) and pH 7.3 (E & F). (A) SEM image of side view of the apertural region showing numerous sharp teeth and tubercles with impaled frustules of the diatom *Navicula sp*. (*) are visible. (B) A side view of test surface of specimen A. (C) SEM image of a side view of the apertural region, showing numerous rounded teeth and tubercles. Some frustules of the diatom *Navicula sp*. (*) are visible. (D) A side view of corroded test surface of specimen C. (E) SEM image of a side view of the apertural region showing clearly a reduction in the number of teeth and tubercles, and also with signs of dissolution and cracking of the test. Teeth and tubercles are less sharp and extremely rounded. Some frustules of the diatom *Navicula sp*. (*) are observed. (F) A side view of the test surface of specimen E, exhibiting corroded septal bridges and sutures. (←) Yellow arrows highlight areas affected by dissolution.

6.3.13 SEM observations of the morphological response of 'live' *H. germanica*

SEM images of intact tests of 'live' *H. germanica* showed a progressive alteration of the foraminiferal morphology when individuals were exposed to high CO_2 concentrations/low pH for 52 days at 15°C. The most outstanding features observed on the test surface are the

presence of cracks, asymmetric and larger test pores with clear signs of dissolution on individuals exposed to all pH levels including a pH of 8.1 (ambient) (Fig. 6.11, A-F). Specimens cultured at pH 7.7 and 7.3 clearly displayed dissolution and cracks around the apertural region, notably on the apertural teeth and tubercles which are extremely corroded. In addition, test surfaces and sutures showed clear signs of corrosion in comparison to those cultured at a pH of 8.1 (ambient) which generally exhibited smooth surfaces and regular shapes of these structures (Fig 6.11, A & B).

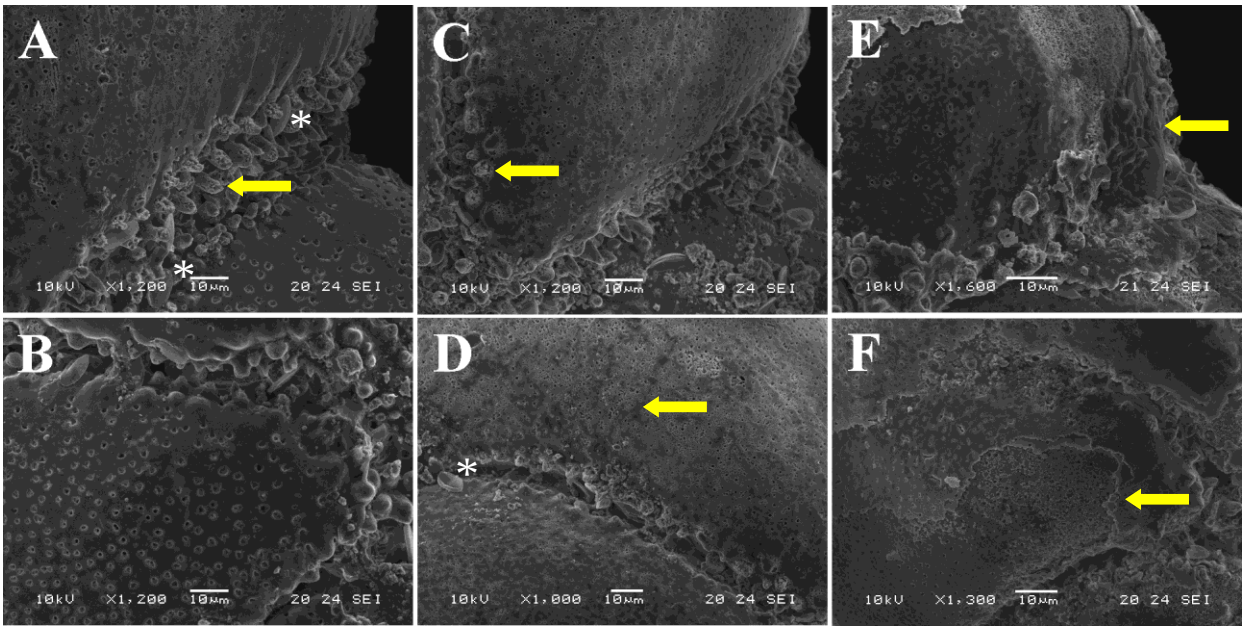

Figure 6. 11 Scanning electron micrographs (SEM) images of 'live' specimens of *Haynesina germanica* cultured at pH 8.1 (A & B), pH 7.7 (C & D) and pH 7.3 (E & G). (A) SEM image of side view of the apertural region showing slight signs of corrosion on numerous teeth and tubercles. Frustule of the diatom *Navicula sp*. (*) and organic detritus are visible on ornamentation. (B) A side view of the test surface of specimen A. (C) SEM image of a side view of the apertural region, showing organic detritus, rounded and corroded apertural teeth and tubercles. (D) A side view of the dissolution effect on test surface of specimen C. (E) SEM image of a side view of the apertural region, where a partial absence of teeth-like structures is observed. (F) A side view of the test surface of specimen E showing several test wall layers strongly affected by dissolution and cracking processes. (←) yellow arrows highlight areas affected by dissolution.

6.4 Discussion

6.4.1 Reproduction events

Contrary to observations from the previous CO_2 experiments at 13°C, in this study at 15°C, the number of specimens retrieved at the end of the CO_2 experiment was higher than the initial number introduced into culturing chambers across pH treatments (Table 6.2). Thus, the increased numbers of *E. williamsoni* from all three treatments and *H. germanica* (only at a pH of 7.7) are attributed to reproduction events during the experimental period, rather than errors in counting of specimens introduced into the culturing chambers at the start of the experimental period. Newly reproduced specimens showed a distinct lack of calcein labelling to distinguish new growth and they were not considered for further analysis to detect the influence of environmental factors on biological parameters of foraminifera.

As temperature is considered one of the major environmental drivers controlling reproduction in the ocean (Edwards & Richardson 2004), our results suggest that specimens of *E. williamsoni* in all pH conditions, including ambient treatment, were capable of reproduction during the experimental period and that a rise in temperature of 2°C may have exerted a greater influence to promote reproduction even under the unfavourable low pH conditions of these experiments. The capacity of postponing the reproductive phase by foraminifera when environmental conditions are not favourable (Murray 1983) may be applied to *H. germanica* cultured at pH 8.1 and pH 7.3, but why reproduction occurred at a pH of 7.7 is unclear. These results are similar to other studies where reproduction events

occurred under high CO_2 concentrations (Bernhard et al. 2009; McIntyre-Wressnig et al. 2014).

6.4.2 Survival rates

Mortality rates of *E. williamsoni*, calculated for both CO_2 experiments at 13°C and 15°C, rose as pH was reduced but with a substantial difference in magnitude of mortality rates between both studies. Mortality rates of up to 33 % at 13°C and up to 20 % at 15°C were estimated, mainly at the lowest pH treatments (Table 4.2 and Table 6.2).

In the case of *H. germanica*, mortality rates obtained from the CO_2 experiment at 15°C clearly showed a negative effect of both decreasing pH and elevated temperature in comparison to mortality rates estimated for the CO_2 experiment at 13°C, where no obvious mortality trend was observed (Table 4.2 and Table 6.2). These results suggest that experimental conditions of lowering pH and rising temperature levels definitely promote a differential and strong influence on the survivorship of *E. williamsoni* and *H. germanica.* Furthermore, these biological responses (mortality trend) of both foraminiferal species to multiple climatic stressors are similar to other calcareous benthic foraminifera which have been reported as negatively impacted by one or more environmental factors such as high pCO_2/low pH levels, salinity, Ω and dysoxia under laboratory and natural acidified setting (Bernhard et al. 2009; Dias et al. 2010; Haynert et al. 2011; Saraswat et al. 2011; Haynert & Schonfeld 2014; van Dijk et al. 2017).

The observed sensitivity (vulnerability) of the benthic foraminifera *E. williamsoni* and *H. germanica* to future OA levels via survival /mortality rates may vary when a wider range of

extreme temperatures (above mean annual values observed in natural environments) are used in future experiments. Furthermore, the calculated survivorship/mortality obtained at the lowest pH conditions for 52 days indicate the difficulty of performing long-term experiments under the present experimental conditions, even when the initial number of 'live' specimens of *E. williamsoni* and *H. germanica* was increased considerably for these two experiments. Therefore, if the aim for future experiments is to assess foraminiferal survivorship under long-term exposure to elevated CO_2 and increased temperature, the near-future experimental designs should take into consideration the high mortality rates observed in both studies, emphasizing also that a new method to concentrate a larger number of 'live' specimens is still needed.

6.4.3 Foraminiferal maximum test diameter

For *E. williamsoni* (Fig. 6.12), two-way ANOVA indicates that the combined effect of decreasing pH and elevated temperature significantly affected the decreased/increased test size, mainly at a pH of 7.7 ($p = 0.0109$). However, despite the slight difference in test size of *H. germanica* in both studies, those changes were not statistically significant and did not display a strong stress response to the interactive effect of both environmental factors on foraminiferal size ($p = 0.3905$) (Fig. 6.12). These results of mean test size are similar to those reported in Chapter 3 of this research, and also in other studies (Keul et al. 2013; Prazeres et al. 2015) where high pCO_2 and concomitants were the environmental factors assessed.

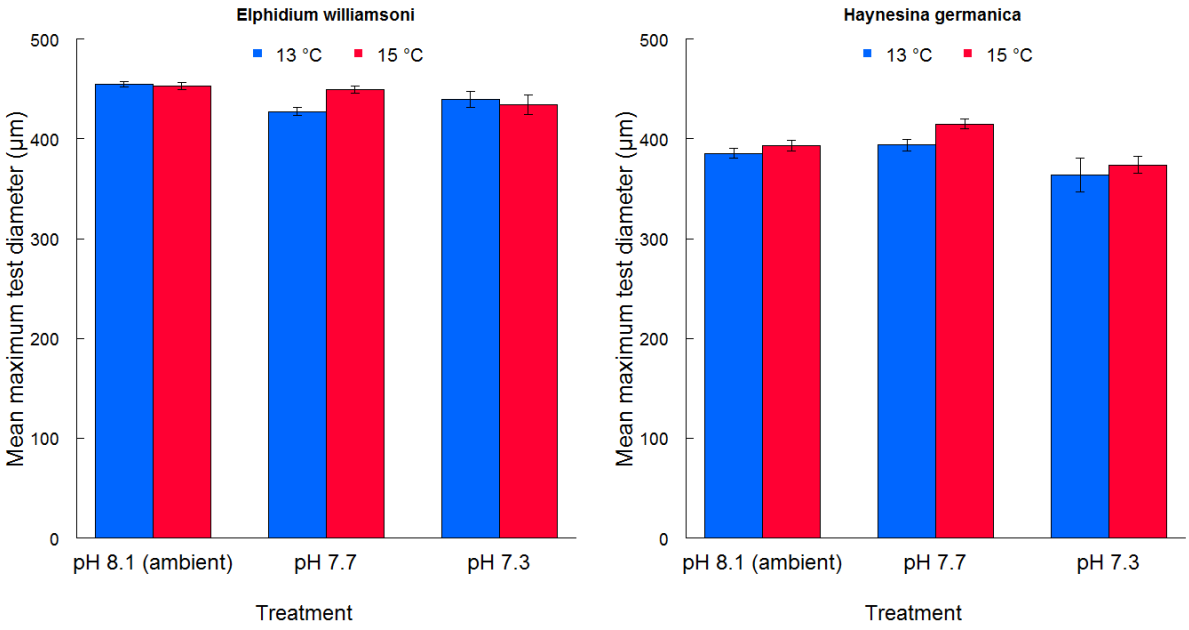

Figure 6. 12 Mean values (± standard error) of maximum test diameter (µm) for 'live' *Elphidium williamsoni* and *Haynesina germanica* cultured at different pH (8.1, 7.7 and 7.3 units) and temperature (13 and 15°C) levels.

6.4.4 Foraminiferal test weight

A two-way ANOVA indicates that a combined effect of decreasing pH and elevated temperature substantially affected the mean weight of *E. williamsoni* (p = 2.39 x 10^{-7}) and *H. germanica* (p = 4.89 x 10^{-6}) (Fig. 6.13).

In general, the decreasing trends of test weight associated with the interactive effect of pH and temperature potentially represent reduced calcification or increased dissolution, notably between the treatments at a pH of 7.7 (Ω_{cal} >1) and pH 7.3 (Ω_{cal} <1). These results are similar to those observations reported on *M. kudakajimensis* (Kuroyanagi et al. 2009) and *A. lessonii* (Prazeres et al. 2015) where pCO_2 was the only environmental factor assessed.

