THE BIOLOGICAL RESPONSE OF FORAMINIFERA TO OCEAN ACIDIFICATION

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The Biological Response of Foraminifera to Ocean Acidification

Nikki Khanna

A thesis submitted for the degree of Doctor of Philosophy

School of Biology, University of St Andrews

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Abstract

Elevated atmospheric concentrations of carbon dioxide (CO_2), partly driven by anthropogenic activity, are decreasing the pH of the oceans. This thesis aimed to assess the biological response of foraminifera to ocean acidification. Foraminifera are single-celled organisms that form the dominant component of many marine communities. A series of laboratory experiments were carried out on benthic intertidal foraminifera from the Eden and Ythan estuaries, NE Scotland, to assess the impacts of ocean acidification.

The responses of two dominant intertidal species of foraminifera (*Haynesina germanica* and *Elphidium williamsoni*) to ocean acidification were initially investigated in a short-term (6 week) experiment. Multiple species and multiple stressors (seasonal temperature regime and elevated CO_2) were then incorporated in a long-term (18 month) mesocosm study to investigate the physiological consequences (e.g. survival, growth) of ocean acidification.

Survival of both *Haynesina germanica* and *Elphidium williamsoni* was significantly reduced under low pH conditions. Live specimens of both these calcareous species were however recorded at low pH, in reduced numbers. Following long-term exposure to ocean acidification, foraminiferal populations were still dominated by calcareous forms. Agglutinated foraminifera were recorded throughout the long-term incubations but their numbers were not high enough in the initial sediment collections to allow them to contribute significantly to the populations. Overall, survival of all foraminifera was greatly reduced in elevated CO_2 treatments. Temperature effects were observed on foraminiferal survival and diversity with the largest CO_2 effects recorded under the seasonally varying temperature regime.

Foraminiferal test damage for all live species was also highest under elevated CO_2 conditions. Test dissolution was particularly evident in *Haynesina germanica* with important morphological features, such as functional ornamentation, becoming reduced or completely absent under elevated CO_2 conditions. A reduction in functionally important ornamentation could lead to a reduction in feeding efficiency with consequent impacts on this organism's survival and fitness. In addition, changes in the relative abundance and activities of these important species could affect biological interactions (e.g. food web function) and habitat quality.

1. Introduction

1.1.1 Ocean acidification

The accumulation of carbon dioxide (CO_2) in the Earth's atmosphere increases the natural greenhouse effect and generates climate changes (IPCC, 2007; IGBP, IOC, SOCR, 2013). Elevated atmospheric concentrations of CO_2, primarily driven by anthropogenic activity, are decreasing the pH of the oceans (Hoegh-Guldberg & Bruno, 2010). This ocean acidification can be defined as the change in ocean chemistry driven by the oceanic uptake of chemical inputs to the atmosphere, including carbon, nitrogen and sulphur compounds, that decrease the pH conditions (Guinotte & Fabry, 2008).

Anthropogenic activities have increased atmospheric concentrations of CO_2 to levels unprecedented for at least 420,000 years and possibly for the past tens of millions of years (IPCC, 2001). Atmospheric CO_2 is currently rising at a rate of 0.5% $year^{-1}$ (Forster et al., 2007) with future levels of atmospheric CO_2 dependant mostly on socio-economic parameters (Gattuso & Hansson, 2011). On current trends, CO_2 emissions could easily be 50% higher by 2030 (Turley et al., 2006).

Measurements and reconstructions of the atmospheric CO_2 history reveal that less than half of these emissions remain in the atmosphere (Sabine et al., 2004), with natural sinks on land and in the ocean removing part of the anthropogenic CO_2 (Canadell et al., 2007). By taking CO_2 away from the atmosphere, the oceanic and terrestrial sinks mitigate climatic changes (Gattuso & Hansson, 2011). Gases are readily exchanged across the air-sea interface due to differences in the partial pressure of CO_2 (pCO_2) between the ocean and the atmosphere (Royal Society, 2005). The oceans have constituted the only true net sink for anthropogenic CO_2 in

the last 200 years, and without this oceanic uptake, atmospheric CO_2 would be about 55 ppm higher today than currently observed (Sabine et al., 2004).

Atmospheric CO_2 levels in the Cretaceous were thought to have been several times higher than levels today (Zeebe & Westbroek, 2003; Ridgwell, 2005; Hönisch et al., 2012). By the Eocene, atmospheric CO_2 levels were approximately 2 - 3 times higher than present (Berner, 1994, 2001). Although past mass extinctions events have been linked to ocean acidification (Guinotte & Fabry, 2008), the rate at which atmospheric CO_2 changed in the past is thought to have been much slower than that compared to modern day (Royal Society, 2005). The chemistry of ocean waters is currently being altered at a faster rate and greater magnitude than is thought to have occurred for at least a million year and possibly up to 40 million years (Barry et al., 2011, and references therein). Consequently, ocean acidification impacts on surface ocean pH and biota will likely be more severe than for example during the Paleocene-Eocene Thermal Maximum (Zachos et al., 2005, Guinotte & Fabry, 2008). The past tells us that there will be both winners and losers in a changing ocean (Knoll & Fischer, 2011).

The mean pH of seawater has probably changed by less than 0.1 units over the last several million years (Turley et al., 2006). From the years 1750 to 2000, the oceans have absorbed about one-third of the CO_2emitted by humans (Zeebe & Ridgwell, 2011). To-date, there has been a drop of 0.1 pH units from the pre-industrial level in surface ocean pH from ~8.2 to ~8.1 (Gattuso & Hansson, 2011), which represents a 30% increase in the concentration of hydrogen ions (Hoegh-Guldberg & Bruno, 2010). A further decrease of 0.3 - 0.4 pH units in the surface ocean pH is predicted by the end of this century (Caldeira & Wickett, 2003). The top-end prediction for atmospheric CO_2 of 1071 parts per million by volume (ppmv) by 2100 (Plattner et al.,

2001) corresponds to a fourfold increase since 1750 (Gattuso & Hansson, 2011), and would equate to an oceanic pH decrease of 0.5 units (Turley et al., 2006).

1.1.2 Changes in ocean carbonate chemistry

Ocean uptake of anthropogenic CO_2 helps to reduce the levels of CO_2 in the atmosphere but it also changes the ocean's fundamental chemistry (Orr, 2011). In the ocean, carbon dioxide exists in three inorganic forms (Fig. 1.1): free aqueous carbon dioxide ($CO_2(aq)$), bicarbonate (HCO_3^-), and carbonate ions (CO_3^{2-}) (Zeebe& Wolf-Gladrow, 2001). A minor form is true carbonic acid (H_2CO_3) with a concentration of less than 0.3% of $CO_2(aq)$ (Zeebe& Wolf-Gladrow, 2001). Under typical seawater conditions, the majority of dissolved inorganic carbon is in the form of bicarbonate (> 85%), followed by carbonate ion (13%), with dissolved carbon dioxide being present in only small concentrations (0.5%) (Zeebe& Wolf-Gladrow, 2001). As a result of the equilibria between the above three species, seawater is weakly buffered with respect to changes in hydrogen ion concentration (Dickson, 2010). All three forms of dissolved CO_2 are important for biological processes in marine organisms.

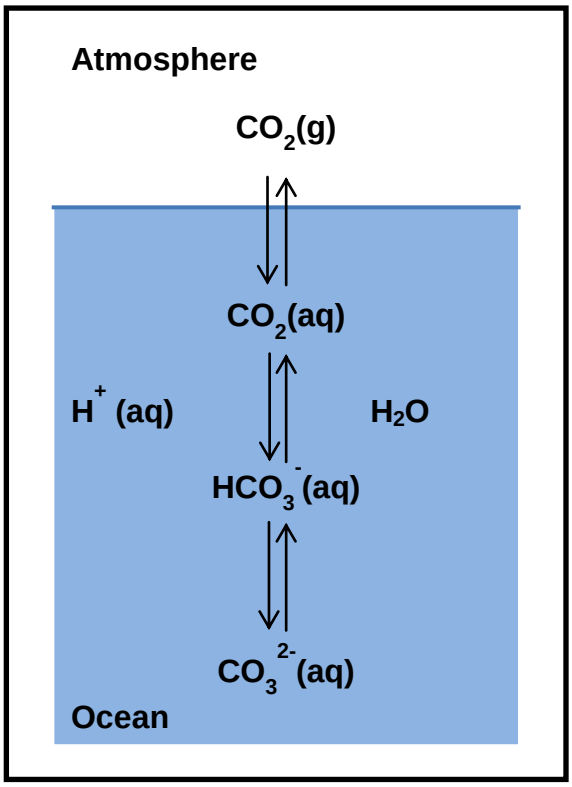

Figure 1.1 Schematic illustration of the chemical equilibria of the carbon dioxide system in seawater (redrawn from Zeebe& Wolf-Gladrow, 2001). CO_2 is exchanged between the atmosphere and ocean via equilibration of CO_2 (g) and CO_2 (aq). Dissolved CO_2 is part of the carbonate system in seawater that includes bicarbonate (HCO_3^-) and carbonate ions (CO_3^{2-}). The notations (g), (l) and (aq) refer to the state of the species, i.e. a gas, a liquid, or in aqueous solution respectively.

A number of changes in carbonate chemistry are generated following dissolution of CO_2 in seawater (Gattuso & Hansson, 2011). In addition to a reduction in pH there is a simultaneous effect on the carbonate ion concentration (Orr et al., 2005). The net effect of dissolution of CO_2 in seawater is an increase in the concentration of bicarbonate ions and dissolved inorganic carbon, while lowering the pH, the concentrations of carbonate ions and the saturation state of the three major carbonate minerals present in shells and skeletons (Gattuso & Hansson, 2011). The

current rate of decline of the CO_3^{2-} ion concentration is ~214 times the average rate of decline for the entire Holocene (Mackenzie & Andersson, 2013).

1.1.3 Biological responses to ocean acidification

Ocean warming and acidification are already having dramatic and wide-ranging effects on the flora and fauna of the oceans (Bijma et al., 2013). Calcium carbonate ($CaCO_3$) is one of the most common building materials used in the formation of skeletons, shells and other protective structures in the marine biota (Zeebe & Sanyal, 2002; Riebesell & Tortall, 2011). There are three major biogenic $CaCO_3$ minerals that occur in seawater: aragonite, calcite and magnesium calcite (Mg-calcite) (Gattuso & Hansson, 2011). The ability of marine animals to produce calcareous skeletal structures is directly affected by seawater chemistry (Fabry et al., 2008). The formation and dissolution of carbonate minerals are represented by the following equation:

$$CaCO_3 \Leftrightarrow [Ca^{2+}] + [CO_3^{2-}]$$

Dissolution of $CaCO_3$ is the reverse process of calcification and results in the chemical disintegration of the solid mineral phase into its individual components (Andersson et al., 2011). The energetic cost of calcification is thought to increase with ocean acidification causing a decrease in pH and carbonate ion concentration (Riebesell & Tortell, 2011).

A determining factor in the formation and dissolution of calcium carbonate is the $CaCO_3$ saturation state of seawater, which is a function of the carbonate ion concentration (Zeebe& Wolf-Gladrow, 2001). The $CaCO_3$ saturation state is defined as the ratio between the observed ion product and the expected ion product when the solution is in equilibrium with a particular calcium carbonate mineral (Gattuso

&Hansson, 2011). Seawater can be supersaturated (Ω > 1), undersaturated (Ω < 1), or in equilibrium (Ω = 1), with the mineral (Gattuso & Hansson, 2011). The further away from equilibrium during undersaturation, the faster the rate of mineral dissolution (Andersson et al., 2011). In addition to atmospheric CO_2, carbonate saturation is also a consequence of local changes in pCO_2 associated with temperature, salinity and diurnal and seasonal cycles of photosynthesis and respiration (Crevison & Hallock, 2007).

Calcite and aragonite minerals both consist of $CaCO_3$ but differ in their crystallography (rhombohedral versus orthorhombic) (Zeebe & Wolf-Gladrow, 2001). Calcite and Mg-calcite have the same basic mineral structure but the calcium ions have been randomly replaced by magnesium ions in the latter (Andersson et al., 2011). The different structures of each of the minerals lead to different physical and chemical properties, including solubility (Zeebe & Wolf-Gladrow, 2001). Aragonite is about 1.5 times more soluble than calcite (Gattuso & Hansson, 2011).

Precipitation of calcium carbonate is controlled by the concentration of carbonate ions and the $CaCO_3$ saturation state, both of which decrease with increasing ocean acidification (Gattuso & Hansson, 2011). One of the most significant implications is a likely reduction in calcifying capacity of marine organisms (Doney et al., 2009; Kroeker et al., 2010) where dissolution may even exceed calcification (Mackenzie & Andersson, 2013).

Although the chemistry of ocean acidification is well understood, its impact on marine organisms and ecosystems remains, in some cases, poorly known (Gattuso et al., 2010). For the past decade numerous studies and experiments have been conducted with a range of different taxa of both benthic and planktic organisms.

Changes in distributions of populations and declines in sensitive species have already been observed (Bijma et al., 2013).