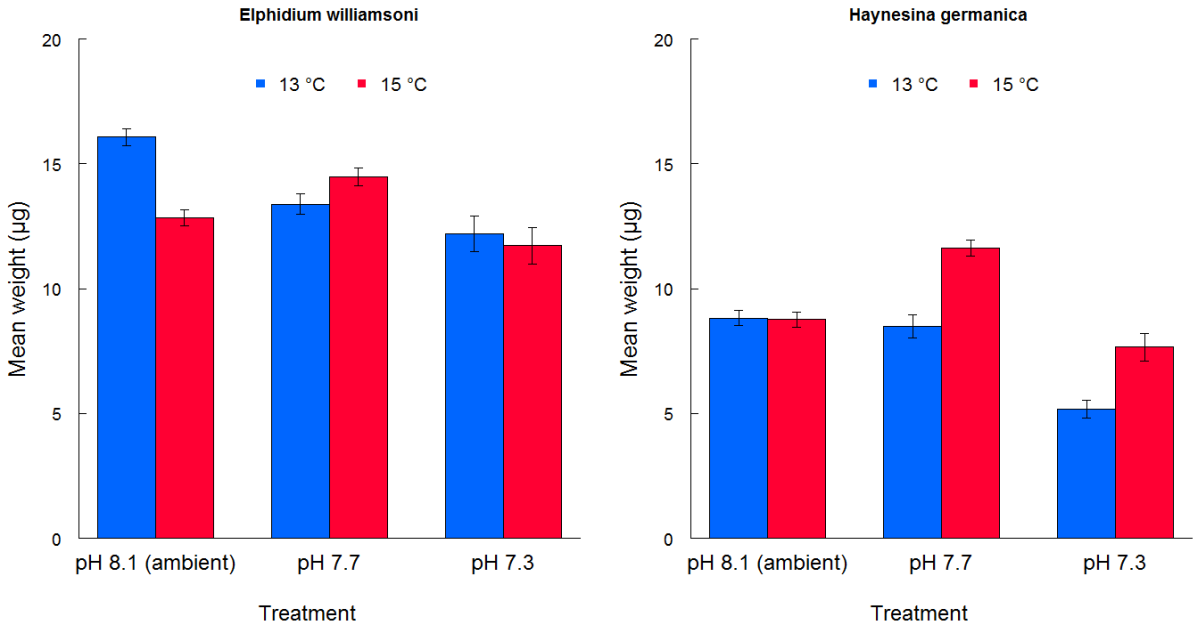

Figure 6. 13 Mean values (± standard error) of test weight (µg) for 'live' *Elphidium williamsoni* and *Haynesina germanica* cultured at different pH (8.1, 7.7 and 7.3 units) and temperature (13 and 15°C) levels.

Future experiments assessing the combined effect of OA and extreme rising temperature on foraminiferal weight should consider estimating both the test thickness of newly added chambers added via non-destructive techniques and estimations of dissolution rates at different pH and temperature levels. This information may help determine whether the changes in weight are linked directly to the production of thinner chambers affected by the combined effect of multiple factors or just as a consequence of high dissolution rates affecting foraminiferal tests.

6.4.5 Growth rate and newly deposited chambers

The results of a two-way ANOVA indicate that a combined effect of decreasing pH and elevated temperature substantially affected the growth rates of *E. williamsoni* (p = 0.00036239) and *H. germanica* (p = 0.000001), which were expressed as the number of new chambers added over a period of 52 days (Fig. 6.14).

In general, the decreasing trends observed in growth rates under future increased temperature and lowering pH levels suggest a potential reduction in foraminiferal growth mainly between pH 8.1 (Ω_{cal} >1) and pH 7.3 (Ω_{cal} <1) over the coming decades and centuries. These results are similar to the trend of decalcification on tests (reduction in production of new chambers) observed in specimens of foraminiferal species *Ammonia aomoriensis* at elevated pCO_2 and reducing Ω_{cal} (Haynert & Schonfeld 2014). In addition, these decreasing growth trends are also similar to those observed in Kuroyanagi et al. (2009) where seawater pH was the only environmental factor assessed. However, these results are totally opposite to those found in Chapter 4 and Khanna (2014) for both foraminiferal species where increasing trends were observed when seawater pH was evaluated as the single environmental factor acting on mean foraminiferal growth.

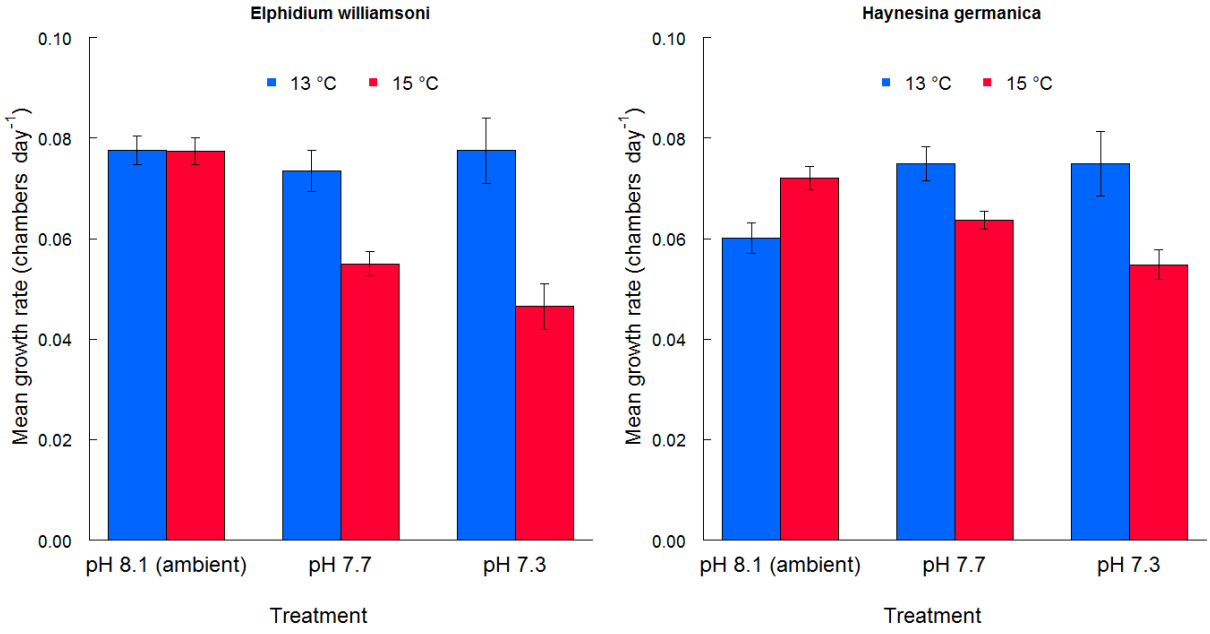

Figure 6. 14 Mean values (± standard error) of growth (chambers days^{-1}) for 'live' *Elphidium williamsoni* and *Haynesina germanica* cultured at different pH (8.1, 7.7 and 7.3 units) and temperature (13 and 15°C) levels.

6.4.6 SEM observations of the morphological response of 'live' specimens

SEM images of 'live' *E. williamsoni* and *H. germanica* exposed to high CO_2 concentrations/low pH levels over a period of 52 days at a temperature of 15°C (Fig. 6.10 and 6.11) showed similar alterations to the foraminiferal morphology as found in experiments performed at 13°C (Chapters 3 and 4). In general, the findings support previous results, showing the vulnerability of living assemblages of *E. williamsoni* and *H. germanica* to changing carbonate seawater chemistry, in spite of possessing low-Mg calcite tests. This foraminiferal test composition is thought to be an essential feature to resist corrosion and dissolution at early stages under lowering pH and calcite seawater undersaturation (Engel et al. 2015).

Differential dissolution susceptibilities on the foraminiferal morphology induced by the interactive effect of OA and small rise in temperature are still difficult to quantify by simple visualization of SEM; however, dissolution features observed in the present study in *E. williamsoni* and *H. germanica* across pH and temperatures levels are similar to those reported in other studies where pCO_2 was assessed as the single environmental factor affecting foraminiferal morphology (Haynert et al. 2011; Khanna et al. 2013).

6.4.7 Final remarks and future work

As climate change includes a whole range of multi- stressors, such as rising temperature, sea-level rise, OA, increased ultraviolet-b radiation, altered nutrients concentrations, and increased frequency and intensity of storms (Harley et al. 2006; Harley & Connell 2009), the comparative framework of this experimental study with a focus on OA (decreasing pH) and temperature provide relevant evidence of the combined effect of both climatic factors on foraminiferal survivorship, weight, growth/calcification and morphology (i.e. fundamental feeding structures). Furthermore, this study demonstrates that even an increase in temperature of 2°C projected for Scottish coastal waters by the year 2100 (Hiscock et al., 2001) also has the potential to impact strongly on benthic foraminiferal assemblages from intertidal habitats in a high CO_2 world. These laboratory results are consistent with projected changes to occur over the coming decades and centuries due to the impacts of climate change, including OA and rising temperature in coastal environments. Under future climatic scenarios, the temporal and spatial distribution of coastal marine species is expected to be altered substantially with unprecedented consequences for ecosystem structure and services (Helmuth et al. 2002; Harley et al. 2006).

Undoubtedly, further research studies focused on the long-term exposure of foraminiferal assemblages to both environmental factors are still required to confirm the findings obtained in this joint study. These future studies should incorporate the use of natural sediments containing natural predators and competitors for foraminifera, to allow trophic interactions. Experiments at higher temperatures might also be conducted in order to evaluate whether the observed morphological alterations are strongly increased as a consequence of the interaction of high CO_2 concentrations with elevated temperatures. The outcomes from long-term research may enable us to better understand the unexplored biological responses of benthic foraminifera from mid-latitude intertidal mudflats to multi-climatic factors associated with global climate change. Furthermore, these new experimental designs may help to reveal clearly the synergistic or additive effects on the biological parameters studied in this research and deliver a more realistic understanding of future OA impacts on marine calcifiers.

6.5 Conclusions

Based on the results of this short-term comparative study on the biological responses of multispecies assemblages of benthic foraminifera to the interactive effects of OA and subtle rising temperature in seawater, the following conclusions may be drawn:

(1) The survival rates of *E. williamsoni* and *H. germanica* were negatively affected by the decreasing pH and elevated temperature levels (+2°C). The differential mortality rates between foraminiferal species studied indicate that *H. germanica* was apparently less affected by both environmental factors, suggesting a species-specific response.

(2) Mean maximum test diameter of *E. williamsoni* was not significantly affected by decreasing pH and elevated temperature levels, suggesting that growth continues under these conditions.

(3) Test weight of *E. williamsoni* and *H. germanica* was not significantly affected at the lowest pH and by rising temperatures.

(4) Decreasing trend observed in the calculated mean growth rates (via new chambers addition method) for *E. williamsoni* and *H. germanica* suggest diminished calcification rates due to decreasing pH and rising temperature levels.

(5) SEM imaging suggest that under future scenarios in a high CO_2 world, living assemblages of *E. williamsoni* and *H. germanica* may be highly vulnerable to OA effects in the short-term, and this may ultimately affect their ecological interactions and

dominance, resulting in alterations to benthic community structure, carbon cycling and total production of $CaCO_3$ in coastal environments such as intertidal mudflats.

(6) The combination of lowered pH and higher temperatures levels (+2°C) clearly showed a greater impact on a large number of biological parameters of *E. williamsoni* and *H. germanica* than did the isolated effect of a single environmental factor observed in earlier experiments (Chapters 3 and 4).

Chapter 7. Short-term effects of high CO_2/ low pH levels on post-mortem dissolution of *E. williamsoni* and *H. germanica*.

7.1 Introduction

Foraminifera, an ubiquitous marine group (Archibald et al. 2003; du Châtelet et al. 2004) with high adaptability and sensitivity to changes in environmental parameters (Linke & Lutze 1993; Lecroq 2014), are expected to be particularly susceptible to future high pCO_2 /low pH scenarios in global oceans. This vulnerability has been naturally and experimentally assessed in the last decades, mainly focused on living assemblages exhibiting a decline in calcification rates. This negative effect may be the most notable and consistent biological response of marine organisms to the adverse effects of OA (Riebesell et al. 2000; Caldeira & Wickett 2003; Orr et al. 2005; Andersson et al. 2008; Maier et al. 2012; Kroeker et al. 2013).