Ocean acidification can lead to contrasting biological responses (Dupont & Pörtner, 2013) with some species and regional faunas being more resilient than others (Byrne, 2011). For example, organisms with aragonite shells will be particularly prone to dissolution (e.g. shelled pteropods and heteropods). A decrease in saturation of aragonite has been shown to decrease the levels of calcification in pteropods shells (Comeau et al., 2010; Bednaršek et al., 2012; Wall-Palmer et al., 2012). Marine calcifiers are on average more sensitive to ocean acidification than non-calcifiers (Dupont & Pörtner, 2013). However, in addition to calcifying organisms, elevated CO_2 levels can affect marine organisms in general (e.g. echinoid reproduction) (Pörtner et al., 2005). It is clear that there will be both organisms that benefit from higher CO_2 and organisms that will be negatively affected by higher CO_2 (Andersson et al., 2011).

The experimental responses of organisms that produce carbonate structures has not been uniform and has varied depending on the type of animal taxa, and the type and length of exposure (Gooding et al., 2009; Form & Riebesell, 2012; Wittman & Pörtner, 2013). There have been large variations in sensitivities reported among different species and between local populations of the same species, e.g. in bivalves (Range et al., 2013). Different sensitivities of life-history stages and species have implications for persistence and community function in a changing ocean (Byrne & Przeslawski, 2013). For some organisms, early life and reproductive stages may be more vulnerable than adult stages (Byrne, 2011). Examples include the intertidal barnacle *Semibalanus balanoides* (Findlay et al., 2009) and the summer flounder *Paralichthys denatus* (Chambers et al., 2013).

Changing calcification rate is one of a number of potential physiological responses to ocean acidification (Knoll & Fischer, 2011) but this is not always the case. For example, a variety of invertebrates and algal species were grown at a range of saturation states and the majority of species showed a decline in skeleton formation with decreasing saturation state (Ries et al., 2009). However, some species showed an initial increase in skeletal mass before declining while others increased skeletal mass with decreasing saturation state (Ries et al., 2009).

Ocean acidification impacts have been reported for a variety of biological processes including growth, reproduction and survival (Garrard et al., 2012). Although acidification may not be lethal to benthic invertebrates, e.g. *Amphiura filiformis*, it could result in behavioural changes that could have longer-term implications for species survival, ecosystem structure and functioning (Murray et al., 2013). Changing calcification rates may be accompanied by decreased performance in aspects such as growth or metabolism (Pörtner, 2008). Some organisms, e.g. brittle stars, may be able compensate by up-regulating their metabolism and calcification but this has substantial energetic costs (Wood et al., 2008). Up-regulation uses energy would have otherwise have been used on other essential processes, e.g. growth and reproduction, and can only be maintained in the short-term (Wood et al., 2008). Common limpets (*Patella vulgata*) were able to maintain extracellular acid-base balance, metabolism and feeding following short-term exposure (Marchant et al., 2010). However, shell-thinning and visible dissolution were observed on the radula which may allow easier predation in addition to compromising the organisms feeding (Marchant et al., 2010).

Most research concerning the effects of ocean acidification has focussed on aspects of the performance and survival of individual species during short-term studies (Barry

et al., 2011). Most experimental incubations have been short-term, typically performed over days to weeks (Kroeker et al., 2013b), but there is a need to capture long-term responses (Dupont & Pörtner, 2013). Both long and short term experiments are important for understanding whether organisms are able to acclimatize in the short-term or adapt over generations to the predicted changes (Andersson et al., 2011). For example, calcification rates in the cold-water coral *Lophelia pertusa* decreased following 8 days exposure to increased CO_2 but increased over a 6 month exposure period (Form & Riebesell, 2012). Responses of the non-calcifying intertidal polychaete (*Alitta virens*) were not present in the short-term (7 days) and only appeared after several months had elapsed in longer term experiments (Godbold & Solan, 2013). These studies emphasise the need for longer term experiments.

In addition to time period, there is also a need for studies to move away from testing single species in simplistic experiments to more complex experimental designs that mimic realistic environmental conditions (Dupont & Pörtner, 2013). At a population level, impaired individual performance and survival have consequences for populations and species (Barry et al., 2011). The effect of ocean acidification on individual species may be either positive or negative when processes such as competition and food availability are also considered (Andersson et al., 2011). For example, the biovolume of two species of cyanobacteria was lower when grown together as compared to grown separately (Karlberg & Wulff, 2013).

In the near future, environmental change could cause ecosystem simplification via changes in community dynamics (Kroeker et al., 2013a) and could potentially lead to long-term shifts in species compositions (Wittman & Pörtner, 2013). There have,

however, only been a limited number of studies conducted at the community scale (Andersson et al., 2011).

Clues to the impacts of ocean acidification on benthic environments may be provided through local environments, e.g. volcanic vents, which already experience high-CO_2 and low-pH conditions (Hall-Spencer et al., 2008; Martin et al., 2008; Dias et al., 2010; Rodolfo-Metalpa et al., 2010) and other places, such as the Wagner Basin (Pettit et al., 2013). At these sites, some organisms may benefit from reduced pH but many more lose out (Fabricus et al., 2011). Shifts in competitive interactions have been observed in addition to severely reduced diversity and structural complexity and resilience (Fabricus et al., 2011).

Environmental variability may not matter too much as long as it stays within the non-limiting range of organisms. If organisms have sufficient energy resources, e.g. food and nutrients, they may be able to compensate for the additional energy demand required to calcify under elevated CO_2 conditions (Andersson et al., 2011). For example, spider crab larvae (*Hyas araneus*) were able to compensate for higher metabolic costs under elevated seawater pCO_2 levels and their survival was not affected (Schiffer et al., 2013).

In addition to CO_2, the additional drivers of ocean warming and hypoxia are having pronounced impacts on the composition, structure and function of marine ecosystems (Doney et al., 2009; Bijma et al., 2013). When multiple stressors act simultaneously there is a greater probability of additive, synergistic and antagonistic effects occurring among them (Ghedini et al., 2013). Organisms that are already living at, or close to, their thermal tolerance levels (Stillman, 2003) may be under the greatest threat from additional ocean acidification (Widdicombe et al. 2011). For example, larvae that calcify are sensitive to both acidification and warming while

larvae that do not calcify are more sensitive to warming (Byrne & Przeslawski, 2013). Dominant effects of temperature have been observed in a number of studies (Bulling et al., 2010; Nguyen et al., 2012; Godbold & Solan, 2013). If organisms are living at the limit of their CO_2 tolerance, just a small change in CO_2 levels could have a detrimental effect on their long-term survival (Widdicombe et al., 2011).

1.2 Foraminifera

Foraminifera are one of the most abundant groups of calcifiers, estimated to precipitate ca. 50% of biogenic calcium carbonate in the open oceans (Faber & Preisig, 1994; Schiebel, 2002). Foraminifera are single-celled organisms protected by a test composed of one or more interconnected chambers (Loeblich & Tappan, 1964). They have a global distribution, inhabit a diverse range of habitats and are one of the most successful groups of protozoa (Lee, 1980).

Foraminifera have also been used extensively in different fields of Earth and environmental sciences due to several factors (Coccioni, 2000). Their hard exoskeleton records fundamental environmental changes and evolutionary processes (Coccioni, 2000) through analysis of assemblage compositions, shell morphologies and geochemistry (Hallock, 2000a). They are also excellent recorders of environmental changes over a short-time span (Coccioni, 2000) and are utilized as ‘ecosystem indicators’ in modern environmental research (Hallock, 2000a). In the following section, aspects of foraminiferal biology are discussed in addition to their suitability as target organisms for ocean acidification research.

1.2.1 Introduction to foraminifera

Owing to their test morphologies and life-cycles, foraminifera are useful indicators of the state of the marine environment (Tarasova, 2006). The long evolutionary life

histories of foraminifera have allowed extensive utilization of their skeletal remains in biostratigraphic and palaeoecological research (Faber &Preisig, 1994). They have an abundant and diverse fossil record spanning nearly 600 million years (Brasier, 1986). They first appeared in the Cambrian and, during the Phanerozoic, invaded most marginal to fully marine environments (Goldstein, 2002). Foraminifera have recorded countless global change events in the geologic record, ranging from subtle to mass extinctions (Hallock, 2000b). Foraminifera are important constituents of most marine environments as result of their abundance and distribution (Lee et al., 1985). Owing to their small size and abundance, statistically significant sample sizes can be collected quickly and inexpensively for assemblage assessment and experimental studies (Hallock, 2000a).

Of ~34,000 species described (Loeblich & Tappan, 1969) more than 12% are alive today (Lee et al., 1985). Foraminifera are grouped into 12 suborders based on the composition and structure of their test and are classified further by means of shell characteristics including chamber shape, arrangement, coiling mode, accessory and internal structures and the aperture (Loeblich & Tappan, 1988). Individual species within suborders are constrained by the types of test they are generally capable of constructing (Hallock et al., 1991).

Benthic foraminifera are extremely varied in morphology and are incredibly diverse. There is some relationship between morphology and habitat of living forms (Brasier, 1986). The great variety of test form suggests that many taxa are particularly well adapted for specialized ecological niches (Marszalek et al., 1969). Observations on trends of morphological changes will allow us sometime in the future to better understand and interpret the fossil record (Boltovskoy et al., 1991). The physical and chemical properties of foraminiferal shells provide a multitude of paleoproxies, based

on the chemical composition and morphology of the test as well as species abundance patterns (Kucera, 2007). Initial shell weights have been used in a number of studies on changing seawater chemistry, for example, as a proxy for bottom- or surface water [CO_3^{2-}] (Bijma et al., 2002; Broecker & Clark, 2001;2002;2003). Additionally, studies have used boron isotope compositions ($\delta^{11}B$) in foraminifera to reconstruct past oceanic pH (Sanyal et al., 2000).

1.2.2 Test construction

The test enables foraminifera to withstand changes in the environmental conditions and provides a highly efficient means of controlling their immediate environment (Marszalek et al., 1969). Foraminifera build an extraordinary variety of tests that range from simple tubes or spheres to complex multichambered forms (Lipps, 1973). Morphology is considered an adaptive characteristic (Hallock et al., 1991). Structural morphological modifications are believed to have arisen as a means to exploit particular environments and resources (Hallock et al., 1991). The complex architecture present in the shells of some smaller foraminifera responds to 'internal', physiological or metabolical needs as well as to the ambient environment (Hottinger, 1986).

All foraminiferal tests are constructed from organic substances (Lipps, 1973), with construction being largely mediated by reticulopod deployment and transport (Travis & Bowser, 1991). Foraminifera protrude a net-like meshwork of pseudopodia with a halo of thin, web-like, granular rhizopodia and pencil-shaped filopodia emerging from the cell body (Anderson & Lee, 1991). Pseudopodia are extremely dynamic and continually extend and retract as they scan the environment (Bowser & Travis, 2000). In general, the organic matrix is formed extracellularly where it is considered to act as a template for mineralization (Jepps, 1942; Towe & Cifelli, 1967).

Foraminifera are thought to promote calcification by elevating their intracellular pH at the site of calcification (Erez, 2003; Bentov et al., 2009; de Nooijer et al., 2008, 2009a, 2009b; Glas et al., 2012). Cultured *Ammonia* spp. were observed to decrease pH within its extracellular microenvironment during calcite precipitation (Glas et al., 2012). The amount of protons removed during calcification was directly related to the volume of calcite precipitated (Glas et al., 2012). In acidified seawater, foraminifera would require more energy to elevate intracellular pH, leading to a possible decrease in calcification and increased energetic cost.

All organisms are affected during growth by the state of their physical environment. Noxious stimuli that interfere with pseudopodial activity can result in catastrophic structural changes (Bowser & Travis, 2000). The carbonate system in the direct surrounding of the foraminifera may also be altered by photosynthesis and respiration (Rink et al., 1998; Glas et al., 2012).

Foraminiferal tests are composed of several types of material (Loeblich and Tappan, 1964). The wall of foraminiferal tests may be organic, agglutinated, or made of calcium carbonate in various chemical and structural modifications (Hemleben et al., 1986). The characteristics of the test do not prevent a taxon from living in a particular habitat but certain wall structures should be more advantageous than others in certain environments (Hallock et al., 1991). The type of shell material can therefore determine where various species can survive (Scott et al., 2001). Agglutinated forms have organic threads with or without carbonate or silica cement (Lipps, 1973). Water chemistry can, therefore, matter quite significantly. Calcium carbonate forms are composed of either magnesium calcite, calcite or aragonite (Hemleben et al., 1986). About 900 described genera deposit tests of calcium carbonate (Lipps, 1973).

Foraminiferal shells are composed of primary and secondary layers of calcite (Erez, 2003). A lamellar structure allows progressive test strengthening with the earlier chambers being continuously re-enforced throughout the life of the individual (Hallock et al., 1991). Primary material consists of high-Mg calcite (Bentov & Erez, 2005), which is less resistant to dissolution than the needles of secondary, low-Mg calcite (Haynert et al., 2011). Tests composed of high-Mg calcium carbonate needles consist of an internal layer arranged in irregular meshwork and a proximal and distal layer of needles arranged approximately parallel to the smooth veneer (Anderson & Lee, 1991). Deformed shells have been found to exhibit increased Mg/Ca ratios (Yanko & Kronfeld, 1992; 1993).

Agglutinated tests of benthic foraminifera consist of accumulated mineral particles cemented together within an organic matrix (Anderson & Lee, 1991). They show a great variety of structure and form. Species with agglutinated tests use rhizopodial streaming to gather particles to be attached to the wall during wall construction (Anderson & Lee, 1991). Particles often appear to be random collections of available material in the environment but some species show discrimination in selection of particle size, composition and shape (Lipps, 1973; Anderson & Lee, 1991).

Readily identifiable families, genera, and morphotypes are often characteristic of certain environments (Hallock, 2000a). The general pattern is that brackish subtidal environments, such as estuaries, have assemblages with a mixture of agglutinated and hyaline walls (Murray, 2006). Normal marine and hypersaline examples plot along the hyaline-porcelaneous side of a ternary diagram, with varying amounts of agglutinated walls (Murray, 2006). Calcareous forms are present from the intertidal to abyssal depths above the carbonate compensation depth (Murray, 1991a).

1.2.3 Foraminifera and ocean acidification research

Some foraminifera exhibit sensitivity to ecological conditions (Faber & Preisig, 1994). For example, ecological sensitivities in *Amphistegina* have been shown to alter the development and degree of ornamentation, decreasing with decreasing salinity (Boltovskoy & Wright, 1976). Saraswat et al. (2011) observed comparatively less growth at salinity higher or lower than 35‰ in the benthic foraminifer *Rosalina globularis* (d'Orbigny). In the planktic foraminifera, *Globigerina bulloides* (d'Orbigny), reductions in shell mass of 30 - 35% have been observed since the pre-industrial Holocene (Moy et al., 2009).

Mesocosms can unravel the key role of natural variability in species responses (Dupont & Pörtner, 2013) with living populations and surface sediment assemblages being used to assess the state of a benthic ecosystem (Hallock, 2000a). Some species can be readily maintained in culture (Arnold, 1954) to determine the responses of selected taxa to physical stresses (Hallock, 2000a). For example, Filipson et al. (2010) successfully cultured benthic foraminifera for several months in an investigation of the use of benthic foraminifera as palaeotemperature proxies.

The majority of ocean acidification research on foraminifera has been published in the last 3 years with a variety of responses recorded (Keul et al., 2013). The observed differences in response have been attributed to both the species studied and the experimental methods used (Keul et al., 2013).

It is probable that there are different pH dissolution limits depending on the foraminiferal species reflecting metabolic capability and the nature of the tests. Inactive and empty tests are the first to be affected by dissolution (Haynert et al., 2011). Live specimens are less easily attacked due to the presence of a "protective"

cytoplasm (Le Cadre et al., 2003). Dissolution depends on wall thickness, wall structure and chemical composition. Boltovskoy and Wright (1976) observed dissolution of calcareous foraminifera beginning at a pH of less than 7.8 but did not indicate which species were affected and whether live or dead specimens were considered.

The first stage of decalcification is an increase in opacity of the test followed by dissolution of the external walls of the last chambers (Le Cadre et al., 2003). Loss of transparency of the test is generally interpreted as a perturbation in the calcification system (Stubbles et al., 1996a, b) and is due to the dissolution of the secondary calcite that is deposited between the needles that make up the test (Debenay et al. 1996, 1999). The last chambers decalcify first because the wall, made up of a smaller number of lamellae, is thinner (Le Cadre et al., 2003). Decalcification then progressively extends over the entire test. When maintained at low pH, the entire test can be destroyed with only the cytoplasm covered by the inner organic layer remaining (Le Cadre et al., 2003).

Patterns of dissolution include etching on the wall surface, breakage of the final chamber, breakage of other chambers, complete loss of the outer test wall exposing underlying septa and chamberlets, followed by total destruction (Cottey & Hallock, 1988; Murray, 2006). Loss of large portions of the outer wall, exposing the underlying chamberlets can therefore indicate dissolution. A star shape test after heavy dissolution, resulting from the prominence of the remaining interlocular walls, is very typical and may indicate low pH conditions (Le Cadre et al., 2003). It can easily be distinguished from shell breaks resulting from strong abrasion, since abrasion also destroys interlocular walls. For example, pitted surfaces and impact depressions in

Archaias angulatus (Fichtel & Moll) tests indicate mechanical breakdown in relative high-energy environments (Cottey & Hallock, 1988).

Test decalcification under acid conditions in the laboratory can be followed by recalcification if conditions became favourable again (Bradshaw, 1961; Angell 1967; Le Cadre et al., 2003). Recalcified walls are easy to recognise because they have a smooth surface, by contrast to remains of previously decalcified walls, indicating that only local recalcification occurs in initial stages (Le Cadre et al., 2003).

Deformed tests occur when the growth plan of the test is disrupted to give an abnormal shape. Effects can range from slight deformity (smaller chambers) to severe deformity with chambers in the wrong place, distorted spirals and additional apertures (Murray, 2006). If solubility is low, calcareous species are able to build larger and more robust tests but as solubility increases, tests get thinner and some calcareous species develop alternate morphologies (Scott et al., 1977; Boltovskoy et al., 1991).

1.2.4 Experimental responses

In ocean acidification research, the studied response variables of foraminifera include shell size, weight and thickness, growth and calcification rates and changes in community structure. For example, culture studies have indicated that shell weights and calcification rates in species of both planktic and benthic foraminifera reduce with decreasing pH (Bijma et al., 1999, 2002; Dissard et al., 2010; Lombard et al., 2010). Responses have however shown non-linear trends with decreasing pH (Vogel & Uthicke, 2012). In the case of *Marginopora*, a large benthic foraminifera, responses to elevated pCO_2 have been varied. Calcification has been observed to both increase (Vogel & Uthicke, 2012) and decrease (Kuroyanagi et al., 2009;

Sinutok et al., 2011) with increasing pCO_2. In *Marginopora kudakajimensis* (Gudmundsson), growth rate, shell weight and chamber addition were reduced after 71 days exposure to low pH (7.7) (Kuroyanagi et al., 2009). However, in other examples, elevated calcification was observed in cultures at intermediate pH (Kuroyanagi et al., 2009; Dissard et al., 2010; Fujita et al., 2011). Diatom-bearing species *Amphistegina radiata* and *Heterostegina depressa* (d'Orbigny) and dinoflagellate-bearing *Marginopora vertebralis* (Quoy & Gaimard) did not show negative effects in exposures up to 1925 µatm pCO_2. *M. vertebralis* displayed significantly increased calcification rates in elevated pCO_2 regimes. Species-specific differences were also observed between the hyaline species *Baculogypsina sphaerulata* (Parker & Jones) and *Calcarina gaudichaudii* (d'Orbigny) and the porcelaneous species *Amphisorus hemprichii* (Ehrenberg) (Fujita et al., 2011). At intermediate levels of pCO_2, calcification decreased for the porcelaneous species while increasing in the hyaline species (Fujita et al., 2011). The short-term ability of these species to cope or even benefit from elevated pCO_2 is however no guarantee for their survival in the long-term (Vogel & Uthicke (2012). For example, *Marginopora* were recorded as completely absent at volcanic vent sites of low pH (7.9) in Papua New Guinea (Uthicke & Fabricus, 2012).

Specimens of the benthic foraminifera *Elphidium williamsoni* (Haynes) cultured at reduced pH (7.6) produced significantly thinner and less dense chamber walls than those of the same species cultured at pH 8.3 and 8.1 (Allison et al., 2010). A population of smaller individuals was also observed at low pH compared to other treatments (Allison et al., 2010). In *Elphidium excavatum* (Terquem) *clavatum* (Cushman), dissolution under low pH (7.5) conditions was visible after just 14 days

(Green et al., 1998). Following 70 days of culturing, mortality rates were three times higher under reduced pH than under control pH (8.1) conditions (Green et al., 1998).

Culturing experiments on *Ammonia aomoriensis* (Asano) from Flensburg Fjord revealed a clear relationship between seawater pCO_2 and shell dissolution (Haynert et al., 2011 Haynert, 2013). The greatest increase in mean shell diameters were observed at the lowest pCO_2 (618 µatm) with decreases (up to 22%) observed at higher pCO_2levels (> 1829 µatm) (Haynert et al., 2011). Size reductions were due to the loss of outer shell wall and partial collapse of inner organic lining. All chambers were destroyed by complete calcium carbonate dissolution with only the inner organic lining staying intact in specimens incubated at 3130 µatm (Haynert et al., 2011). In the benthic foraminifera *Rosalina globularis*, death of all specimens at low pH (7.2 and 7.5) was preceded by dissolution of the entire test (Saraswat et al., 2011). The drop in pH severely hampered the calcification capacity and reproduction of this species (Saraswat et al., 2011).

Keul et al. (2013) adopted an experimental setup that allowed them to distinguish between, and identify a single variable of the carbonate system causing observed effects on the benthic foraminifera *Ammonia* sp. Over a period of 59 - 96 days, they observed an increase in size-normalised weight and growth rate caused by increasing carbonate ion concentration (Keul et al., 2013).

1.2.5 Natural pH gradients

Potential ecosystem impacts in benthic foraminiferal communities as a result of ocean acidification include compromised fitness of these calcifying organisms with a possible shift in competitive advantage towards non-calcifiers (Fabry et al., 2008). The structure and diversity of benthic communities are strongly controlled by the

interactions that occur between species (Barry et al., 2011). Exposure times have typically been short for most calcification studies and therefore often too short to detect possible acclimatization (Barry et al., 2011). With ocean acidification occurring at a slower timescale than can be replicated in the laboratory, the capability, if any, of organisms to adapt has been questioned (Zeebe & Ridgwell, 2011). Natural CO_2 vent sites can provide natural laboratories in which to study foraminiferal responses to rising CO_2 levels.

The impact of ocean acidification in still unclear following a mixture of responses from the few studies examining benthic foraminiferal assemblages (Dias et al., 2010; Fabricius et al., 2011). Reduced benthic foraminiferal diversity and abundance has been recorded as markedly reduced at vent sites of lowered pH (Dias et al., 2010; Uthicke et al., 2013). In one case, assemblages shifted to one completely dominated by agglutinated individuals in low pH conditions (Dias et al., 2010). However, at another site, both calcifying and non-calcifying foraminifera were absent from sites of lowered pH (Uthicke et al., 2013). In the northern Gulf of California live calcareous foraminifera were present in low numbers under low pH conditions (Pettit et al., 2013). Dissolution was however evident and could have been a result of the foraminifera not being able to meet the increased energy requirements to maintain calcification rates under low pH conditions (Pettit et al., 2013).

In addition to vent fauna, shallow-marine organisms are often stressed on multiple levels and they must either adapt or will be replaced by opportunistic taxa (Hallock, 2000b). Intertidal species are generally regarded as being particularly tolerant to changes in temperature and pH as they are subjected to environmental (e.g. tidal and seasonal) fluctuations (Godbold & Solan, 2013; Kitidis et al., 2012). The processes of decalcification under acidic conditions and recalcification under

favourable conditions may also take place in natural environments (Geslin et al., 2002). Numerous species of benthic foraminifera have adapted to the high environmental variability in marginal marine habitats (Sen Gupta, 2002). For example, benthic foraminiferal communities are subject to relatively large changes in pH and salinity on a daily cycle and are still able to survive. For example, Almogi-Labin et al. (1992) found a living population of *Ammonia beccarii* (Linné) *tepida* in an inland hypersaline pool, where salinities ranged from 39.7 - 54.5‰ (winter-late summer). Dominance of adult and intermediate specimens suggests a great number of juveniles reached maturity under favourable conditions (Almogi-Labin et al., 1992). Many intertidal organisms may however already be at their upper tolerance limits. Any further increase in environmental stress associated with ocean acidification or warming could have significant physiological and ecological impacts (Pörtner & Farrell, 2008).

1.3 Aims of Research

All research within this thesis is from a series of lab-based experiments. The following approaches and related hypotheses will be addressed in order to further elucidate the biological response of intertidal benthic foraminifera to ocean acidification and temperature.

1. Determine the responses (survival & growth) of two dominant intertidal foraminiferal species (*Haynesina germanica* (Ehrenberg) and *Elphidium williamsoni*) during short-term exposure to ocean acidification (Chapter 3).

Hypothesis: Survival and growth of calcareous foraminiferal species (*Haynesina germanica* and *Elphidium williamsoni*) will be reduced with decreasing pH.

2. Identify community level foraminiferal responses to ocean acidification within benthic intertidal communities through long-term mesocosm experiments. In addition to the species-specific responses above it is necessary to investigate how the structure and diversity of benthic communities will be impacted by ocean acidification. Quantitative analyses of foraminiferal diversity, assemblage structure, population abundance and test morphology will be undertaken (Chapter 4).

Hypothesis: The proportion of agglutinated foraminiferal species will increase while calcareous species will decline under the long-term exposure of natural mixed assemblages to elevated CO_2.

Hypothesis: Overall survival of foraminifera will be reduced by elevated CO_2 and temperature.

Hypothesis: Multiple stressors will have a greater detrimental effect on community structure and population survival than single stressors.

3. Categorise foraminiferal test deformation and damage following long-term exposure to ocean acidification (Chapter 5)

Hypothesis: Damaged and deformed foraminiferal specimens will increase in frequency with elevated CO_2.

4. Identify impacts of long-term exposure to ocean acidification on foraminiferal functional morphology in *Haynesina germanica* (Chapter 6).

Hypothesis: Calcareous morphological features and ornamentation of functional importance will be reduced under elevated CO_2 conditions.

5. Establish if foraminiferal feeding in *Haynesina germanica* is impacted following dissolution of functional morphological features (Chapter 7).