A reduced calcification rate may render foraminifera more vulnerable to erosion (both biological and physical) and dissolution processes (Orr et al. 2005; Raven et al. 2005), thereby remarkably reducing surface biogenic $CaCO_3$ production and sedimentation rates with implications on the marine carbon cycle in shallow waters (Fink et al. 2017) where benthic foraminifera can contribute up to 30 % of total annual carbonate production.

Although progress has been made in understanding biological responses of coastal benthic foraminifera to OA, mainly focused on projecting decreased calcification and growth rates (Le Cadre et al. 2003; Kuroyanagi et al. 2009; Allison et al. 2010; Reymond et al. 2011; Saraswat et al. 2011; Khanna et al. 2013; Haynert et al. 2014; Prazeres et al. 2015), the

effects of increased carbonate dissolution rates as a consequence of ongoing OA on living and dead benthic foraminiferal fauna have received relatively little attention.

Evidence from earlier *in-situ* and laboratory work on dissolution effects on living and dead foraminiferal assemblages does, in most cases, only provide a general description of dissolution effects to better understand the interaction between foraminiferal population dynamics and coastal environmental stressors including high CO_2 concentrations, declining carbonate saturation state (Ω), oxygen, salinity and environmental contaminants (Alve & Murray 1994; Murray & Alve 1999; Alve & Murray 2001; Le Cadre et al. 2003; Buzas-Stephens & Buzas 2005; Crevison & Hallock 2007; Souder et al. 2010; Haynert et al. 2011; Haynert et al. 2012; Engel et al. 2015).

As carbonate dissolution of foraminifera has been commonly associated with post-mortem effects under the natural process of diagenesis, the substantial ongoing impact of dissolution on living foraminiferal individuals has been overlooked (Buzas-Stephens & Buzas 2005; Crevison & Hallock 2007). However, it is important to start focusing quantitatively on enhanced post-mortem dissolution as a major taphonomic process induced by OA, potentially affecting the long-term fossilization process (i.e. accumulation, preservation and burial) of the recently dead calcareous assemblages in the coastal sedimentary records which are usually used for palaeoenvironmental studies (e.g. Murray & Alve 1999).

Results from CO_2 experiments performed in Chapter 3 highlight a notably negative trend of weight-size relationship of *E. williamsoni* at a pH of 7.3 throughout the transition from a

living assemblage (e.g. initial state at the start of experiments) to a dead group within 4 weeks of the experimental period. These outcomes provided relevant evidence but still only descriptive of progressive post-mortem dissolution enhanced by OA on benthic foraminifera. Hence, the present study aims to conduct a short-term laboratory CO_2 experiments to quantify post-mortem dissolution rates based on alteration of morphology of *E. williamsoni* and *H. germanica* associated with changes in test weight. Subsequently, data obtained from this post-mortem dissolution experiment will be combined with data provided in previous chapters of this research and other researchers' data to model potential changes in net $CaCO_3$ production by *E. williamsoni* and *H. germanica* under future CO_2 scenarios. This integrative approach will help evaluate future ecological changes and carbon cycle mainly driven by OA in benthic foraminifera populations from coastal ecosystems such as intertidal mudflats.

7.2 Materials and Methods

7.2.1 Selection of target foraminifera and experimental conditions

For this post-mortem dissolution experiment, intact tests of *E. williamsoni* and *H. germanica* from two different sources were used: 1) pre-conditioned foraminifera (dead specimens from previous CO_2 experiments), and 2) recently dead foraminiferal assemblages from natural sediment collected in July 2017 from the high intertidal mudflats of the Eden Estuary, N.E. Scotland.

Pre-conditioned foraminifera of both species previously exposed for 31 days (Chapter 3) and 52 days (Chapter 4) to high CO_2/low pH levels were randomly selected and picked out using a fine paint brush. Isolated specimens of each species were managed separately. A group of 50 individuals of *E. williamsoni* and *H. germanica* from a specific pH treatment was counted and transferred into pre-weighed aluminium capsules. The low number of specimens available with intact tests left from previous CO_2 experiments meant that no replicate experiments were run.

In the case of recently dead assemblages, they were easily recognised under the binocular microscope by the colour and the reduced distribution of their cytoplasm within tests. A group of 100 dead individuals for each species were identified, isolated, cleaned carefully with a fine paint brush and left for two weeks in Petri dishes with natural seawater until most of the tests were completely empty. Subsequently, tests were picked, rinsed twice with distilled water and counted again before being transferred into pre-weighed aluminium

capsules. For these samples, the number of replicates per pH treatment for each species was also equal to 1.

All samples with pre-conditioned and recently dead assemblages placed into pre-weighed aluminium capsules are referred hereafter as bulk samples. They were dried in an oven at 50°C for 24 hours; mainly to evaporate the excess of distilled water possibly remained inside the tests during cleaning, picking and transfer processes. Subsequently, initial measurements of dry weight (µg) of each bulk sample were obtained by employing a microbalance (Sartorius M2P Microbalance, with a precision of ±1µg).

Each oven-dried bulk sample was independently transferred into a culturing insert and covered with a mesh of 110 µm which was previously tested to allow seawater to flow through freely. A plastic cable tie around the insert edge helped keep the mesh attached to the insert. This system prevented the loss of specimens out of the system by flotation. Subsequently, inserts were placed in a 6-well plate (Fig. 7.1) and immersed for 6 weeks in a series of transparent Tupperware containers of 700 cm^3 prefilled with seawater with specific pH levels and carbonate chemistry. These containers with airtight lids to prevent evaporation were connected to the same manipulative mesocosm explained in previous Chapters (2-5). However, unlike previous CO_2 experiments, in this study, the experimental setting consisted of only three pH levels (8.1 (ambient), 7.7 and 7.3) kept in a temperature-controlled room at 15°C.

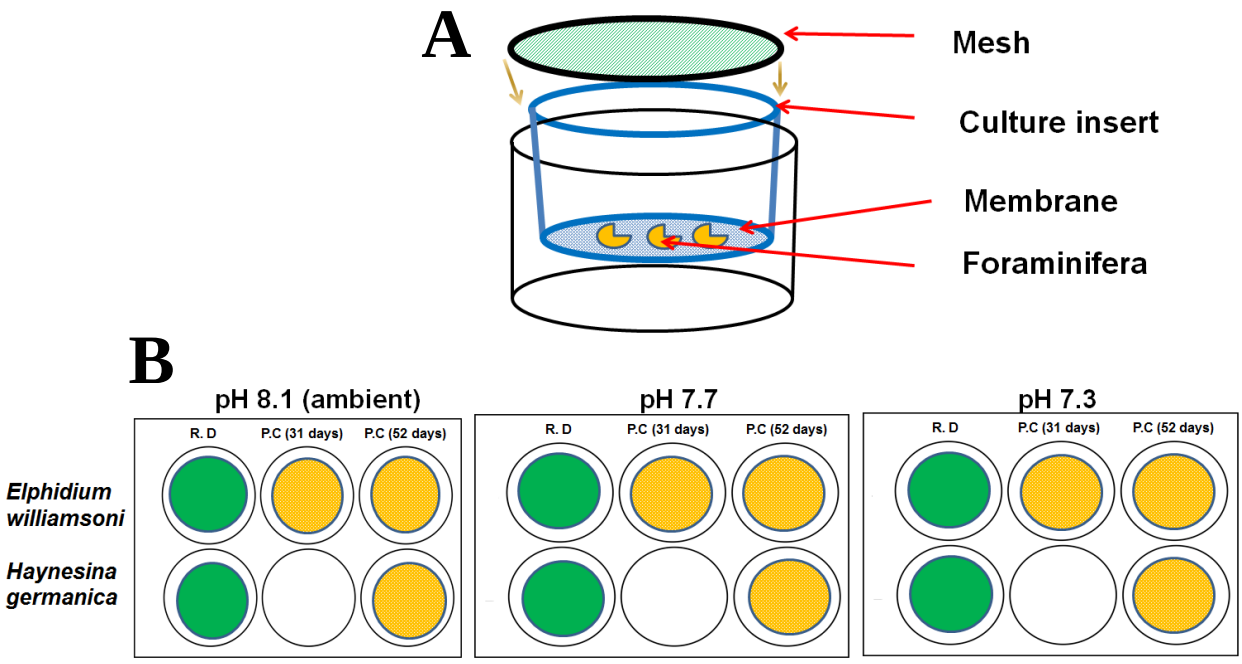

Figure 7. 1 Schematic diagram of the dissolution experiment design for three pH scenarios. A) Dead assemblages of *E. williamsoni* and *H. germanica* were introduced into inserts independently**.** B) Inserts with Recently dead (R.D, in green) and pre-conditioned specimens (P.C, in orange) of both foraminiferal species placed in a series of 6-well plates before being connected to the CO_2 manipulative mesocosm for 42 days at 15°C.

At the end of the experimental period of 6 weeks, all inserts were removed from the experimental system and placed in clean Petri dishes and opened. Foraminifera were counted and transferred to new Petri dishes to be rinsed carefully with distilled water. Specimens were placed onto new pre-weighed aluminium capsules and dried in an oven at 50°C for 24 hours. Subsequently, final bulk weight measurements of each oven-dried bulk sample were recorded.

7.2.2 Dissolution rates

At the end of the experiment, in the event that the number of tests retrieved was lower than the number used at the onset of the experiment (mainly due to some of the specimens being completely altered or dissolved), the final bulk weight of each sample was normalized or adjusted with respect to the initial number of specimens used in each initial bulk weight (Kotler et al. 1992). This approach prevented an overestimation of the net loss of foraminiferal mass (µg) throughout the experimental period of 42 days.

Dissolution rates for each pH treatment were calculated by subtracting the final normalized bulk weight from the initial bulk weight, and divided by both the experimental period in days and the initial number of individuals used in each bulk sample (see Equation 7.1):

$$\textbf{Dissolution rate} = \frac{\text{Initial bulk weight - final normalized bulk weight}}{\text{(number of days) x (initial number of individuals)}} \quad \text{(Eq. 7.1)}$$

Thus, the dissolution rate is expressed as average change in mass (net carbonate loss) of each individual over an average daily timescale (µg day^{-1}). Subsequently, to facilitate further calculations, dissolution rates of all groups of dead assemblages exposed to a specific pH condition were averaged and used for estimating mean dissolution rates for each foraminiferal species.

7.2.3 Morphological response of dead foraminiferal assemblages to dissolution

SEM images of specimens belonging to assemblages of preconditioned and recently dead specimens of *E. williamsoni* and *H. germanica* allowed us to determine potential morphological responses to each pH treatment. Specimens were classified according to the

physical condition observed on tests as detailed in Chapter 2-4, where Level 1, 2 and 3 corresponded to intact, slightly dissolved or minor alterations and broken tests, respectively. Relative contribution or counting of specimens within a specific level of test modification was not carried out because SEM images were only used to link observed test damages and measured loss of foraminiferal mass. The latter was deduced from changes in tests weight measurements at the start and the end of the experiment (see Equation 7.1).

7.2.4 From post-mortem dissolution process to net $CaCO_3$ production by living benthic foraminifera assemblages affected by OA

In this study, net foraminiferal $CaCO_3$ production was defined as the difference between gross foraminiferal $CaCO_3$ production and gross dissolution rates estimated throughout this chapter. In this context, the challenge is to understand the process of transition between the live assemblages, the net $CaCO_3$ production and the long-term accumulation of $CaCO_3$ on the sea floor. The following equation (model) for net foraminiferal production requires the incorporation of the gross post-mortem dissolution estimated above and calculations of gross foraminiferal productivity which are detailed below (see Equation 7.2). This approach has taken into consideration only some components of equations mentioned in other studies (Andersson et al. 2006; Andersson et al. 2009; Eyre et al. 2014) to explain the balance between gross $CaCO_3$ production and destructive processes (i.e. dissolution) mainly in coral reef ecosystems, However, this type of model is used here as an approach for estimating carbonate productivity by calcareous benthic foraminifera as follows (see Equation 7.2).

Net $CaCO_3$ production = Gross $CaCO_3$ production (P) – gross dissolution rates*

(Eq. 7.2)

Gross carbonate dissolution rate (*) is defined as the product between mean dissolution rate µg day^{-1}) (see Equation 7.1) in each pH treatment and annual test production (N) (# tests / m^2) obtained from recalculated standing stock datasets for *E. williamsoni* and *H. germanica* available in Austin (2003) for the Eden Estuary for the years 2000-2001. This standing stock was previously defined as a number of "living" individuals within a unit area of substratum (Murray 1983; Austin 2003).