Hypothesis: Feeding function will be compromised in calcareous foraminiferal species under elevated CO_2.

2. Materials and Methods

This chapter is separated into two discrete sub-sections. Section 2.1 details methodology for the short-term foraminiferal culturing experiment (Chapter 3). Section 2.2 describes the long-term foraminiferal culturing time-series (upon which Chapters 4 to 7 are based).

2.1 Short-term foraminiferal culturing

2.1.1 Field collection of specimens

Surficial mud scrapes (1 – 2 cm depth) were collected from the Eden Estuary (Fig. 2.1), N.E. Scotland (56°22'N, 2°50'W) in May 2011. To isolate live benthic intertidal foraminifera for culturing, only the upper layers of mud were taken during low tide.

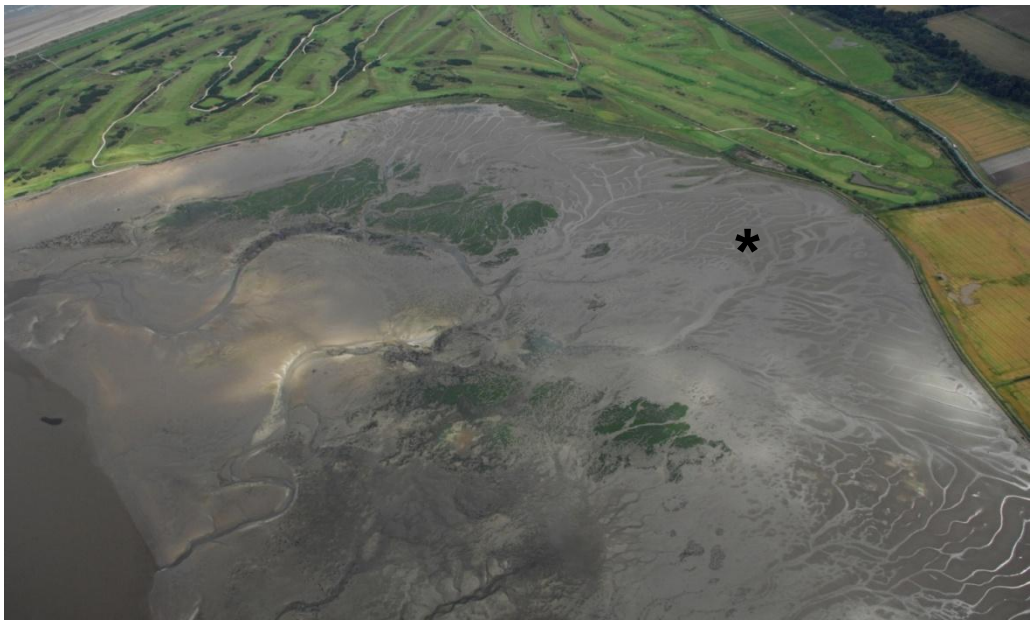

Figure 2.1 Aerial photograph of the Eden Estuary indicating sampling site (*). Image courtesy of RAF Leuchars.

The sediment was transferred into plastic containers (250 ml) containing seawater to transport back to the laboratory. Once at the laboratory, the container was left to settle for three to four hours. The flocculent layer was siphoned off using a pipette into a Petri dish containing seawater (previous observations show that foraminifera tend to settle out in this layer if left for a number of hours).

2.1.2 Foraminiferal isolation

The foraminifera were picked into a shallow Petri dish containing seawater using a fine (000) sable brush under an Olympus® SZ stereo-microscope. The brush was then used to gently remove any visible traces of sediment before transferring the foraminifera to culture chambers. Ice packs were placed on the trays alongside the Petri dishes containing live specimens to keep the seawater cool.

2.1.3 Identification of live specimens

A number of weeks were spent developing a protocol for the identification of live specimens, with the task of distinguishing live individuals from dead ones retaining protoplasm proving to be both difficult and time consuming. The presence of foraminiferal cytoplasmic colour is a method that has been used extensively to distinguish live individuals. However, colour can be retained in dead foraminiferal cytoplasm for weeks or years (Bernhard, 2000, and references therein). In other cases, apertural bolus, where debris accumulates around the apertural region of the foraminifer, alongside pseudopodial activity has been used to identify live specimens.

The following protocol was adopted to ensure all foraminiferal specimens were alive prior to culturing. Foraminifera were selected as ‘potentially live’ through observation of cytoplasmic colour (Fig. 2.2A) and apertural bolus. For each selected individual

the apertural bolus was then removed using a fine paintbrush. Previous observations revealed that live specimens are more likely to move and/or extend their pseudopodia if adherent materials are removed.

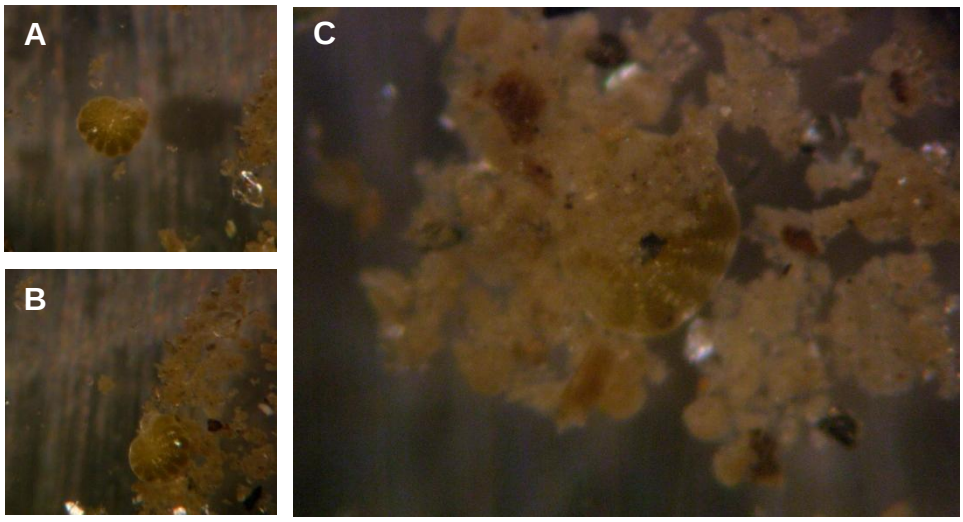

Figure 2.2 Movement and collection of detrital material by an individual specimen of *Elphidium williamsoni* (imaged over a 1 hour period). A) Specimen showing clear cytoplasmic colouration after being cleaned of detrital material. B) Specimen had moved from original position in Petri-dish towards detrital material. C) Creation of apertural bolus/cocoon. Approximate size of individual imaged was 430 µm.

Each of the foraminifera were then left overnight in a Petri dish with seawater to see if they moved from a pre-marked position (Fig. 2.2B) and attached to either the Petri dish or detritus (Fig. 2.2C). Movement ensured that the foraminifera were without doubt alive at the time of final selection.

2.1.4 Calcein staining

After being identified as live, the pre-existing (i.e. field grown) foraminiferal calcite was labelled by exposure to the fluorescent stain calcite label calcein. Foraminifera were incubated in seawater containing 5mg/L calcein for two weeks (Bernhard et al., 2004) allowing us to define pre-existing material. Calcein was incorporated into the

lamellar test during calcein incubation which fluoresced yellow-green when viewed with epifluorescence. Subsequent calcite incorporated into the test during the experiment did not fluoresce (Fig. 2.3A). During the calcein incubation period, the culture chambers were connected to 1 litre glass bottles (Fig. 2.3B), with seawater changed once a week (Allison et al., 2010). At the end of the incubation, the culture chambers were opened and the foraminifera picked out to be placed in clean culture chambers. Following the experimental period, the foraminifera were viewed under an Olympus® BX51 epifluorescence microscope to record any experimental growth.

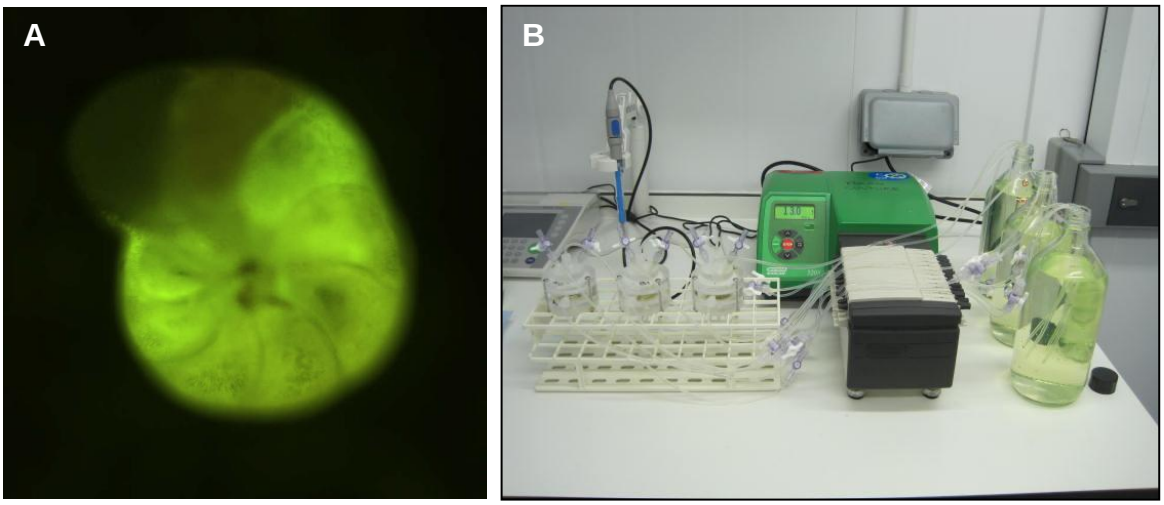

Figure 2.3 Calcein staining and incubation. A) Individual of *Haynesina germanica* showing pre-existing calcite (fluorescent chambers) and experimentally precipitated chambers (non-fluorescent ultimate and penultimate chambers). B) Photograph of calcite staining setup, showing calcein labelled seawater bottles and foraminiferal culture chambers.

During the calcein staining and throughout the experimental period, the foraminifera were fed once a week with ~ 100 µL of concentrated algae (*Dunaliella tertiolecta*) (typically 1 x 10^7 cells ml^{-1}). The food was introduced during a low flow regime to ensure the food was not immediately washed out. After 1 hour, standard flow

conditions were resumed to allow time for the food to settle. All chambers were fed at the same time.

2.1.5 Foraminiferal culturing system

All culture components, with the exception of the silicone tubing, were thoroughly cleaned with 1 - 2 M HCl for >24 hours and rinsed with deionised water prior to use (Allison et al., 2010).

The foraminiferal culture system (Fig. 2.4) was based on a modification of the recirculating system of Hintz et al. (2004). The system was housed in an environmental control room set at 15°C, with a 12hr light:dark cycle to represent local conditions at the time of the experiment. Three large (400 l) high density polyethylene reservoir tanks were filled with filtered seawater (1 µm) drawn from a shallow site in St Andrews Bay by a HDPE pipe. The seawater was aerated for one week prior to the experiment. The reservoir tanks were sealed to prevent off-gasing and to minimise the interaction between the seawater with atmospheric gases. Sealed tanks also reduced possible salinity changes that would result due to evaporation. The outside of the tanks were covered in dark plastic to minimise bacterial and algal growth.

To separate the foraminifera from bubbling seawater, they were housed in acrylic culture chambers that had recirculating seawater flowing through them from the reservoir tanks. Multi-channel peristaltic pumps were used to circulate the seawater from the reservoir tanks to the culture chambers.

Platinum cured silicone rubber tubing (0.16 cm inner diameter, 0.24 cm outer diameter) connected the reservoir tanks, peristaltic pumps and culture chambers. Tubing connecting both the peristaltic pumps and culture chambers to the reservoir

tanks were plumbed through the tank lids. Tubing used to draw the water from the reservoir tank was submerged 0.5 m below the water surface, weighted down with PTFE-coated stir bars (Hintz et al., 2004). Water returning from the culture chambers was plumbed through the tank lid but left to drip without submerging. The pump flow rate was set to 3 mL min^{-1} (Hintz et al., 2004).

Figure 2.4 Photograph of culturing system showing the reservoir tanks, culture chambers and carbonate chemistry manipulation system.

2.1.6 Chamber setup

Acrylic culture chambers, custom milled following the design of Hintz et al. (2004), were used to house the foraminifera for the duration of the experiment. The design allowed seawater to flow in and around a cell culture insert housed inside each culture chamber. Polystyrene culture inserts (8 µm pore size, diameter 25 mm), fitted

with a polycarbonate membrane at the base, sealed the culture to minimise foraminiferal migration out of the culture chamber. Polystyrene feet on the base of the insert allowed the membrane to be suspended above the chamber floor. Seawater inputs and outputs located on both the top and bottom half of the culture chambers allowed seawater to be pumped into the chambers both above and below the culture inserts. The seawater residence time was 37 s inside the cell culture insert and 6 min below the cell culture insert (Hintz et al., 2004). For substrate, a 1 mm thick layer of silica was added to the base of the insert. This was extensively cleaned before use following the protocol of Hintz et al. (2004).

2.1.7 Carbonate chemistry manipulation system

The pH of the reservoir tanks was monitored constantly throughout the experimental period using a pH controller (IKS Aquastar, IKS ComputerSysteme GmbH, Germany) via 3 pH modules, one placed in each large reservoir tank. To maintain the same starting point of CO_2 across all treatments, the seawater in the reservoir tanks was continuously bubbled with natural air through a separate line connected to an air pump. This buffered the system against natural fluctuations of seawater pCO_2 (Findlay et al., 2010).