Gross $CaCO_3$ productivity (g/m^2 per year), defined below as P, is the product of annual (i) test production (**N**), (ii) turnover rate (τ) defined as the fraction of the total $CaCO_3$ produced by living foraminiferal populations that will subsequently accumulate in the sediments upon foraminifera death over a given length of time (Hallock 1981). Average turnover rate (**τ)** equal to 11 (year $^{-1}$) used for this study was estimated from other benthic foraminifera (Hallock 1981) and (iii) an average foraminiferal test weight of 12 µg (**m**) measured for specimens *E. williamsoni* and *H. germanica* cultured at ambient pH of 8.1 in Chapter 4 (see Equation 7.3).

$$\mathbf{P} = N \times \tau \times m \qquad \text{(Eq. 7.3)}$$

7.2.5 Statistical Method

The assumptions of homogeneity of variance and normality of mean dissolution rates were independently assessed by Shapiro-Wilk and Levene tests. When the assumptions of these

two tests matched values of $p > 0.05$ or $p < 0.05$, either a One-way ANOVA (parametric analysis) or Kruskal-Wallis rank sum (non-parametric) tests were performed, respectively. The statistical significance level of 95% ($p < 0.05$) was set for all tests.

All statistical analysis was performed using the statistical programme R 3.1.2 (R Development Core Team. 2014).

7.3 Results

7.3.1 Post-mortem dissolution rates

In general, dissolution rates (µg day^{-1}) of tests for pre-conditioned and recently dead assemblages followed a similar positive non-linear relationship across all pH treatments, with greater values of carbonate dissolution with lowering pH.

E. williamsoni, however, displayed varying dissolution rates within the three groups of dead assemblages and between pH treatments. Specimens of all groups exposed to a pH of 7.3 showed the greatest increase in dissolution rates in comparison to other pH conditions (Fig. 7.2).

For *H. germanica,* recently dead groups showed similar dissolution rates across all pH treatments (~10 µg day^{-1}), except for pre-conditioned specimens pre-exposed for 52 days which displayed a 5-fold higher dissolution rate at a pH of 7.3 (~ 0.21 µg day^{-1}) and 1.5-fold higher at a pH of 7.7 (~ 0.062 µg day^{-1}) than ambient treatment (~0.043 µg day^{-1}) (Fig. 7.2). Furthermore, recently dead specimens of *H. germanica* in all groups showed 2-fold higher dissolution rates than those observed in *E. williamsoni* when exposed to ambient pH of 8.1 (Fig. 7.2); all these results suggesting that *H. germanica* may be more prone to test dissolution than *E. williamsoni.*

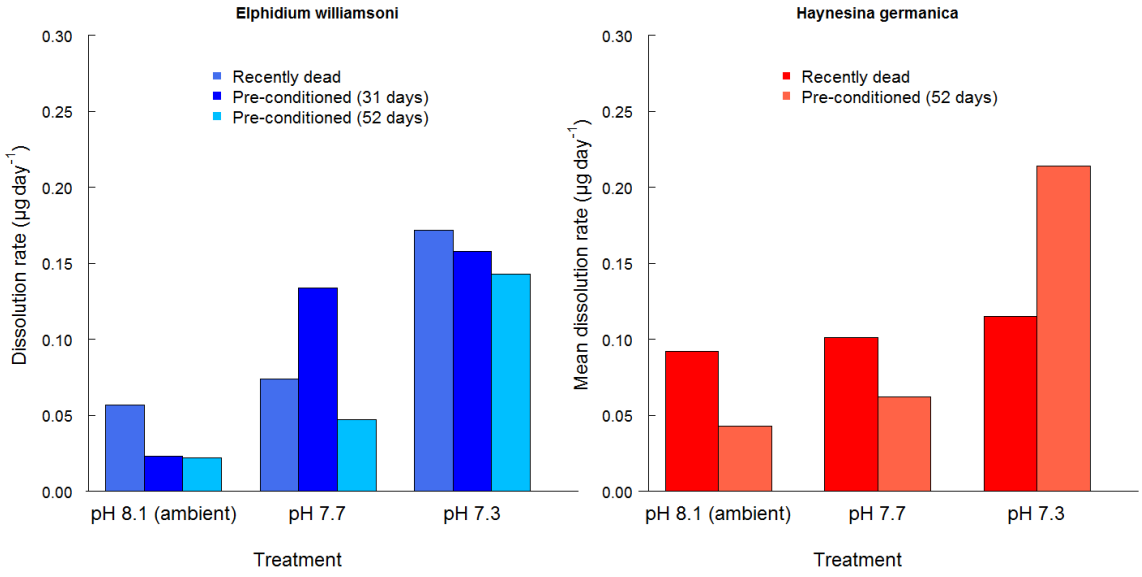

Figure 7. 2 Dissolution rates of pre-conditioned and recently dead specimens of *Elphidium williamsoni* and *Haynesina germanica*. All groups were exposed for a period of 42 days to different experimental pH conditions: ambient: pH 8.1/ ~400 µatm CO_2; pH 7.7/ ~950 µatm CO_2, and pH 7.3/ ~2600 µatm CO_2 at 15°C.

7.3.2 Mean dissolution rates for each foraminiferal species

Calculated mean dissolution rates (µg day^{-1}) for both species followed a non-linear relationship across pH treatments. For *E. williamsoni*, One-way-ANOVA indicates that the mean dissolution rates are significantly different between the pH treatments (p = 0.0061), being 5-fold higher at a pH of 7.3 (~ 0.16 µg day^{-1}) and 3-fold higher at a pH of 7.7 (~ 0.085 µg day^{-1}) than ambient treatment (~0.034 µg day^{-1}) (Fig. 7.3).

In contrast, for *H. germanica*, despite mean dissolution rates being 2.5-fold higher at pH 7.3 (~0.1645 µg day^{-1}) and 1.2 -fold higher at pH 7.7 (~0.0815 µg day^{-1}) than ambient treatment (~0.0675 µg day^{-1}) (Fig. 7.3), Kruskal-Wallis test indicates that this slight

difference in mean dissolution rates between pH treatments is not significant (p = 0.1561) (Fig. 7.3).

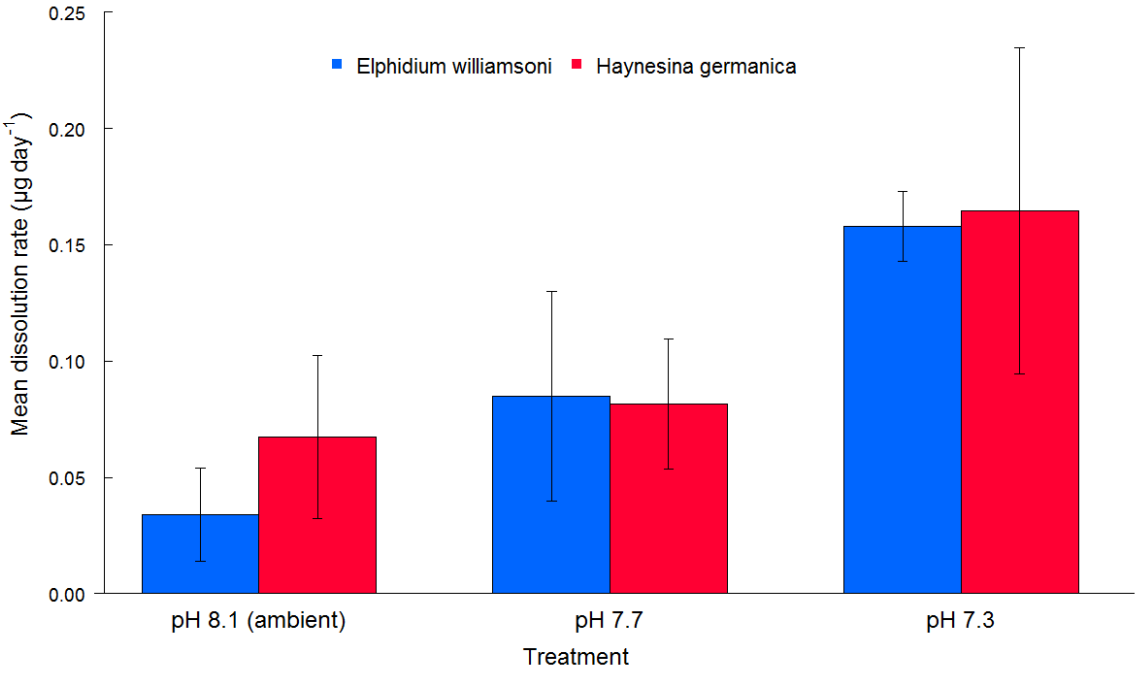

Figure 7. 3 Mean values (± standard deviation) of dissolution rates (µg day^{-1}) for *Elphidium williamsoni* (in blue) and *Haynesina germanica* (in red) exposed for 42 days to different experimental pH conditions: pH 8.1/~ 400 µatm CO_2; pH 7.7/~ 950 µatm CO_2, and pH 7.3/ ~2600 µatm CO_2 at 15°C.

7.3.3 SEM observations and morphological changes

At the end of the experimental period, tests morphological changes were observed in all assemblages of preconditioned and recently dead specimens of *E. williamsoni* and *H. germanica*. Theses test modifications range from apparently non-adverse signs to severe modifications, suggesting a clear response to the experimental pH conditions after an experimental period of 42 days.

For both foraminiferal species, although all groups of dead assemblages displayed the three levels of morphological responses (Fig. 7.4 A-F), the proportion of broken tests (Level 3) increased with lowering pH. Thus, at a pH of 7.3, most of the tests displayed severe alterations around the apertural region (Fig. 7.4 E & F). On the contrary, the highest proportions of intact tests (Level 1) (Fig. 7.4 A & B) and slightly dissolved tests (Level 2) (Fig. 7.4 C & D) decreased with lowering pH.

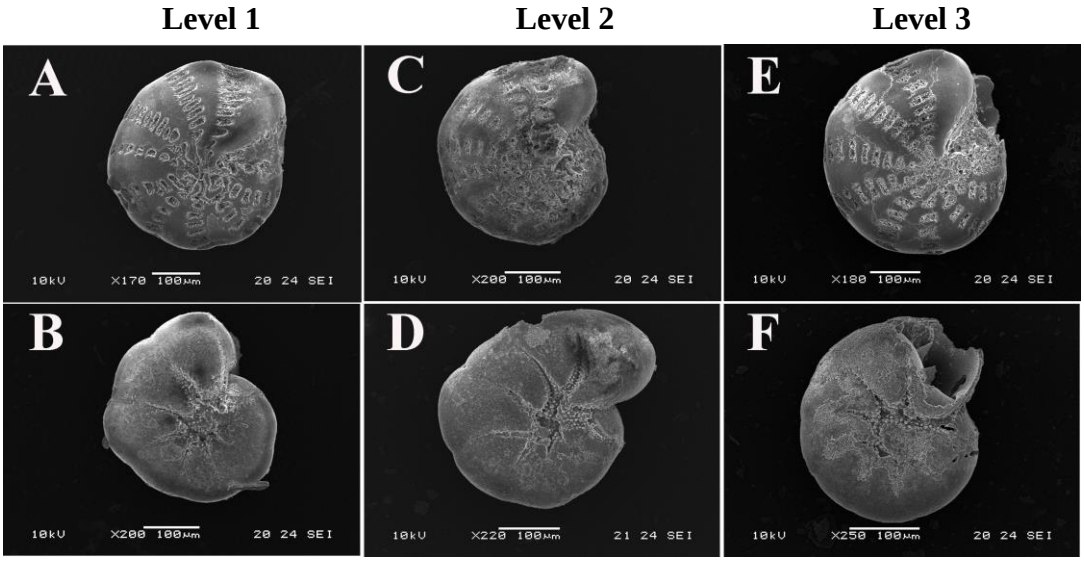

Figure 7. 4 Scanning electron micrographs (SEM) images to illustrate the level of morphological responses displayed by dead *Elphidium williamsoni* (A, C & E) and *Haynesina germanica* (B, D & F) exposed for 42 days to different CO_2/pH conditions. Level 1= intact test, Level 2 = slightly dissolved test, Level 3= broken test.

7.4 Discussion

7.4.1 SEM observations of the morphological response of dead assemblages

Considerable responses of dead assemblages to the experimental pH conditions were observed via SEM images after an experimental period of 42 days. Morphological changes ranging from slight to severe alteration of foraminiferal tests were consistent with net carbonate mass loss deduced from measured changes in test weight which were used for subsequent estimates of dissolution rates (Fig. 7.2). Thus, in this study, an increasingly poor state of test preservation (Level 3) was linked to a greater effect of OA on foraminiferal tests due to higher dissolution rates estimated at the lowest pH treatments. These results are contrary to the morphological changes induced by OA observed experimentally in live assemblages detailed in Chapter 3 and 4, where despite the observation of the three levels of altered tests across the treatments, no obvious trend of the effect of high CO_2/low pH on test morphology (i.e. prevalence of one level of test damage over others) was clearly observed.