In a near identical setup to Widdicombe & Needham (2007), the pH controller continuously monitored the pH and controlled the CO2 bubbling via an electrovalve feedback system to a CO_2 cylinder (Fig. 2.5). The CO_2 was passed through the water as fine bubbles using a pipette tip. Small bubbles increased both the contact time and surface area, enabling the gas to pass rapidly into solution. The flow rate of CO_2 was adjusted via a solenoid valve on the regulator. The supply of CO_2 was haltered via an automatic feedback relay system once the pH in each reservoir tank reached the required levels. This allowed large volumes of seawater to be

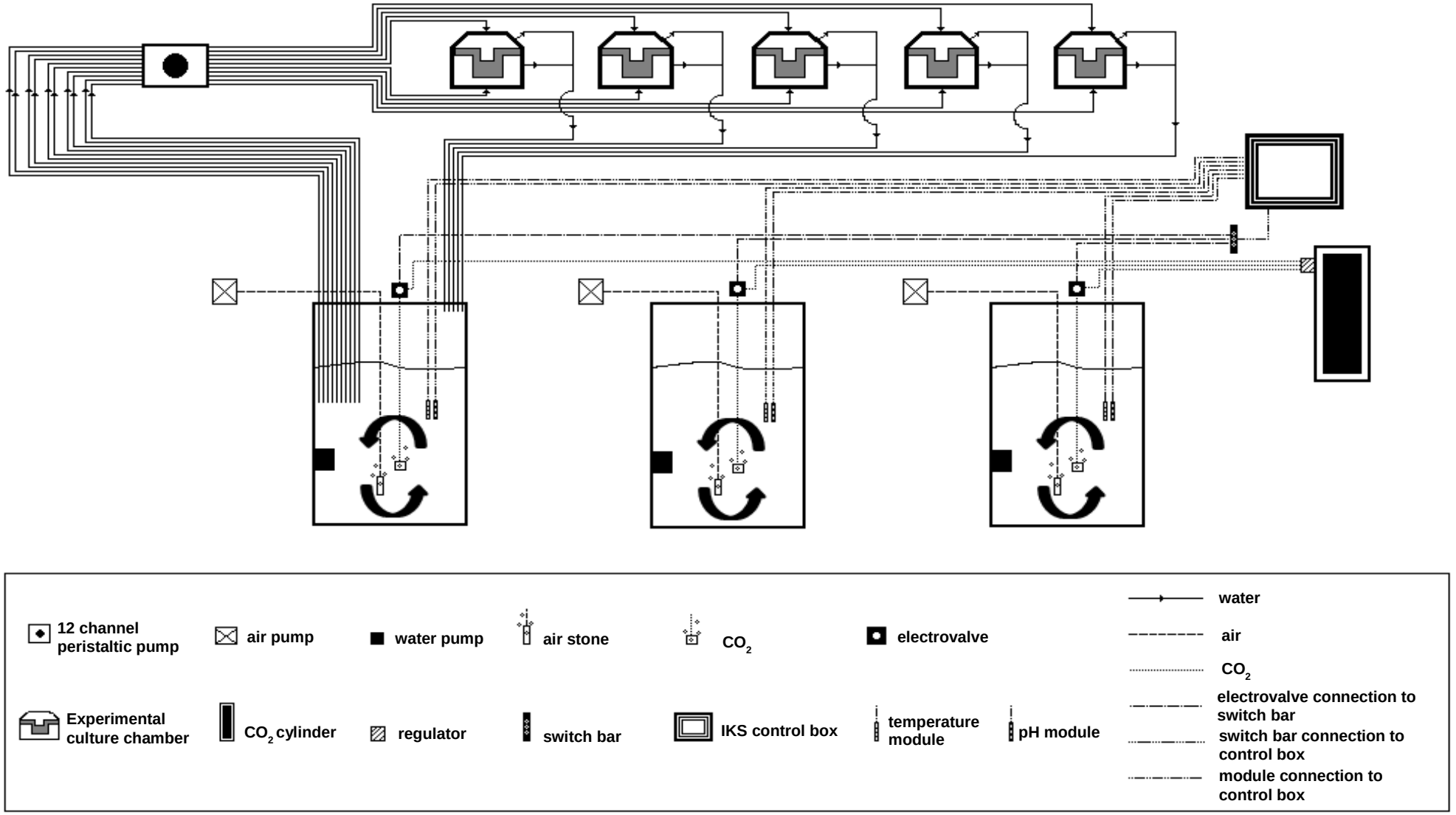

Figure 2.5 Schematic diagram of experimental system (adapted and redrawn from Hintz et al. 2004). Diagram only shows the replicate experimental chambers connected to one reservoir tank.

maintained at the desired pH and supplied to the experimental culture chambers housing the foraminifera. The three pH levels maintained were 7.7, 7.5 and 7.3 (accuracy ± 0.05 pH units).

To prevent acidity shock, the foraminifera were subject to an acclimation period of 5 days. The pH was reduced gradually over this period, with the experiments beginning once the final treatment levels had been reached.

Water pumps in each reservoir tank allowed mixing and ensured a balanced system. In addition, a temperature sensor connected to the control system was placed in each reservoir tank to provide continuous monitoring and record any fluctuations. A re-circulating system was adopted, where the same water pumped out was filtered (Eheim®) and recirculated into reservoir tanks to be adjusted back to the desired pH.

In addition to pH and temperature being continuously recorded by the control system, point measurements of pH, temperature and alkalinity were taken throughout the experiment. The temperature and pH were measured several times weekly using Mettler Toledo Seven Multi pH meter with pro-glass electrode. pH modules were calibrated using pH buffers 4.00 and 7.00. Salinity was measured weekly and nutrient samples were taken every 2nd week. Alkalinity was measured every 2nd week using a Metrohm 888 Titrando (software: Tiamo) at The Scottish Association for Marine Science (SAMS). Samples taken for alkalinity were poisoned with mercury chloride and stored in borosilicate glass Labco exetainer vials (12 ml) prior to analysis. The measured values of temperature, salinity, pH (Total) and Alkalinity (A_T) were used to calculate the values of dissolved inorganic carbon (DIC), pCO_2, saturation states of calcite ($\Omega_{Calcite}$) and aragonite ($\Omega_{Aragonite}$), bicarbonate (HCO_3^-) and carbonate concentration (CO_3^{2-}) using CO_2Calc with appropriate

solubility constants (Mehrbach, 1973, refit by Dickson & Millero, 1987) and KSO_4 (Dickson,1992).

1.8 Foraminiferal counts and measures

The maximum diameter and number of chambers of each individual were counted following calcein staining and prior to the experimental incubation. A series of five replicate culture chambers were prepared for each pH treatment, each containing between 45 and 49 individual specimens (Chapter 3). Under each pH treatment, the average number of foraminiferal specimens per replicate was 46. Consistency in each chamber population allowed potential density and competition factors to be eliminated whilst ensuring that overcrowding did not affect foraminiferal feeding or growth (Lee et al., 1966; Muller, 1975).

Mixed assemblages of *Haynesina germanica* (Fig 2.6A) and *Elphidium williamsoni* (Fig 2.6B), representing the two dominant, naturally co-occurring species at the collection site, were placed in each culture chamber (numbers of each species per replicate are detailed in Chapter 3).

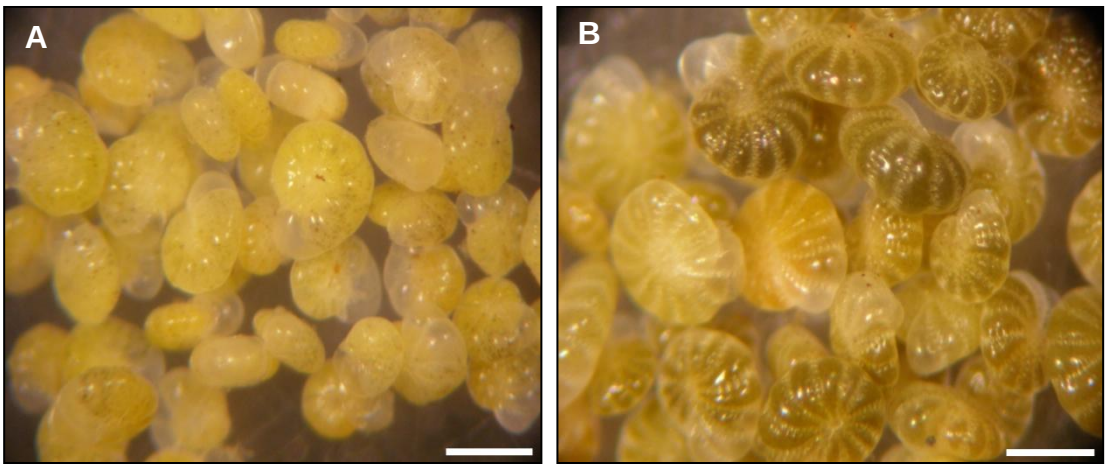

Figure 2.6 Foraminiferal specimens prior to experimental incubation. A) *Haynesina germanica*. B) *Elphidium williamsoni*. Scale bars = 200 µm.

Foraminifera were maintained in the culture system for six weeks. The maximum test diameters were measured for each population at the end of the experiment. Addition of new chambers formed during the experimental period was also quantified according to the calcein staining method described in Section 2.1.4.

2.2 Long-term foraminiferal culturing

The long-term culturing experiment produced a time-series dataset based on regularly collected samples and forms the basis of Chapters 4-7.

2.2.1 Field collection of specimens

Sediment was collected from the Ythan Estuary, N. E. Scotland (57°20'N, 01°57'W), in December 2010. After sieving (500 µm), fine sediment was housed in mesocosms (12 X 12 X 33 cm) with a sediment depth of 12 cm and 20 cm of overlying seawater (Fig. 2.7). The cores were exposed to a seasonally varying diurnal light cycle and maintained at either 10°C (average annual conditions for Scotland) or seasonally varying ambient temperature (Table 2.1).

2.2.2 Experimental system and sampling regime

Seawater pH was controlled by bubbling air with a known concentration of CO_2 (380, 750, 1000 ppm) into each mesocosm. The CO_2 treatment 380 ppm represents current ambient CO_2 levels and served as a control. CO_2 treatments 750 ppm and 1000 ppm represent the mid- and high-CO_2 predictions of Caldeira and Wickett (2003), for the years 2100 and 2300 respectively. The experimental system is outlined in detail in Godbold & Solan (2013) with the carbonate chemistry data reported in Appendix I.

Table 2.1 Details of temperature and light regimes for the experimental period. The ambient temperatures were based on 5 year (2004 - 2009) min-max ranges for the Ythan Estuary. Sunrise and sunset were based on the average monthly mean times and were adjusted on the first of each month. Data provided by J. A. Godbold, University of Southampton.

Month	Ambient Temperature (°C)	Sunrise	Sunset
January	5.5	08:47	15:36
February	5.1	08:08	16:36
March	5.9	07:02	17:40
April	8.6	06:38	19:47
May	11.3	05:20	20:52
June	13.7	04:22	21:51
July	15.2	04:17	22:06
August	14.9	05:05	21:21
September	13.4	06:09	20:05
October	10.8	07:11	18:43
November	7.9	07:18	16:24
December	5.9	08:22	15:32

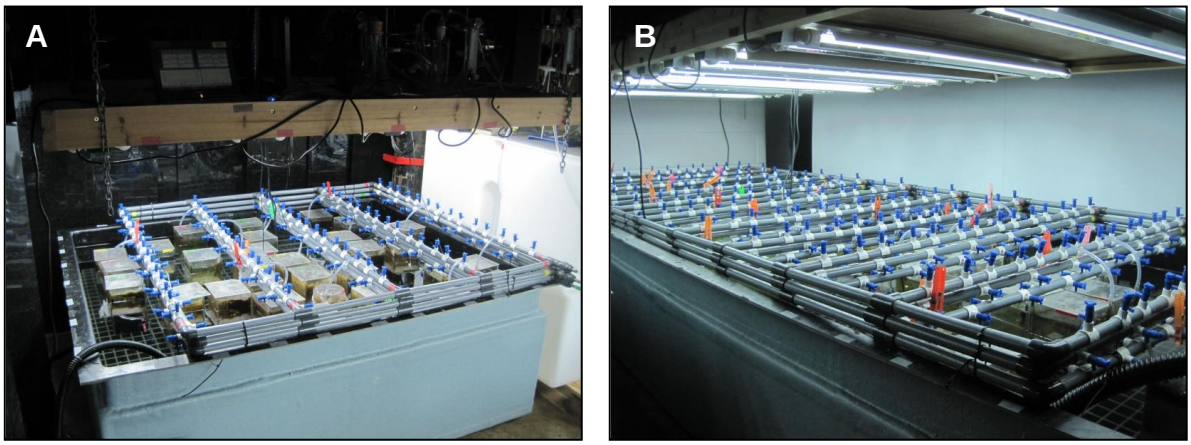

Figure 2.7 Photographs of experimental mesocosms.

2.2.3 Foraminiferal staining and isolation

Time-series samples were taken from the mesocosms throughout a 64 week time period (Table 2.2) to study both short- and long-term variability in the foraminiferal populations.

Three separate replicate cores were sampled at each time point to determine variability and for statistical rigor (Murray, 2006). Each sample combined scrapes from three separate areas on the cores surface to reduce possible effects of patchiness. Care was taken to retain the top 1 cm during sampling as the majority of live specimens are commonly found in the 0 cm to 1 cm interval (Murray, 2000).

Table 2.2 Sampling time points and corresponding dates. (*) are samples not yet analysed.

Time point (week)	Date
T0	10 January 2011
T2*	27 January 2011
T4	11 February 2011
T8	10 March 2011
T12*	4 April 2011
T18*	25 May 2011
T24	6 July 2011
T30	18 August 2011
T36	23 September 2011
T42*	28 October 2011
T50	19 December 2011
T64	19 March 2012

2.2.4 Rose Bengal staining

Samples were fixed and stained immediately after sampling. Foraminifera that were alive at the time of sampling were distinguished from those that were dead using rose Bengal (Walton, 1952). Rose Bengal is a biological stain that absorbs onto proteins, which are major cytoplasmic components, and stains them rose colour

(Bernhard, 2000). Only those individuals that were bright red in at least one chamber were counted as stained (Fig. 2.8). Agglutinated specimens were observed with inverted light to assess whether or not they were stained.

There are number of negative aspects regarding the use of rose Bengal staining (Bernhard, 2000; Bernhard et al., 2006). These include adherence of the stain to dead as well as living cytoplasm, in addition to staining of the organic lining or bacteria attached or located inside the test (Bernhard et al., 2006). Despite its well-known limits, the rose Bengal method remains the most practical way to quantify the living foraminiferal fauna. According to Lutze and Altenbach (1991), if carefully employed, it leads to 96% correct identifications (Debenay et al., 2000).

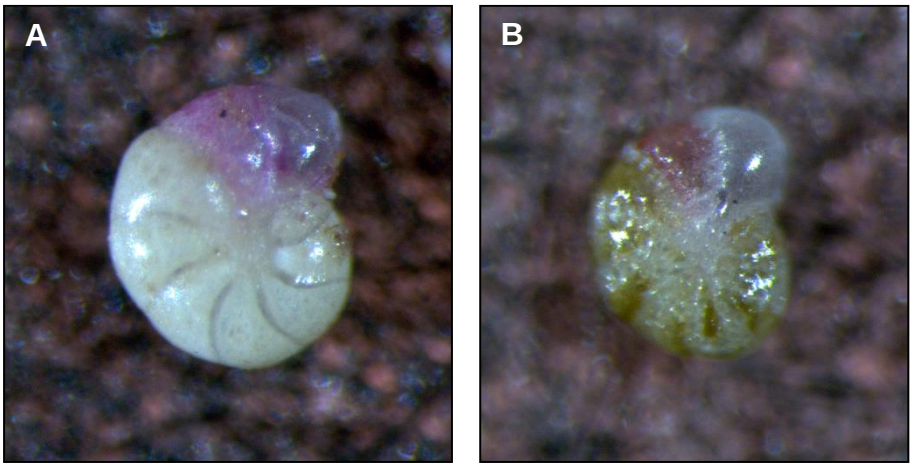

Figure 2.8 Images of foraminiferal specimens showing characteristic rose Bengal staining of the penultimate chamber. A) *Haynesina germanica* (~550 μm). B) *Elphidium williamsoni* (~447 μm).

The sediment samples were gently washed through a 63 μm sieve to remove any excess stain (retaining > 63 μm fraction). The remaining sediment was dried overnight (< 40°C) and the dry weight of each sample was recorded.

2.2.5 Foraminiferal isolation

Foraminifera were extracted from the processed samples after drying. Using a spatula, a portion of the sample was spread thinly over a picking slide and the foraminifera were picked using a fine (000) sable brush under an Olympus® SZ stereo-microscope. The picked individuals were mounted onto pre-glued cardboard slides and an archive collection of this material retained. To achieve statistically reliable assemblage data a minimum of 300 specimens were picked per sample. The portion of each sample picked was measured by weight. In the absence of a known sample wet volume, subsequent foraminiferal concentrations were normalised as number per gram of dry residue.

2.2.6 Foraminiferal measures

To investigate the effects of the target variables (pCO_2 and temperature) on foraminifera the following physical properties were measured for each of the foraminiferal populations. Firstly, census data was extracted (e.g. foraminiferal presence, absence and survival rates). Absolute and relative abundances were then calculated following foraminiferal identification. Finally, measurements of calcareous structures, e.g. functional morphology, were taken on dominant species (*Haynesina germanica*). Both the numbers and sizes of ornamental features within the apertural region of each specimen were quantified and any modifications or changes following experimental exposure were recorded.

2.3 Scanning electron microscopy

Specimens were selected using a fine paintbrush under an Olympus® SZ stereo-microscope and dry-stored on micropalaeontological slides prior to imaging with a scanning electron microscope (SEM). The foraminiferal specimens were mounted

onto SEM stubs using double-sided adhesive tabs. Samples were coated with a thin layer of gold using an Emscope® SC 500 sputter coater. A current of 15 mA at 1.2kV was applied for 3 - 5 minutes (Austin et al., 2005). Specimens were imaged with a scanning electronic microscope (Joel® JSM-35CF SEM).

3. The effect of short-term exposure to ocean acidification on intertidal benthic foraminifera.

3.1 Introduction

Foraminifera have strategic advantages over many other organisms used as environmental proxies because they often occur in large numbers and relatively small samples of sediment often yield statistically significant populations (Schafer, 2000). Their short reproductive cycles and rapid growth make their community structure particularly responsive to environmental change (Yanko et al., 2002).

The ability of foraminifera to grow and their ability to produce calcareous structures is directly affected by changing seawater chemistry. For example, calcification in the benthic foraminifera *Rosalina globularis*, is affected by elevated or reduced salinity, with specimens taking twice as long to reach maturity under these conditions than those kept within a normal range (Saraswat et al., 2011). Larger individuals with more chambers are characteristic of environments approaching minimum limit tolerance for each species (Poag, 1978). This is explained in terms of delayed reproduction which results in the production of larger individuals (Poag, 1978). Growth is achieved by the construction of a new chamber and an increase in number of chambers is also used as an indicator of relative activity under different conditions (Moodley & Hess, 1992).

pH changes as a result of ocean acidification have been shown to have significant impacts on foraminiferal growth and survival (reviewed by Keul et al., 2013). For example, populations of smaller individuals of *Elphidium williamsoni* were observed at low pH compared to other treatments (Allison et al., 2010). Small specimens (150

– 250 µm) have also been observed to sustain greater damage at high pCO_2 levels than larger specimens (Haynert et al., 2011).

Variation in foraminiferal responses can be attributed to the species studied and to the different methods used to manipulate the seawater carbonate chemistry (Keul et al., 2013). Subjecting different species to the same changes in experimental seawater chemistry can allow the detection of species-specific responses (Keul et al., 2013). In this chapter we investigate the short-term response of two dominant intertidal foraminiferal species to reduced pH.

Haynesina germanica and *Elphidium williamsoni* are known to be dominant foraminiferal species in the Eden Estuary, NE Scotland (the sampling site for this study). At different times of the year and over a seasonal cycle, dominance between these two species varies (Austin, 2003). *E. williamsoni* is an important temperate intertidal species that often dominates low-marsh-living populations (Scott et al., 2001). Species of *Elphidium* are well-known for their tolerance to salinity variations (Sen Gupta, 2002). *H. germanica* is common and broadly distributed in temperate coastal settings (Goldstein & Alve, 2011) and is considered one of the most opportunistic brackish foraminifera in Southern England (Murray, 2006). *H. germanica* grows quickly and is capable of reproducing from at least 12 to ~22°C (Goldstein & Alve, 2011). There is no obvious difference in mobility between *H. germanica* and *E. williamsoni* and each of these species are known to thrive equally well under a range of salinities (Alexander & Banner, 1984).

With the biological impacts still poorly constrained, it is unclear if intertidal benthic foraminifera have the ability to acclimate or adapt to the changes that are predicted to occur to surface seawater carbonate chemistry. Any impacts of ocean acidification

on these dominant species could have implications for intertidal foraminiferal communities. Here we perform a short-term incubation to examine the effects of reduced pH on survival and growth in the above two calcareous species.

3.2 Materials and methods

3.2.1 Sediment collection and foraminiferal isolation

Surface scrapes for foraminiferal isolation were collected from the Eden Estuary, N.E. Scotland (56°22'N, 2°50'W) in May 2011. Live specimens of *Haynesina germanica* and *Elphidium williamsoni* were removed from the sediment with a fine paintbrush and subsequently cleaned in shallow Petri dishes containing seawater. Detailed sampling and isolation methods are outlined in Chapter 2.1.

3.2.2 Experimental culture system

The culture system used for this chapter was based on the recirculating system of Hintz et al. (2004). The pH of three large reservoir tanks was manipulated by CO_2 bubbling via an electrovalve feedback system. Details of the culture system are outlined in Chapter 2.1. A series of five replicate culture chambers were prepared for each pH treatment. Reservoir tank 1 (culture chambers 1.1 - 1.5) was set at pH 7.7, reservoir tank 2 (culture chambers 2.1 – 2.5) was set at pH 7.3 and reservoir tank 3 (culture chambers 3.1 – 3.5) was set to pH 7.5. Mixed assemblages of *Haynesina germanica* and *Elphidium williamsoni* were placed in each culture chamber (Fig. 3.1). Each culture chamber housed a total of between 45 and 49 live foraminiferal specimens (Table 3.1). A reproduction event occurred during calcein staining. Each of the 4 - 5 chambered juveniles were distributed evenly into the experimental culture

chambers. All specimens were alive and active at the beginning of their experimental incubations. Before experimental incubation, the maximum diameter and total number of chambers was counted and recorded for each of the 688 foraminiferal specimens (Table 3.2).

Table 3.1 Total culture chamber contents prior to experimental incubations.

Reservoir Tank	pH	Replicate	Number of individuals
1	7.7	1	49
1	7.7	2	46
1	7.7	3	45
1	7.7	4	46
1	7.7	5	46
2	7.3	1	45
2	7.3	2	46
2	7.3	3	47
2	7.3	4	45
2	7.3	5	45
3	7.5	1	45
3	7.5	2	46
3	7.5	3	46
3	7.5	4	46
3	7.5	5	45
Total number of specimens			**688**

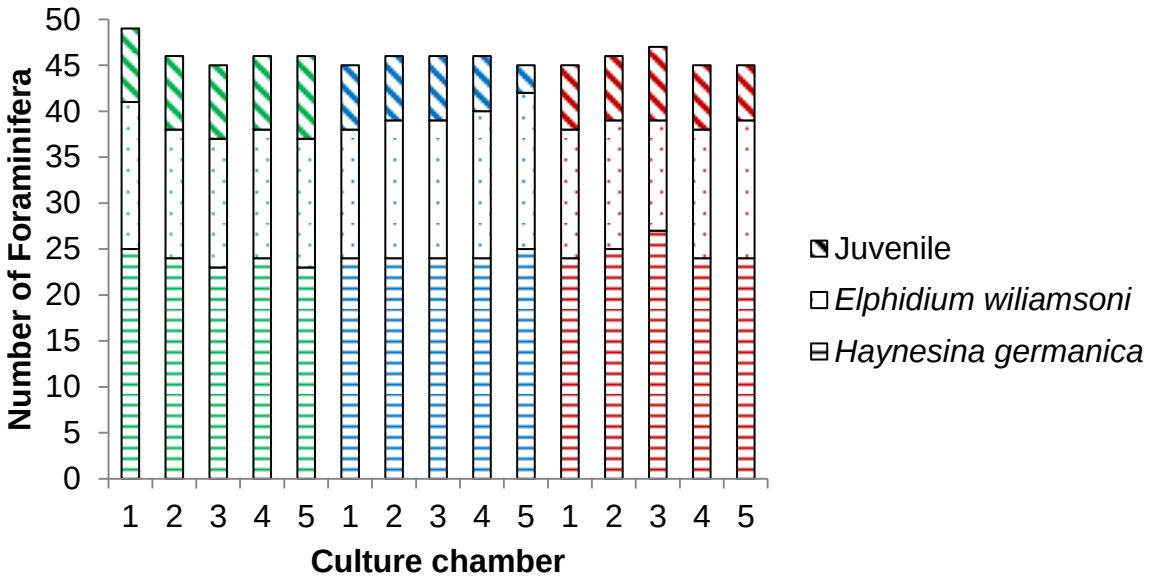

Figure 3.1 Foraminiferal species compositions of culture chambers prior to experimental incubation. pH treatments are represented by green (pH 7.7), blue (pH 7.5) and red (pH 7.3). Specimens of *Haynesina* are represented by horizontal lines, *Elphidium* by dots and juveniles by diagonal lines.

Foraminifera were maintained in the culture system for six weeks. At the end of the experimental incubations foraminifera were classified as live or dead. The maximum test diameters were measured for each population in addition to determining the number of new chambers formed during the experimental period.

3.2.3 Statistical analyses

To determine possible associations between foraminiferal survival and growth statistical analyses were performed using the statistical programming software 'R' (R Development Core Team, 2012). Once assumptions (normally distributed data and homogenous variances) were confirmed, a one-way ANOVA was used to determine the effects of pH on survival and growth (maximum diameter and number of chambers). Post-hoc tests were performed to determine which means differed from each other.