In terms of different stages of morphological alterations due to undersaturated seawater driven by high CO_2 levels, our results are similar to those features observed in other studies on live *A. aomoriensis* and *Archaias angulatus* (Crevison & Hallock 2007; Haynert et al. 2011; Haynert et al. 2014).

7.4.2 Post-mortem dissolution effect on two benthic calcareous foraminifera

The results obtained from the present 42 day-experiment at a temperature of 15°C using pre-conditioned specimens and recently dead specimens of *E. williamsoni* and *H.*

germanica indicate no considerable differences in dissolution rates between all groups studied, at least over a short-term period (Fig. 7.2). Therefore, for further purposes, the use of mean test dissolution rates (µg day^{-1}) for each foraminiferal species certainly provide new insights into the potential vulnerabilities that dead assemblages and carbonate sediments may face under near-future high CO_2 /low pH scenarios.

As there are no remarkable differences in calculated dissolution rates between *E. williamsoni* and *H. germanica*, an average of mean dissolution rates of both species appears to be a good representative value of dissolution effects on dominant calcareous species representing ~ 90% of total calcareous foraminifera production in the intertidal mudflats of the Eden Estuary, Scotland. Consequently, by combining these new values of dissolution rates with recalculated foraminiferal standing stock data available in Austin (2003), annual carbonate dissolution rates or gross carbonate dissolution rates were calculated for each pH scenario (Table 7.1).

Table 7. 1 Estimates of mean dissolution rates (µg day^{-1}) for two dominant calcareous species. Gross dissolution rate (*) is defined as a product of recalculated foraminiferal standing stock data available in Austin (2003) and mean dissolution rates estimated in this study.

Treatment	Mean Dissolution rate (µg day^{-1})	Gross dissolution rate* (g m^{-2} $year^{-1}$)	Gross dissolution rate* (Kg m^{-2} $year^{-1}$)
pH 8.1 (ambient)	0.05	38.10	0.04
pH 7.7	0.08	60.96	0.06
pH 7.3	0.16	121.93	0.12

The mean dissolution rate observed at a pH of 8.1 clearly reflects the corrosive capacity of ambient seawater to dissolve foraminiferal tests of dead assemblages but at lower rates compared to those rates estimated at lower pH conditions (Fig. 7.2 and Table 7.1).

7.4.3 Estimation of net $CaCO_3$ production by living benthic foraminifera: A case study of foraminifera from intertidal mudflats under elevated CO_2 conditions

The available standing stock estimated by Austin (2003) for *E. williamsoni* and *H. germanica* from the Eden Estuary was essential to estimate both annual test production and gross $CaCO_3$ production of the dominant benthic foraminifera. Thus, annual test production of 1890 tests/10 cm^2 corresponded to 90% of the contribution of *E. williamsoni* and *H. germanica* to the total annual accumulation of benthic foraminiferal tests observed in the Eden Estuary for the years 2000-2001. This percentage contribution by both foraminiferal species agreed with subsequent field sampling carried out throughout this research in different months (April and July) for the years 2015-2017 when other CO_2 experiments were carried out (Chapter 3-6). As foraminiferal test production varies with location and seasonality, annual test production of 1890 tests/10 cm^2 in the Eden Estuary notably differed from estimations reported in other coastal environments (Murray 1983) where *E. williamsoni* and *H. germanica* are also important contributors to carbonate sediments.

In terms of gross productivity, 22.70 g $CaCO_3$ m^{-2} $year^{-1}$ was estimated to be calcified by *E. williamsoni* and *H. germanica* at ambient pH of ~ 8.1. This contribution of 22.70 g $CaCO_3$ m^{-2} $year^{-1}$ to the total local $CaCO_3$ budget (not estimated in this study) is considerably lower than that estimated for benthic foraminifera from coral reefs which ranged between 150 and

2800 g $CaCO_3$ m^{-2} $year^{-1}$ (Hallock 1981). By assuming that this estimated value of gross foraminiferal productivity has remained relatively constant over the last two decades, we were able to estimate the potential impacts of carbonate dissolution driven by future coastal OA on net $CaCO_3$ production by *E. williamsoni* and *H. germanica* (Fig. 7.5).

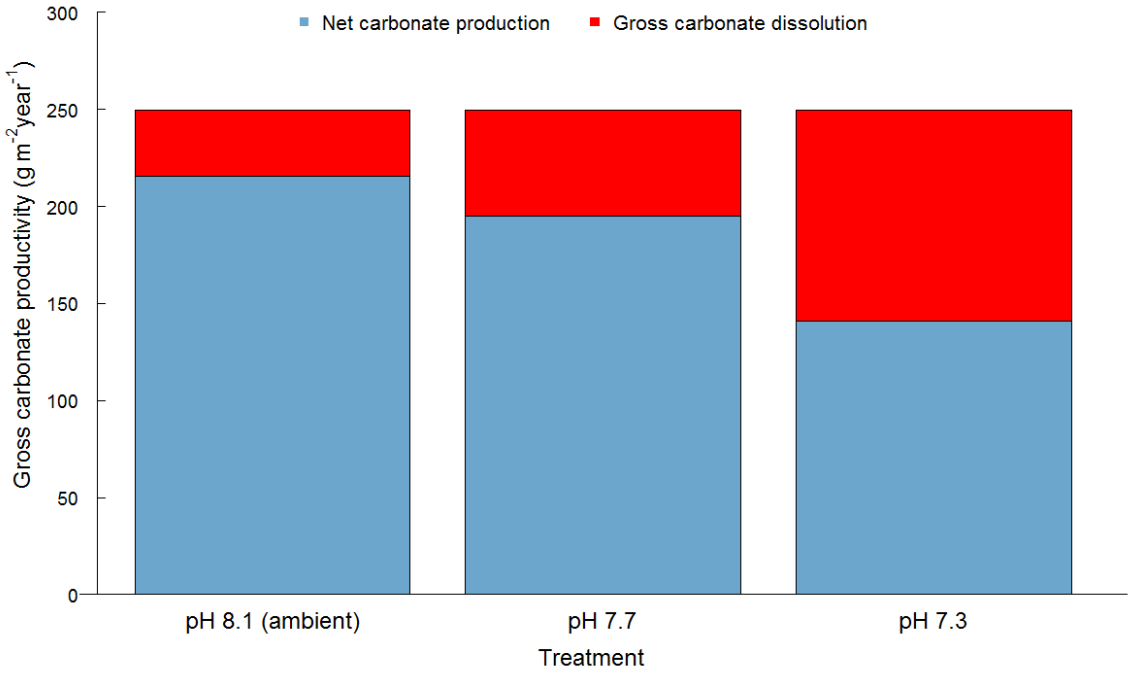

Figure 7. 5 Estimations of net carbonate productivity (g m^{-2} $year^{-1}$, in blue) and gross carbonate dissolution (g m^{-2} $year^{-1}$, in red) for *Elphidium williamsoni* and *Haynesina germanica* under projected high CO_2/low pH levels.

Estimates indicate that net carbonate productivity of living foraminiferal assemblages may be reduced by up to 44 % due to a notable disruption in the balance between gross $CaCO_3$ production and dissolution rates induced by elevated CO_2/low pH levels and undersaturated seawaters (Fig. 7.5). This estimated negative productivity (~44%) resembles those predicted for other calcifying communities and ecosystems under future high atmospheric

CO_2 scenarios (Langdon 2002; Andersson et al. 2006; Kleypas et al. 2006; Eyre et al. 2014; Comeau et al. 2015).

Although, these experimental results suggest the capacity of calcareous foraminifera to maintain positive net carbonate production under future coastal OA conditions, cumulative or synergistic impacts by other coastal environmental drivers alongside OA changes may also strongly influence the net foraminiferal productivity estimated in this study, rendering foraminiferal communities more vulnerable to a higher net dissolution under projected lowering pH and seawater concomitants. For example, an elevated temperature may exacerbate destructive processes such as dissolution, which may become increasingly important alongside $CaCO_3$ production, leading to a decline in coastal biogenic production by up to 40% by 2100 (Langdon 2002; Andersson et al. 2006; Kleypas et al. 2006; Eyre et al. 2014).

In terms of the possibility that *E. williamsoni* and *H. germanica* might disappear as a consequence of future acidification and calcite undersaturation of seawater in coastal oceans, our findings indicate a notable impact of OA on foraminifera over the coming decades and centuries but not as deleterious as already predicted for other foraminiferal species such as *A. aomoriensis* (Haynert et al. 2011).

7.4.4 Final remarks and future studies

Although the carbonate system accounts for only a minor part of the global carbon cycle (Milliman 1993), shallow water carbonates can become extremely important for biogeochemical cycles since they can contribute up to 40% of global oceanic carbonate

production and accumulation within these coastal ecosystems (Milliman 1993). This important carbonate contribution may be strongly affected by future OA conditions if carbonate dissolution rates estimated in this study (via experimental measurements on dominant benthic calcareous foraminiferal species) were attained in the near future. The results presented in this Eden Estuary case study have opened-up the possibility of forward modelling the impacts of OA on net foraminiferal productivity which may be one the most important local carbonate inputs on intertidal mudflats in mid-latitudes.

The outcomes suggest that severe changes in morphology and reduced net carbonate productivity (~44%) of *E. williamsoni* and *H. germanica* projected to occur due to lowering pH and seawater chemical concomitants may be highly deleterious for coastal ecosystems taking into consideration that natural carbonate dissolution associated with abiotic (i.e. temperature, salinity) and biotic (i.e. aerobic and anaerobic respiration processes) factors can reduce between 20 and 50% of gross carbonate productivity in shallow-water ecosystems (Walter et al. 1993; Ku et al. 1999). This fact may considerably alter the accumulation, preservation and burial of foraminiferal tests in the sedimentary records in coastal benthic ecosystems. Such changes would also have important implications for the long-term storage of inorganic carbon in intertidal sediments under increasing coastal OA over the next decades and centuries.

However, mean dissolution rates and subsequent gross carbonate dissolution rates estimated in this study on dead assemblages should be carefully applied to living assemblages when estimating future net carbonate production by foraminifera. Firstly, because living foraminiferal communities are more resilient to high concentrations of pCO_2

since they have the capacity to moderate dissolution effects via inbuilt compensatory mechanisms to maintain (up to a limited level) the integrity of their tests (Haynert et al. 2011). Secondly, because quantitative studies on thresholds of dissolution on living foraminiferal communities, assemblages and individual organisms are still limited, constraining our results to support only findings detailed in previous Chapters (3-6) of this research. In these cases, the vulnerability of "live" specimens appeared to be biologically controlled to cope with unfavourable experimental conditions such as experimental low pH, decreased carbonate concentration and decreased carbonate saturation state (Ω).

New estimations of dissolutions rates on dead and living foraminiferal assemblages are required to confirm our results and also to determine differential dissolution susceptibilities on morphology and calcification between *E. williamsoni* and *H. germanica* under future corrosive seawater conditions. These future experimental studies should also take into consideration test composition, architecture, microstructure, test porosity, ornamentation, size, surface/volume ratio, test thickness and reproductive cycles as intrinsic drivers that may all regulate dissolution processes in foraminiferal tests (Herrero & Canales 2002). These further studies will help to better understand the carbonate dissolution process on specific foraminiferal species and communities.

Furthermore, as the solubility of $CaCO_3$ is inversely related to temperature (Andersson et al. 2006; McClintock et al. 2009), future CO_2 experiments should incorporate projected changes in seawater temperatures to assess combined effects of multiple environmental factors to understand how OA will affect carbonate dissolution rates of foraminiferal species and communities. Such experiments in conjunction with integrative studies on other

calcifying communities would provide a better understanding of the overall local $CaCO_3$ budget.

Finally, in this study, a simple model with a few assumptions (explained above) helped to estimate present day and potential changes in net foraminiferal productivity due to ongoing and future coastal OA via enhanced dissolution rates. However, further long-term *in-situ* ecological studies are needed to obtain present-day estimates of standing stocks of benthic foraminifera which are fundamental to validate our model and outcomes presented in this chapter.

7.5 Conclusions

Based on the results of this study on the short-term effects of OA on dead assemblages of benthic foraminifera *E. williamsoni* and *H. germanica*, the following conclusions may be drawn:

(1) Post-mortem test dissolution rates suggest that dead assemblages of *E. williamsoni* and *H. germanica* from natural environments may be more vulnerable to OA effects in comparison to living assemblages that possess biological mechanisms to moderate dissolution effects. This notable impact by OA may alter accumulation and preservation of $CaCO_3$ in shallow-water benthic ecosystems such as intertidal mudflats, ultimately affecting the carbon cycling over the coming decades and centuries.