Table 3.2 Measurement data for foraminiferal specimens prior to experimental incubations. Average maximum diameters and number of chambers per specimen are displayed for each species (values represent mean ± SD). n = number of individuals.

Treatment		*Haynesina*			*Elphidium*			Juvenile		
pH	Replicate	Size (µm)	Chambers	n	Size (µm)	Chambers	n	Size (µm)	Chambers	n
7.7	1	292.04 ± 50.96	9.36 ± 0.70	25	313.13 ± 66.98	12.06 ± 1.34	16	117.63 ± 10.60	4.63 ± 0.92	8
7.7	2	291.13 ± 52.51	9.08 ± 0.72	24	325.07 ± 63.24	12.07 ± 1.27	14	109.00 ± 12.09	4.88 ± 0.64	8
7.7	3	281.61 ± 46.14	9.35 ± 0.65	23	313.29 ± 60.24	12.07 ± 1.14	14	115.50 ± 39.65	4.75 ± 0.46	8
7.7	4	276.75 ± 48.57	9.33 ± 0.92	24	300.64 ± 44.60	12.00 ± 1.47	14	107.63 ± 12.79	4.75 ± 0.71	8
7.7	5	273.30 ± 39.70	9.26 ± 0.62	23	287.36 ± 49.58	11.69 ± 0.85	14	121.11 ± 24.53	5.44 ± 1.24	9
7.5	1	272.83 ± 43.31	9.21 ± 0.72	24	275.07 ± 64.46	11.57 ± 1.50	14	121.43 ± 26.44	4.71 ± 0.76	7
7.5	2	279.04 ± 54.93	9.33 ± 0.70	24	311.87 ± 59.87	11.73 ± 1.33	15	114.86 ± 9.69	5.14 ± 0.69	7
7.5	3	286.61 ± 43.32	9.30 ± 0.70	23	314.00 ± 57.84	12.00 ± 1.20	15	104.86 ± 10.24	4.43 ± 0.53	7
7.5	4	295.42 ± 63.99	9.50 ± 0.66	24	302.44 ± 60.81	12.06 ± 1.18	16	135.17 ± 44.05	5.83 ± 2.14	6
7.5	5	285.20 ± 48.32	9.20 ± 0.65	25	351.71 ± 58.82	12.47 ± 1.12	17	157.33 ± 55.87	7.00 ± 2.65	3
7.3	1	280.75 ± 67.91	9.63 ± 0.97	24	309.57 ± 58.05	12.21 ± 1.58	14	113.14 ± 16.62	4.86 ± 0.90	7
7.3	2	278.72 ± 55.26	9.56 ± 0.92	25	307.64 ± 52.11	12.07 ± 0.92	14	121.43 ± 32.09	4.86 ± 0.69	7
7.3	3	267.96 ± 50.14	9.38 ± 0.85	26	345.58 ± 46.60	12.00 ± 1.13	12	103.38 ± 10.23	4.50 ± 0.53	8
7.3	4	278.46 ± 44.42	9.08 ± 0.83	24	325.31 ± 43.98	11.92 ± 1.04	13	143.50 ± 96.62	5.63 ± 2.77	8
7.3	5	280.17 ± 52.94	9.29 ± 0.86	24	309.20 ± 62.45	11.87 ± 1.06	15	115.83 ± 16.56	5.00 ± 0.63	6

3.3 Results

3.3.1 Seawater carbonate chemistry

Table 3.3 Average seawater measurements from experimental culture chambers (values represent mean ± SD, n = 3). The measured values of temperature, salinity and pH (Total) and total alkalinity (A_T) were used to calculate the values of dissolved inorganic carbon (DIC), pCO_2, saturation states of calcite ($\Omega_{Calcite}$) and aragonite ($\Omega_{Aragonite}$), bicarbonate (HCO_3^-), and carbonate concentration (CO_3^{2-}) using CO_2Calc with appropriate solubility constants (Mehrbach, 1973, refit by Dickson & Millero, 1987) and KSO_4 (Dickson, 1990).

Target pH	Temp (°C)	Salinity	$pH_{(Total)}$	A_T (µmol kg^{-1})	DIC (µmol kg^{-1})	pCO_2 (µatm)	$\Omega_{Calcite}$	$\Omega_{Aragonite}$	HCO_3^-	CO_3^{2-}
7.7	16.57 ± 0.15	29.1 ± 0.20	7.69 ± 0.02	2379.2 ± 2.42	2314.16 ± 4.91	535.90 ± 33.19	1.68 ± 0.09	1.04 ± 0.06	2211.20 ± 6.50	68.20 ± 3.71
7.5	16.60 ± 0.17	29.2 ± 0.06	7.50 ± 0.03	2396.3 ± 23.11	2389.71 ± 29.52	892.13 ± 76.89	1.08 ± 0.08	0.67 ± 0.05	2341.19 ± 23.03	44.01 ± 3.17
7.3	16.73 ± 0.25	29.1 ± 0.06	7.28 ± 0.05	2404.4 ± 28.10	2471.07 ± 19.81	1607.21 ± 175.64	0.63 ± 0.07	0.39 ± 0.05	2287 ± 27.71	25.67 ± 3.01

3.3.2 Foraminiferal survival (loss and mortality)

Following the experimental incubation, each culture chamber was opened and specimens were categorised as alive or dead (Fig. 3.2). Any specimens that were inactive or contained no cytoplasm were considered as dead. The number of missing specimens in each culture chambers was also calculated.

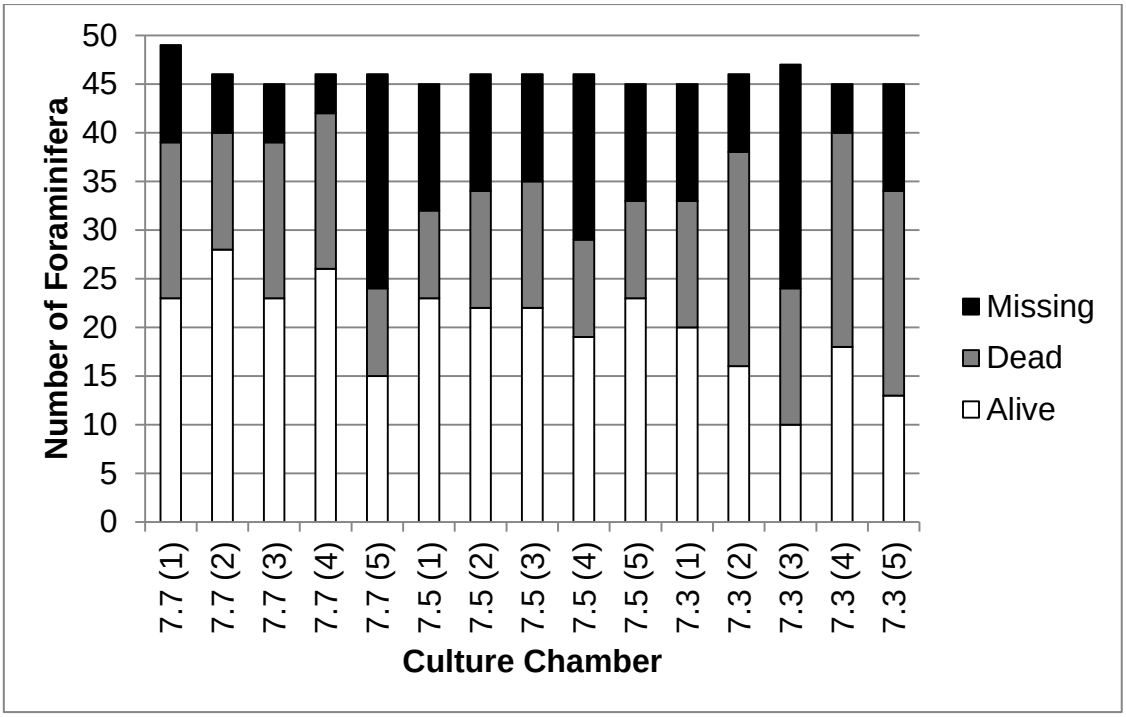

Figure 3.2 Survival, loss and mortality of foraminiferal specimens. Each bar represents an individual culture chamber. Replicate culture chambers are labelled 1 - 5 for each pH treatment (7.7, 7.5 & 7.3).

The average loss and mortality for each of the pH treatments revealed the lowest survival at pH 7.3 (Table 3.4). The lowest number of live specimens (15.40 ± 3.97) and highest number of dead specimens (18.40 ± 4.51) were both found in the lowest pH treatment. The highest survival rates were observed under normal pH (7.7) conditions where, on average, 23.00 ± 4.95 specimens were recorded as alive. On average, between 9.60 ± 7.27 and 13.00 ± 2.35 specimens were recorded as missing.

pH treatment had a significant effect on the number of live specimens recorded following the experimental incubations ($P < 0.02$). Post-hoc tests revealed significant

differences between the number of live specimens recorded at pH 7.3 and those recorded in each of the other pH treatments (pH 7.5 and pH 7.7).

Table 3.4 Average numbers of live, dead and missing foraminifera from each experimental treatment (values represent mean ± SD, n = 5).

	Foraminiferal Specimens		
pH Treatment	Live	Dead	Missing
7.7	23.00 ± 4.95	13.80 ± 3.19	9.60 ± 7.27
7.5	21.80 ± 1.64	10.80 ± 1.64	13.00 ± 2.35
7.3	15.40 ± 3.97	18.40 ± 4.51	11.80 ± 6.83

3.3.3 Test growth

The average final diameter varied between 302.64 ± 101.95 µm and 256.24 ± 31.40 µm for *Haynesina germanica* and 401.50 ± 12.02 µm and 230.70 ± 84.47 µm for *Elphidium williamsoni* (Table 3.5). For *H. germanica*, the highest average maximum diameter was recorded at normal pH (7.7). For *E. williamsoni*, the highest average maximum diameter was recorded at the lowest pH (7.3). pH treatment had no significant effect on the average maximum diameter ($P > 0.7$) or average number of chambers ($P > 0.6$) in specimens of *H. germanica*. pH treatment did however have a significant effect on the average maximum diameter ($P < 0.02$) and average number of chambers ($P < 0.02$) in specimens of *E. williamsoni*. Post-hoc tests revealed a significant difference in the average maximum diameter of *E. williamsoni* incubated at pH 7.7 to those at the two reduced pH (7.5 and 7.3) levels. The average number of chambers on *E. williamsoni* specimens significantly differed between the highest (7.7) and lowest (7.3) pH treatments.

Table 3.5 Measurement data for foraminiferal specimens following experimental incubations. Average maximum diameters and number of chambers per specimen are displayed for each species (values represent mean ± SD). n = number of individuals.

Treatment		*Haynesina*			*Elphidium*		
pH	**Replicate**	**Size (µm)**	**Chambers**	**n**	**Size (µm)**	**Chambers**	**n**
7.7	1	302.64 ± 101.95	8.29 ± 0.61	14	320.56 ± 77.58	11.22 ± 0.67	9
7.7	2	285.70 ± 42.82	8.40 ± 0.50	20	288.88 ± 71.52	10.88 ± 2.17	8
7.7	3	286.62 ± 38.39	8.31 ± 0.63	13	230.70 ± 84.47	10.00 ± 2.16	10
7.7	4	270.58 ± 44.06	8.25 ± 0.75	12	240.29 ± 84.31	9.86 ± 2.48	14
7.7	5	263.15 ± 62.59	8.00 ± 0.91	13	307.50 ± 53.03	11.00 ± 0.00	2
7.5	1	256.24 ± 31.40	7.94 ± 0.43	17	341.50 ± 52.99	11.33 ± 0.82	6
7.5	2	278.87 ± 50.41	8.40 ± 0.63	15	312.14 ± 52.66	10.71 ± 1.50	7
7.5	3	283.95 ± 44.64	8.16 ± 0.76	19	322.00 ± 45.30	11.33 ± 1.53	3
7.5	4	295.18 ± 52.15	8.59 ± 0.51	17	306.50 ± 176.07	10.50 ± 2.12	2
7.5	5	270.00 ± 49.52	8.18 ± 0.95	17	363.83 ± 66.37	11.33 ± 1.51	6
7.3	1	287.83 ± 60.05	8.83 ± 0.83	12	315.00 ± 55.60	11.38 ± 1.19	8
7.3	2	278.27 ± 62.18	8.45 ± 1.13	11	373.60 ± 51.15	11.80 ± 0.84	5
7.3	3	276.25 ± 24.27	8.25 ± 0.46	8	401.50 ± 12.02	12.00 ± 1.41	2
7.3	4	295.80 ± 36.37	8.40 ± 0.52	10	341.13 ± 56.14	11.00 ± 1.07	8
7.3	5	267.42 ± 73.58	8.00 ± 1.21	12	332	12	1

Frequency distribution charts display the maximum diameter data for both *H. germanica* (Fig. 3.3) and *E. williamsoni* (Fig. 3.4) at the start and end of the experimental incubations. For *H. germanica*, there was a decrease in the mean and mode average maximum diameter between the start and end of the experiment at normal pH (Fig. 3.3A). The median value increased by 3 to 281 µm. At pH 7.5, the mean, median and mode values for average maximum diameter of *H. germanica* all

decreased between the start and end of experiment (Fig. 3.3B). At the lowest pH, the mean, median and mode values for average maximum diameter all increased (Fig. 3.3C). The median increased from 242 µm to 264 µm and the mode increased from 273 µm to 280 µm.