(2) Gross dissolution rates driven by OA may have the potential to reduce by up to 44% of the annual net carbonate produced by living assemblages of *E. williamsoni* and *H. germanica*. These negative impacts may cause unknown consequences for foraminiferal abundance, distribution and diversity, food web-dynamics and their role in the biogeochemical processes (e.g. nutrient fluxes, carbon sink, etc.) of coastal environments.

Chapter 8. Discussion

8.1 General Discussion

Two dominant (~90 % in abundance) and co-occurring benthic foraminifera species (*E. williamsoni* and *H.* germanica) were collected from local intertidal sediment in the Northeast of Scotland and used for this experimental programme of research. Their dominance and occurrence have been reported in live and dead assemblages in many intertidal habitats, including UK coastal waters (Murray 1983; Cearreta 1988; Alve & Murray 1994; Austin 2003; Müller-Navarra et al. 2016). This suggests that their ecological role and their contribution to biogeochemical processes (e.g. nutrient fluxes, carbon sink, etc.) in coastal benthic environments are likely to be highly important at local and regional scales.

Their ecological and biogeochemical importance, combined with limited evidence describing multiple biological responses of benthic foraminifera from intertidal mudflats to future OA scenarios, led to a number of increasingly complex laboratory experiments (Chapters 3-7). This allowed the quantification of the impacts of future declining pH associated with elevated atmospheric CO_2 concentrations on benthic calcifying organisms, particularly on *E. williamsoni* and *H. germanica*.

The focus of each experimental chapter and the specific hypotheses stated at the start of the thesis allow some broad general outcomes to be identified, as detailed below. Each hypothesis is addressed in turn, referring to the relevant data chapter.

1. Determine the initial effects of short-term high CO_2 concentrations and low pH in seawater on survival, growth/calcification and taphonomic processes (e.g. dissolution) of the benthic foraminifera *E. williamsoni* and *H. germanica* (**Chapter 3).**

Hypothesis One: OA will negatively affect survival rate, test size and weight, growth/calcification rate, morphology and post-mortem dissolution **(Chapter 3).**

The hypothesis that OA would have a measurable impact on benthic foraminiferal species was partially supported for *E. williamsoni* but not for *H. germanica*. The latter was excluded from any analysis due to the extremely low number of retrieved specimens at the end of experiments.

In the case of *E. williamsoni*, parameters such as maximum test diameter, test weight and growth rate of surviving specimens did not exhibit substantial changes across different pH levels, suggesting that *E. williamsoni* was efficiently adapted to cope with the experimentally elevated short-term CO_2 conditions. Only further longer-term CO_2 experiments (over 4 weeks, as in Chapter 4) would confirm the potential adaptability of *E. williamsoni* to future OA conditions. Longer-term studies also allow the observation of more deleterious effects in morphology and other biological parameters. These effects have been reported for other coastal benthic foraminifera, where survival rates, maximum test diameter, test weight and growth rates changed due to a longer exposure to high CO_2 concentration seawater (Kuroyanagi et al. 2009; Sinutok et al. 2011; Khanna et al. 2013, Khanna 2014; Prazeres et al. 2015).

In the present study, despite the decreasing trend observed in survival rates of *E. williamsoni* and *H. germanica* across lowering pH, the low number of surviving specimens in each pH condition (Chapter 3 Section 3.3.4) is clearly a limiting factor to determine reliable effects of short-term OA on survival rate. Consequently, it was suggested that further CO_2 studies should considerably increase the number of initial numbers of specimens used in each experiment. This simple approach does not attempt to reduce the high mortality rates observed; however, it will help retrieve from experiments a greater number of surviving specimens allowing the distinction between natural mortality and OA-induced mortality across lowering pH levels.

In addition, the extremely high mortality rates of *E. williamsoni* and *H. germanica* in a natural pH of 8.1 (control treatment) may potentially be linked to a direct response of foraminifera to controlled-temperature conditions of 13°C in the seawater, as they typically inhabit tidal environments that experience diurnal temperature fluctuation. If further experiments are performed and similar mortality rates observed at pH 8.1 still remain as high at a constant temperature, then this may be the major limiting factor to successfully obtain a larger amount of surviving specimens at the end of experimental periods.

Despite this short time period, SEM imaging, most notably at a pH of 7.3, demonstrates that 4 weeks was sufficient for observing alterations in early stages of test morphology induced by OA (i.e. test surface and feeding ornamentation) of *E. williamsoni*. These morphological alterations were similar to those reported for other calcareous benthic foraminifera exposed to longer-term CO_2 experiments (Haynert et al. 2011; Haynert et al. 2012; Khanna et al. 2013; Khanna 2014).

Changes observed in test weight-size relationship of live and recently dead assemblages, again mainly at a pH of 7.3, clearly confirmed the differential test dissolution susceptibility between both types of foraminiferal groups under similar CO_2 and low pH levels as previously reported for other benthic foraminifera (Haynert et al. 2011; McIntyre-Wressnig et al. 2013). These initial results not only support the suggestion that live and dead foraminiferal assemblages will be severely affected by future OA conditions, but also indicate that taphonomic process such as carbonate dissolution of calcifiers, which naturally occurs during diagenesis, may be enhanced by future high CO_2 levels. This fact may modify the accumulation and preservation of benthic foraminifera in intertidal sediments, with implications for the marine carbon cycle, as this is an important carbon sink. This first experimental chapter shows clear qualitative results, but future dissolutions experiments (carried out later in Chapter 7) may help determine quantitatively the implications of higher OA-induced dissolution rates on dead foraminiferal assemblages and associated effects on marine carbon cycle.

In general, findings from this experimental study (Chapter 3) provide an initial insight into the threshold (i.e. minimum exposure time, pH level) at which future OA impacts can be measured in benthic calcareous foraminifera such as *E. williamsoni*. However, care should be taken when these preliminary results are used to compare the short-term impacts of OA on survival, growth and calcification with other benthic foraminiferal species or communities.

2. Determine multiple biological responses of *E. williamsoni* and *H. germanica* exposed to short-term high CO_2/ low pH levels and concomitant low carbonate ion concentrations [CO_3^{2-}] (**Chapter 4).**

Hypothesis Two: Multiple biological parameters will help determine that foraminiferal growth and calcification of benthic foraminifera *E. williamsoni* and *H. germanica* are adversely affected as a result of short-term exposure to high CO_2 concentration seawater **(Chapter 4).**

Based on the uncertain outcomes from Chapter 3 and following suggestions for future experiments, initial numbers of specimens to be used in each pH treatment were considerably increased; consequently, the numbers of surviving specimens were also greater. This experimental improvement not only allowed benthic foraminifera *E. williamsoni* and *H. germanica* to be exposed to a longer timescale (7 weeks) of high CO_2/low pH conditions at a constant temperature of 13°C in seawater, but also enabled estimation of natural mortality and OA-induced mortality. The latter strongly differed between both species across lowering pH levels, suggesting that *H. germanica* may be less sensitive/better adapted to future OA scenarios. In general, these mortality results may suggest a potential decline in total production and preservation of biogenic $CaCO_3$ in marine sediments of intertidal habitats. This would directly modify carbon cycling, matching present expectations for other coastal environments as a consequence of future OA conditions (Sinutok et al. 2011).

Despite this clear trend in mortality with decreasing pH, the observed mortality for both foraminiferal species in a natural pH of 8.1 (control treatment) is still high (~50%). This suggests that the experimental conditions of constant temperature (13°C) rather than other environmental stressors (e.g. salinity) may be again a limiting factor, reducing the numbers of surviving specimens in a much longer-term experiment than 7 weeks. A salinity of ~34 ppt was maintained throughout the experiment, matching the salinity measured at the sampling site; therefore, this environmental factor is unlikely to be a potential contributor to the high mortality rates observed at natural pH conditions.

Results in this chapter also indicated that despite the similarities in multiple ecological features (i.e. test composition, habitat, etc.) between *E. williamsoni* and *H. germanica*; short-term CO_2 exposure appeared to trigger species-specific responses. These differential responses observed in key biological parameters such as growth, test weight and SNW appear to contradict the initial hypothesis two which implied that growth/calcification of both benthic foraminiferal species would be similarly impacted by OA.

In contrast to *E. williamsoni*, *H. germanica* displayed an increased growth under OA conditions, which may be a potential indicator of an ecological advantage for *H. germanica* over *E. williamsoni* and other co-occurring calcareous species under future coastal OA scenarios in intertidal habitats. These contrasting findings (i.e. species-specific responses) in growth and calcification for *E. williamsoni* and *H. germanica* are similar to those reported for other co-occurring calcareous foraminiferal species from coral reef habitats, where one foraminiferal species fares better under OA conditions than other foraminifera (Hikami et al. 2011; McIntyre-Wressnig et al. 2013; Prazeres et al. 2015).

The differential sensitivity observed between *E. williamsoni* and *H. germanica* to OA in this and previous work has been linked to biochemical mechanisms such as Ca-ATPase and Mg-ATPase activities (Prazeres et al. 2015). These enzymatic activities are directly linked to intracellular biomineralization processes, which can notably differ between calcareous species under the same environmental stressors as previously reported by Prazeres et al. (2015). In the present study, although growth-related enzymatic activities were not measured in the benthic foraminifera species, it is likely that an altered underlying biomineralization mechanisms combined with the low availability of carbonate ions in seawater due to lowering pH, may strongly influence the differential responses observed (growth and test weight) measured between the two species.

In terms of test weight, although *E. williamsoni* and *H. germanica* both showed declining trends with reduced pH levels, *E. williamsoni* exhibited a more remarkable decline in test weight. These results are more likely to be linked to a modification of internal test structure (e.g. altered thickness due to reduced biomineralization process) rather than only a greater effect of test dissolution rates, both induced by OA. In addition to this, as only intact tests of live specimens were used for this analysis, thinner chambers would explain a reduction in test weight at lowest pH conditions as reported for *E. williamsoni* under experimentally acidified seawater (Allison et al. 2011).

SEM imaging indicated that live assemblages of *E. williamsoni* and *H. germanica* were similarly vulnerable to OA and associated dissolution rates in the short-term (7 weeks), supporting previous studies on comparable timescales illustrating similar effects for other benthic foraminifera (Haynert et al. 2011; McIntyre-Wressnig et al. 2013). In this study,

test morphology (i.e. test surface and feeding ornamentation) was severely affected by lowered pH, matching observations in Chapter 3. This is likely to affect the feeding behaviour of benthic foraminifera (experiment carried out in Chapter 5), consequently affecting their carbon uptake derived from primary producers (e.g. diatoms) with severe implication for food web carbon processing (Middleburg et al. 2000; Middelburg 2018).

3. Estimate the feeding efficiency and carbon (^{13}C) uptake by benthic foraminifera exposed to short-term high CO_2 concentrations and low pH seawater under laboratory conditions with ^{13}C-labelled food supply (**Chapter 5).**

Hypothesis Three: Diatom consumption and ^{13}C uptake by *E. williamsoni* and *H. germanica* will be substantially affected due to damaged feeding structures as a consequence of short-term exposure to high CO_2 concentrations and low pH in seawater (precondition). This will alter foraminiferal feeding/sequestration mechanisms of primary production and also the subsequent amount of energy transfer within the marine food web **(Chapter 5).**

In this study, the observations of corroded feeding ornamentation of *E. williamsoni* and *H. germanica* linked to short-term exposure to increasing CO_2 and lowering pH conditions (precondition) were identical to those reported only for *H. germanica* exposed to a long-term CO_2 experiment (32 weeks) (Khanna et al. 2013). However, subsequent feeding experiments between both studies clearly differed in results. For instance, Khanna's feeding experiments exhibited mostly uncertain results of changes of carbon uptake by *H. germanica* driven by OA, likely due to the relatively low number of specimens and

replicates used in each pH treatment. In the case of the present study, this was addressed by improving the experimental design, making it easier to quantify the negative impact of future OA conditions on diatom-derived-carbon uptake mainly by *E. williamsoni*.

Common ecological features between *E. williamsoni* and *H. germanica*, widely discussed across several chapters of this research (e.g. Chapter 4, Section 4.4.6) assumed that feeding ornamentations and diatom-derived carbon uptake of both foraminiferal species would be similarly compromised by short-term exposure to high CO_2 concentrations and low pH in seawater. However, this underlying assumption that similar impacts of OA on both foraminiferal species (hypothesis three) is not supported by the quantitative outcomes from feeding experiments carried out in this study. Clear species-specific responses were observed as a result of a single pulse of labelled-diatom feeding. This highlights the opportunistic features of *E. williamsoni* (e.g. greater ^{13}C-uptake rates during the first hours of feeding experiment), and also suggests that *H. germanica* would prefer other carbon sources such as large diatoms (Austin et al. 2005) or bacteria (Brouwer et al. 2016) rather than *Navicula sp.*

In the case of *H. germanica*, occasional shifts of their diet (i.e. preference for bacteria over diatoms) can be triggered by unfavourable environmental conditions (i.e. experimentally induced hypoxia) in shallow water habitats (Brouwer et al. 2016). However, in this study, besides experimental conditions, the combination of type (i.e. smaller size than usually naturally selected) and condition-derived quality of diatoms provided (i.e. fresh and active cells) may have exacerbated the food selectivity of *H. germanica*. For instance, *H. germanica* was fed with bundles of freshly collected labelled-diatom *Navicula sp.* with

10µm diameter rather than being fed with artificial labelled-phytodetritus (Moodley et al. 2000; Wukovits et al. 2017) or freshly large diatoms of approx. 100 µm diameter (Austin et al. 2005). The latter appears to fit better with food preferences and feeding behaviour of *H. germanica* reported by Austin et al. (2005).

The natural features showed by labelled-*Navicula sp.* (small size and active cells) may also have implied a higher demand of energy on food acquisition (grazing), rendering *Navicula sp.* less desirable at least for *H. germanica*. This fact might partially explain the low ^{13}C uptake rates of *H. germanica* across pH treatments reported in this experimental feeding study (Chapter 5, Section 5.3.2). In addition, it is possible that OA conditions could also have altered internal structures of *H. germanica* linked to main carbon storage in foraminifera (e.g. lipid droplets). It is widely recognised that the alterations in size of cellular ultrastructures of other benthic foraminifera *Ammonia spp.* and *Ammonia parkinsoniana* are results of being directly exposed to unfavourable environmental conditions such as heavy metal contamination (Frontalini et al. 2015; Frontalini et al. 2018) or anoxia (Koho et al. 2018). Although this was not directly measured in this study, modified primary internal structures of *H. germanica* might also have led to a change of both habit-based diet and increased difficulty in foraging activities to obtain diatom-organic material such as chloroplasts.

In addition, based on information provided by previous feeding experiments on low preference of *H. germanica* for *D. tertiolecta* (Wukovits et al. 2017) and *Navicula sp.* (Chapter 5 of this study); it is likely that growth of *H. germanica* observed in this study is almost certainly related to the uptake of *Rhodomonas salina* rather than *D. tertiolecta* and

Navicula sp. which were other food sources provided throughout the CO_2 experiments performed for this research (Chapter 3-6). This potential food affinity of *H. germanica* for *R. salina* would support the current experimental design and procedures followed at the onset this research, mainly linked to feeding *E. williamsoni* and *H. germanica* with three different food sources to avoid high mortality rates associated with starvation or non-feeding periods due to food selectivity.

The alteration of feeding behaviour and concomitant feeding preferences of benthic foraminifera under future OA conditions still needs to be profound studied to precise the implications for carbon cycle in coastal temperate environments.

4. Examine the impacts of a combined effect of elevated temperature and increased CO_2 concentrations on benthic foraminifera growth and calcification (**Chapter 6).**

Hypothesis Four: A synergistic effect, of combined warmer and more acidic seawater, will reduce growth/calcification in benthic foraminifera **(Chapter 6).**

It has been clearly demonstrated that intertidal zones from temperate regions experience continuous temporal and spatial variabilities of temperature; hence, a potential rise of 2°C in the average seawater temperature projected for ocean upper layer (Collins et al. 2013) and Scottish coastal waters (Hiscock et al. 2001) by 2100, certainly will be within the thermal tolerance window of intertidal benthic taxa, including foraminifera. However, this study highlights that the combined effects of OA and warming (2°C higher than comparative experiments conditioned at 13°C) have the potential to cause variable biological responses in benthic foraminifera. For instance, reproduction (e.g. observed

through the increasing initial number of specimens in each pH condition) was positively enhanced under experimental conditions whereas test weight, survival rates and growth/calcification rates of both benthic foraminifera were negatively affected. The outcomes from these experiments agreed with the initial hypothesis 4 that an interactive effect of elevated temperature and increased CO_2 concentrations reduced growth/calcification rates of *E. williamsoni* and *H. germanica.*

Similar negative impacts induced by these two environmental stressors on growth and other biological parameters have also been reported for coral reef-associated foraminifera (Sinutok et al. 2011; Schmidt et al. 2014), echinoderm species (Gooding et al. 2009) and a wide range of other marine organisms from different ecosystems (Hoegh-Guldberg & Bruno 2010; Byrne 2011; Kroeker et al. 2013; Li et al. 2016) including transitional marine environments such as intertidal zones (Findlay et al. 2008; Russell et al. 2013; Meadows et al. 2015; Sarmento et al. 2017).

For instance, in the case of intertidal benthic foraminifera, Meadows et al. (2015) reported variable responses in foraminiferal abundance across pH levels between 8.0 and 6.7 and warming seawater (4°C higher than control treatment). These uncertain results are most likely explained by the high variability in abundance found across all treatments at the end of the experiment (e.g. a greater abundance at the lowest pH than at natural pH treatment). This fact was potentially associated with predation effect of other benthic taxa upon foraminifera rather than direct effects of experimental pH conditions (Meadows et al. 20015).

In the case of the present study, in order to avoid natural predation effects affecting foraminiferal abundance throughout the CO_2 experiments, *E. williamsoni* and *H. germanica* were isolated from natural sediment where potentially co-occurring meiofaunal taxa may have preyed upon foraminifera (see Chapter 2, Section 2.4). By excluding predation, this experimental design maximised the potential of collecting more conclusive data of direct effects of changing climate on multiple parameters of intertidal benthic foraminifera.

Previous work studying constant temperature and CO_2 concentrations appear to be applied to ecosystems with relatively low temperature and CO_2 variability rather than intertidal zones exhibiting extreme physicochemical conditions. However, under climate change scenarios, current coastal environments including intertidal habitats will remain flooded due to future sea level rise (Nicholls & Cazenave 2010), reducing notably the amplitude of variability of several environmental parameters observed at different scales. This may mean intertidal benthic habitats may become relatively more stable due to a reduction between the minimum and maximum values of environmental stressors such as temperature. These environmental changes may drastically disturb physiological mechanisms of intertidal benthic communities widely adapted to wide fluctuations of temperature and CO_2, ultimately detrimentally affecting parameters such as metabolism, survival/mortality rates, growth rate and reproduction (Ceballos-Osuna et al. 2013; Sarmento et al. 2017).

If this would be the case, experimentally prolonged constant temperature rather than varying conditions applied to all CO_2 experiments with benthic foraminifera may partially explain the underlying cause of high mortality rates observed across all pH treatments, particularly in ambient pH of 8.1(control) and CO_2 levels of ~ 380 µatm. This assumption

is supported by experimental evidence showing that higher mortality of two types of invertebrates occurred at a constant temperature rather than diurnally varying conditions (Cox & Rutherford 2010)

Since survival rates observed across all treatments performed at 15°C were lower than those estimated in a similar experiment at 13°C, results from this study were conclusive in identifying a synergistic effect rather than an antagonistic effect of elevated temperature and CO_2 in seawater on benthic foraminifera. In addition, although *H. germanica* was notably less negatively affected by the combination of both environmental factors when compared to *E. williamsoni*, the relatively small difference in survival rates between both species is not conclusive in suggesting a potential species-specific response to OA and ocean warming.

Reproduction events observed particularly at 15°C were potentially triggered by the interaction of ocean acidification and warming, resulting in ecologically positive effects on foraminifera. However, caution should be taken when foraminiferal reproduction occurred within experimental settings (e.g. controlled-culturing systems) because an overestimation of survival rates may be due to newly unexpected young specimens that alter the number of foraminifera used at the onset of experiments. This problem is usually solved by recording the initial number of specimens and other parameters (e.g. tests size and weight) before experiments (Hintz et al. 2004; Kuroyanagi et al. 2009; Haynert et al. 2011; Prazeres et al. 2015); thus, any increase/decrease in numbers of individuals can be easily detected.

In the present study, the recognition of new specimens derived from sporadic reproduction events was carried out via detection of an increased initial number of individuals and observation of small individuals (i.e. <100µm) with characteristic lack of calcein-labelling signature. These new individuals also displayed either empty tests or cytoplasm degradation which helped record them as dead specimens to be used for further analyses. Undoubtedly, pre-labelling incubation with calcein, widely used in foraminifera experiments (e.g. Bernhard et al. 2004; Dissard et al. 2009; Dissard et al. 2010), was clearly helpful in distinguishing live specimens and avoiding overestimations or underestimations of survival rates.

Finally, results from this study contribute new evidence to the existing literature about the vulnerability that may be experienced by foraminifera under projected OA and rising temperature, both linked to the increasing atmospheric CO_2 emissions (Canadell et al. 2007; Ingels et al. 2018). Furthermore, it is expected that climate change scenarios (e.g. sea level rise, increased temperature and elevated CO_2 concentration) may impact negatively intertidal habitats by declining biomass, diversity and ecological interactions with other taxa (e.g. prey-predators) and processes which ultimately would alter food web dynamics and would simplify benthic community structures of calcifying species such as benthic foraminifera.

5. Quantify the influence of **s**hort-term high CO_2/low pH levels on post-mortem dissolution of *E. williamsoni and H. germanica* (**Chapter 7).**

Hypothesis Five: Enhanced post-mortem dissolution rates induced by high CO_2/ low pH levels will severely affect the morphology of benthic foraminifera *E. williamsoni* and *H. germanica* **(Chapter 7)**.

Findings from this chapter support initial hypothesis five, which indicated that benthic foraminiferal morphology of dead assemblages would be substantially impacted by OA-induced dissolution. Furthermore, the outcomes of this work on altered foraminiferal morphology due to future changes in carbonate seawater chemistry are based on a quantitative approach rather than only a qualitative approach. As such, short-term test dissolution rates, firstly calculated for dead assemblages of benthic foraminifera (post-mortem effect), were used in a simple model to estimate the potential changes in net $CaCO_3$ productivity (living assemblages) of dominant benthic foraminifera under future scenarios of high CO_2 and low pH levels in seawater (see Chapter 7 Section 7.4.3).

As these types of calculations are rare in current literature for foraminifera and other calcifiers, quantitative outcomes from this study have helped point out that OA and associated dissolution processes have the potential to strongly affect living and dead assemblages of foraminifera, ultimately affecting local production and accumulation of biogenic carbonate in intertidal mudflats habitats.

In the case of living calcifying taxa, the integrity of their shells is maintained by active biochemical mechanisms of compensation in response to environmental stressors (Haynert et al. 2011). However, these evolutionary trade-offs may induce changes in energy allocation, carbonate deposition rates, mineralization processes (e.g. calcification), growth,

survival rate and reproduction (Talmage & Gobler 2010; Gaylord et al. 2011; Pan et al. 2015; Duquette et al. 2017). Some of these direct effects were observed in benthic foraminifera Chapters 3-6. Additionally, due to increasing dissolution rates, calcifiers may exhibit a considerable reduction in elasticity, inner toughness of skeletons and crystallographic control (Fitzer et al. 2014; Duquette et al. 2017). These may be the underlying factors causing severe structural damage observed as anomalies in internal and external morphology such as cracks and fractures in live benthic foraminifera (Le Cadre et al. 2003; Allison et al. 2011; Haynert et al. 2011; Khanna et al. 2013).

This altered foraminiferal morphology mainly induced by increasing CO_2 levels was also confirmed across several chapters of this research (see Chapters 3-6). In general, the modification of morphology may render calcifying organisms more vulnerable to predators and lead to a greater dissolution effect. These ecological interactions with other taxa (indirect effects) rather than direct effects of CO_2-induced acidification may largely contribute to the vulnerability of calcifying organism and their reduced ecological success (e.g. abundance).

In terms of productivity, taking into account that the projected decline of up to 44% in $CaCO_3$ production of benthic foraminifera was calculated based on post-mortem dissolution rates for *E. williamsoni* and *H. germanica*, this projection may vary between dead and living assemblages of calcareous foraminifera, as they possess different test degradation rates under the same levels of high CO_2 /low pH levels (McIntyre-Wressnig et al. 2013). This fact was experimentally demonstrated in this study because dead (mostly empty tests) exhibited substantially more dissolved and corroded tests in comparison to surviving

specimens from previous experiments (Chapters 3-6). The observation of an elevated number of completely dissolved tests may help support the use of bulk test weight rather than size distribution of benthic foraminifera at the start and at the end of dissolution experiments. Thus, changes in bulk weight of foraminifera across different pH levels were useful in demonstrating quantitatively the impacts of OA on intertidal foraminifera.

Finally, regardless of the substantial difference in test degradation in living and dead foraminiferal assemblages directly linked to prevailing environmental conditions acting at different temporal and spatial scales in coastal zones, future OA conditions may induce a considerable increase in dissolved inorganic matter which could be easily transported off-shore. This would substantially reduce the local capacity of accumulation of biogenic carbonate in intertidal sediments widely recognize as biologically productive habitats, and directly affect the marine carbon cycle.

8.2 Limitations and future work

Experimental findings from this thesis have provided substantial and quantitative information about the potential impacts of OA on two dominant foraminiferal species *E. williamsoni* and *H. germanica*. This builds on current knowledge, and also provides additional insight into which processes are likely to be affected by OA, and how this may vary according to species. However, improvements in methodology can always improve deductions from research, and due limitations, caution must be taken when interpreting experimental results. Despite this, the work presented here has helped improve overall understanding of the future impact of high CO_2/ low pH levels at a individual level using

benthic foraminifera as a model organism.

Suggested future benthic foraminifera studies already mentioned within each chapter of this thesis may be strengthened by addressing the limiting factors experiencing during this research. Some key points that may help to provide new insights into the impacts of OA on intertidal foraminiferal species are detailed below.

1. As observed in this study and other studies (i.e. Austin 2005), the mortality of benthic foraminifera may exceed 50 % of the initial number of specimens used in experiments at a natural pH of 8.1. Undoubtedly, these low survival rates clearly constrained the present research to perform only short-term CO_2 experiments (no longer than 7 weeks) with a limited number of replicates. The potential explanation for these high mortality rates observed in laboratory controlled-conditions was previously discussed above (Chapter 8 Hypothesis 4).

The observed difficulty in implementing large-scale approaches (e.g. longer-term experiments) is still unsolved at least for *E. williamsoni* and *H. germanica*. However, the number of replicates with greater numbers of specimens for each pH treatment could be overcome via either prolonged field-work sampling to collect more natural sediment containing living foraminiferal assemblages or, in turn, culturing natural benthic foraminiferal assemblages positively influenced by a range of increased incubation temperatures to induce reproduction under laboratory conditions. Consideration should be given to determine the optimal temperatures and food source for foraminiferal reproduction, and these factors are not well understood yet for most species including for

E. williamsoni and *H. germanica*.

These two potential solutions proposed may help surpass the initial number of specimens used in each experiment of this research (~20.000 specimens in total). However, this implies intensive laboratory work to be done by several available people instead of one or two persons to isolate a much larger number of living specimens at the onset and the end of experimental periods. Therefore, the availability of more people to collaborate throughout all experimental stages is critical.

2. The difficulty in culturing several natural diatom species to provide benthic foraminifera during the experimental period limited the present feeding experiment to provide only *Navicula sp.* for *E. williamsoni* and *H. germanica*. However, outcomes from experiment helped demonstrate that mainly *H. germanica* feeds on specific diatom species (see Chapter 5). Therefore, in other to complement these findings, laboratory protocols for diatom culture should be modified in order to be successful in culturing several strains of natural diatoms which could be subsequently used to determine food preferences via carbon and nitrogen uptake rates by *E. williamsoni* and *H. germanica* assemblages under specific pH level. Additionally, in a more realistic approach, as primary producer (i.e. diatoms) and primary consumers (e.g. foraminifera) will face the same future OA conditions, diatoms should be also culture under projected pH conditions to determine initially whether their physiology and nutritional quality change under changing environmental conditions before feeding experiments. These physiological changes were reported for marine diatoms *Thalassiosira weissflogii* and *Dactyliosolen fragilissimus* which exhibited high sensitivity to near-future OA and warming scenarios (Taucher et al.

2015).

3. In this study, results from experiments demonstrated mostly negative impacts of future OA on benthic foraminifera *E. williamsoni* and *H. germanica*. However, observations were only limited to changes in morphology and biological parameters such as growth, weight, diameter, feeding behaviour (species-specific response) and reproduction. Thus, future design experiments should also consider measures of biochemical parameters (i.e. enzymatic activity) (i.e. Prazeres et al. 2015), complementary observations of internal structures of benthic foraminifera (i.e. images of test structure and cell ultrastructures) (i.e. Nomaki et al. 2015; Prazeres et al. 2015; Eder et al. 2016; Koho et al. 2018) which may be also compromised as a consequence of environmentally unfavourable conditions. These techniques were not applied in this study due to the high cost involved in the analysis processes.

The study of the alteration of complementary parameters mentioned above is critical for an integrative understanding of how biological and biochemical mechanism (e.g. at a cellular level) of *E. williamsoni* and *H. germanica* will respond to future increasing CO_2 and lowering pH conditions.

4. When a high abundance of 'live' specimens was no longer a limiting factor for CO_2 experiments, the impact of OA on multiple biological parameters of specific size fractions could be assessed to determine differential effects among different foraminiferal growth stages. Findings from experiments performed in Chapters 3, 4 and 6 indicated a reduction in size, weight and chamber addition due to declining pH, so it would be worth performing

more experiments following this approach to understand the specific responses to OA at different stages of foraminiferal life cycle, as similar to those negative impacts reported for different development and growth of other foraminifera (Haynert et al. 2011), oyster (Bylenga et al. 2017; Lemasson et al. 2017), sea urchin (Pan et al. 2015; Lamare et al. 2016), crab (Ceballos-Osuna et al. 2013), etc.

5. A reduction in the experimental conditions (from four to three pH levels) occurred in Chapter 6 mainly due to laboratory remodelling work appears not to have an impact on the interpretation of the results obtained on interactive effects of short-term OA and warming experiment on growth of *E. williamsoni* and *H. germanica.* However, as the negative impacts of OA conditions are in some cases observed as non-linearly responses (i.e. parabolic response), it is potentially important to have as many pH levels as possible (see Chapters 3-5) not only to notice how specific parameters slightly change across several lowering pH conditions but also to be comparative to other similar laboratory-based experimental conditions (e.g. Kuroyanagi et al. 2009).

6. The difference in temperature of only 2°C between CO_2 experiments appears not to be a limitation to observe negative impacts on growth/calcification rates of both benthic foraminifera *E. williamsoni* and *H. germanica*. However, future studies may require the increase of the temperature range similar to those observed in a natural setting to determine temperature tolerance limits for each foraminiferal species under specific pH level.

7. As temperature variability would help solve the mortality issue linked to constant temperature, current laboratory facilities and experimental designs both should be adjusted

to install new equipment allowing temperature regime that fluctuates over a 24 cycle/ mimics tidal flux.

8.3 Conclusions

This research has been successful in identifying and quantifying the impacts of future OA on biological parameters of two dominant benthic foraminiferal species *E. williamsoni* and *H. germanica*. Hence, the data obtained across different chapters (3-7) could help to better understand potential changes for benthic calcifying communities of intertidal habitats, which may be similarly affected by future lowering pH as projected for other coastal ecosystems, such as coral reefs (Kuroyanagi et al. 2009; Fujita et al. 2011; Hikami et al. 2011; Sinutok et al. 2011; Uthicke & Fabricius 2012; Fabricius et al. 2011; Uthicke et al. 2013).

Outcomes from short-term CO_2 experiments performed for 4 and 7 weeks (Chapter 3-7) provide important information that is currently limited in the literature for benthic foraminiferal species from coastal habitats, such as intertidal mudflats (Khanna et al. 2013; Khanna 2014). Furthermore, findings from this present study broadly support results from previous studies on benthic foraminifera, where varying responses to OA were observed (Kuroyanagi et al. 2009; Fujita et al. 2011; Vogel & Uthicke 2012; McIntyre-Wressnig et al. 2013; McIntyre-Wressnig et al. 2014; Prazeres et al. 2015; Wit et al. 2016). These impacts ranged from positive, unaffected and negative responses of benthic foraminifera to future CO_2/low pH levels. These biological responses depended on the species used in the experiments, exposure time and other environmental factors acting alongside CO_2/pH conditions.

However, the data generated also raise new research questions that should be assessed in future short- and long-term studies. Furthermore, outcomes from this study should be applied carefully to other calcareous foraminiferal species from other marine habitats because these findings are from laboratory studies and do not attempt to predict ecological consequences in natural settings where biotic (e.g. predation, competitors, food availability, etc.) and abiotic factors (e.g. temperature, salinity, oxygen, dissolution processes, etc.) interact simultaneously alongside high CO_2/low pH levels at different temporal and spatial scales.

Based on the results of short-term studies on the biological responses of multispecies assemblages of benthic foraminifera to OA, the following conclusions may be drawn:

1. The minimum exposure time to high CO_2/low pH levels for *E. williamsoni* and *H. germanica* to display a negative impact on test morphology (i.e. test surface and feeding ornamentation) was 4 weeks.

2. Test weight and SNW of *E. williamsoni* were more negatively affected by OA than *H. germanica* at 13°C, mainly between a seawater pH of 7.7 and 7.3.

3. For *E. williamsoni*, survival rates were reduced by up to 40 % under lowering pH conditions at 13°C, whereas *H. germanica* was unaffected, displaying a species-specific response to OA. However, survival rates of both foraminiferal species were reduced by up to 20% due to the combined effect of elevated CO_2 and increased temperature (+2°C with respect to previous experiments).

4. Growth rates (estimated via a chamber addition method of assessment) for *E. williamsoni* and *H. germanica* were reduced by up to 40 % by the synergistic effect of elevated CO_2 and increased temperature (i.e. at 15°C, +2°C with respect to previous experiments). These findings are at odds with observations of species-specific responses, such as unaffected growth for *E. williamsoni* and enhanced growth of up to 20 % for *H. germanica* due to lowering pH conditions at 13°C. Observations of these negative and positive impacts from OA on foraminifera were more pronounced at a range of seawater pH between 7.7 and 7.3.

5. Stable isotope experiments demonstrate that despite the pre-condition of compromised feeding structures, *E. williamsoni* and *H. germanica* were able to feed on labelled diatoms *Navicula sp.* but at considerably different rates. For example, uptake rates for *E. williamsoni* were one order of magnitude higher than *H. germanica* which showed a species-specific response to OA due to food selectivity.

In addition, the carbon uptake for *E. williamsoni* was reduced up to 50% at lowest pH levels during the first 3 hours of experiments. However, these carbon uptake rates were reduced by one order of magnitude across pH treatments 72 hours after starting the experiments. This demonstrates the opportunistic capacity of *E. williamsoni* to feed on the diatom *Navicula sp.* provided as the only food source during the feeding experiments.

6. The dissolution rates calculated in this study have increased our knowledge of the future impacts of OA on benthic calcareous foraminiferal populations composed of dead and living assemblages. For example, OA can enhance post-mortem test dissolution rates, rendering seawater with a pH of 7.7 and 7.3 more corrosive (2- and 3-fold, respectively) for

dead assemblages than seawater at an ambient pH of 8.1. This may negatively affect their morphology, net accumulation and overall preservation in sediments. These results will ultimately alter both the long-term storage of inorganic carbon and the carbon cycling in coastal benthic ecosystems.

7. In terms of negative impacts of OA-driven dissolution on living assemblages, the model used for estimations of foraminiferal productivity under future OA scenarios indicated a decline in net foraminiferal production of up to 44 % if a pH of 7.3 was attained.

8. This study provides important information to support the suggestion that high test dissolution rates combined with reduced calcification rates and elevated temperature driven by near-future high CO_2 concentrations will negatively impact foraminiferal calcareous species, potentially affecting their role in biogeochemical cycles, food-web dynamics (i.e. predation, foraging of diatoms, etc.) and ecological services provided by coastal habitats, such as intertidal mudflats.

9. Coastal habitats play a key role in the global carbon cycle (Wollast 1998; Moodley et al. 2000; Van Colen et al. 2014), and in intertidal habitats such as estuaries, much of this is mediated by the activity and presence of benthic calcifying organisms such as foraminifera. This work illustrates the variety of responses of these species to future climate scenarios, through a number of short- and medium-term experiments at predicted CO_2 and temperature levels. The overall response was negative, with reduced calcification and increased dissolution, which has implications for carbon sequestration in intertidal habitats. Future environmental changes, driven by elevated atmospheric carbon dioxide levels, will

directly impact carbon cycling in these habitats and may have consequences for the global carbon cycle, tipping the balance between sequestration and production of carbon.

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