Frequency distributions for *E. williamsoni* also showed variation between the start and end of the experimental incubations (Fig. 3.4). Under normal pH (7.7) conditions, the mode value increased from 240 µm to 263 µm while the median value decreased from 305.50 µm to 268 µm (Fig. 3.4A). At pH 7.5, the mode value decreased from 307 µm to 276 µm and the median value increased from 313 µm to 330 µm (Fig. 3.4B). Similarly, the results observed for *H. germanica* show the mean, median and mode values for average maximum diameter in *E. williamsoni* also all increased at the lowest pH treatment (Fig 3.4C). Mode values increased from 340 µm to 393 µm and median values increased from 319 µm to 339.50 µm.

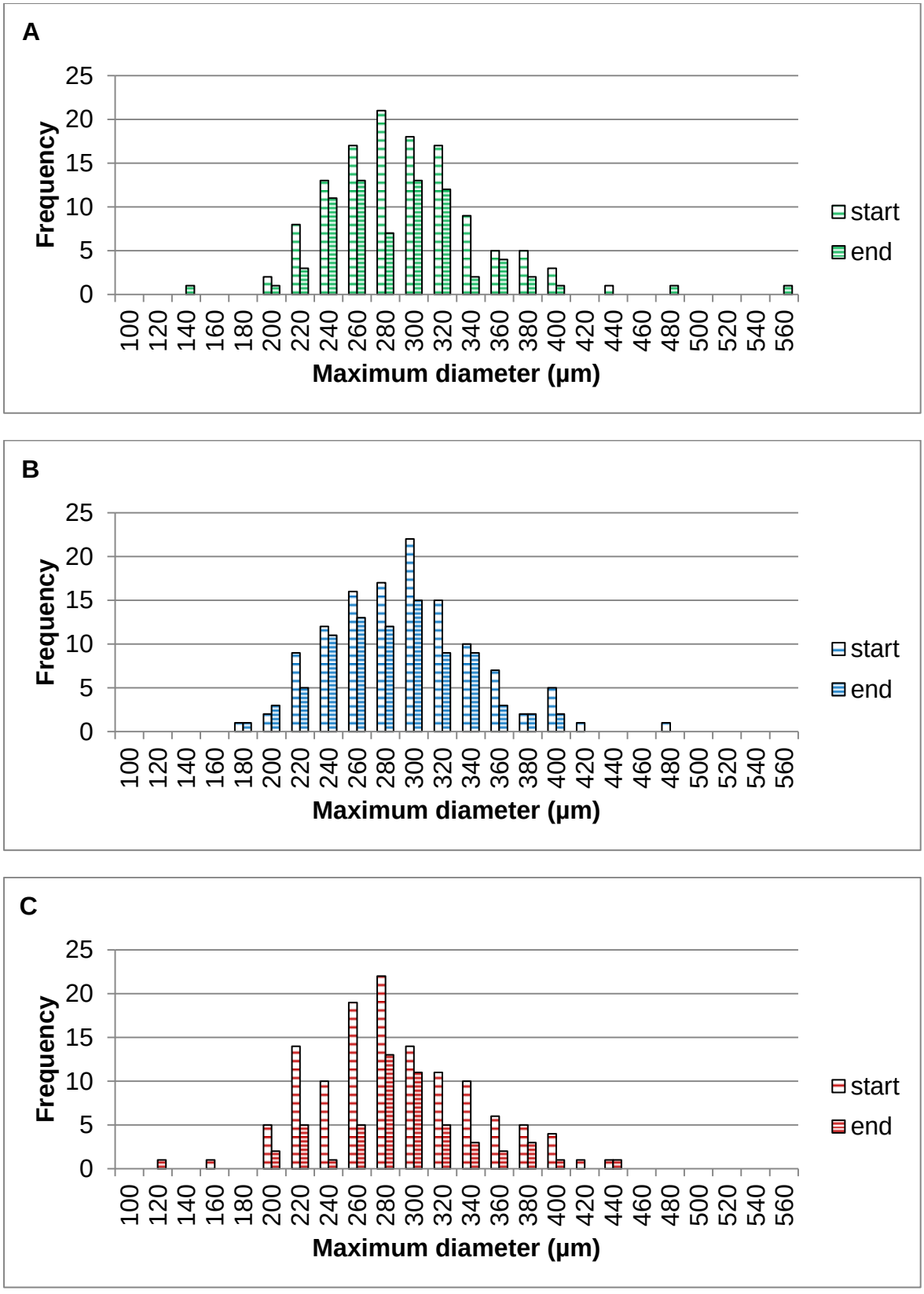

Figure 3.3 Size distributions for live *Haynesina germanica* at the start and end of the culture period. pH treatments are represented by green (pH 7.7), blue (pH 7.5) and red (pH 7.3).

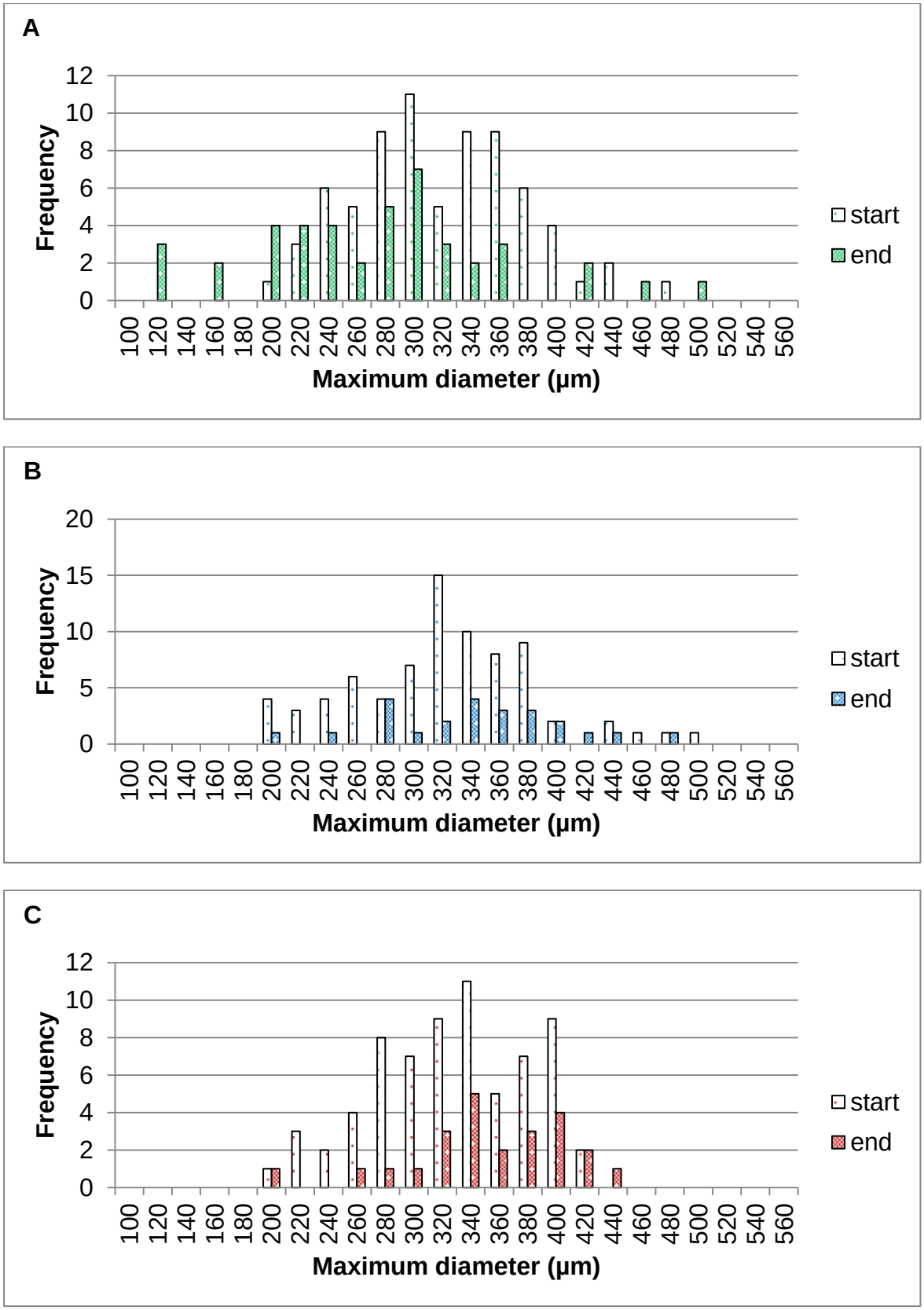

Figure 3.4 Size distributions for live *Elphidium williamsoni* at the start and end of the culture period. pH treatments are represented by green (pH 7.7), blue (pH 7.5) and red (pH 7.3).

3.4 Discussion

3.4.1 Foraminiferal survival

3.4.1.1 Live specimens

Live specimens of both *Haynesina germanica* and *Elphidium williamsoni* were recorded in all 3 pH treatments. The average number of total live specimens was reduced under the lowest pH (7.3) treatment. Predictions based on other intertidal foraminiferal species, e.g. *Ammonia* spp., suggest possible disappearance of these calcareous species due to vulnerability to ocean acidification (Haynert et al., 2011). The responses here however indicate that these dominant calcareous species are able to withstand and survive short-term exposure to ocean acidification. This is comparable to observations in the northern Gulf of California where live calcareous foraminifera were present in low numbers at sites of low pH. Dissolution was evident on calcareous specimens from natural vent sites of lower pH (Pettit et al., 2013). Impacts of ocean acidification on the foraminiferal test are addressed in chapters 5 and 6. Reductions in numbers could lead to changes in community structure and favour non-calcareous foraminiferal species. This is explored further in Chapter 4.

3.4.1.2 Reproduction

Although there was a reproduction event during calcein staining there was no evidence of reproduction during the experimental incubations. Reproduction typically takes place by mature individuals under favourable conditions. Under unfavourable conditions, reproduction may be delayed and growth can slow or completely cease (Austin, 2003). For some species of foraminifera, reproduction is initiated by favourable temperatures (Murray, 2006). It is possible that as the experiment was

carried out over a constant temperature regime that the foraminifera did not have the temperature cue required for reproduction.

3.4.1.3 Dead specimens

Typically, *E. williamsoni* are not recovered from the fossil record of organic-rich intertidal sediments (Scott et al., 2001). Their tests dissolve in these low pH sediments shortly after death (Scott et al., 2001). Typically, a star shaped test in benthic foraminifera is thought to indicate low pH conditions (Le Cadre et al., 2003). However, in this case, both live and dead specimens of *H. germanica* and *E. williamsoni* were recorded as fully intact following six weeks exposure to low pH conditions (Fig. 3.5).

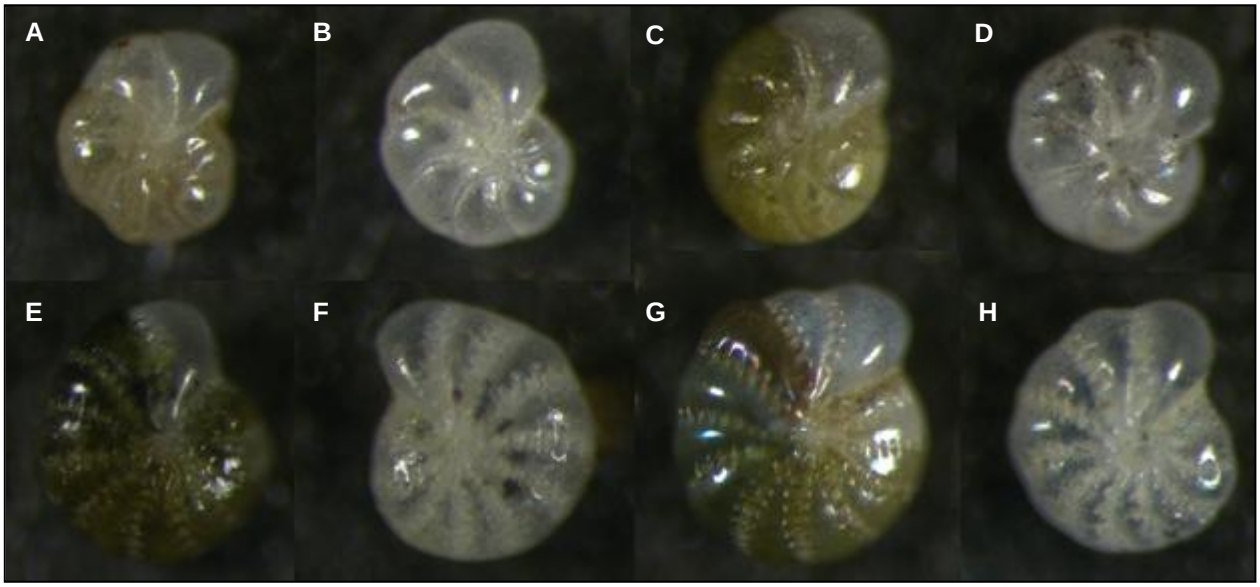

Figure 3.5 Images of typical live (A, C, E, G) and dead (B, D, F, H) individuals of *Haynesina germanica* (A-D) and *Elphidium williamsoni* (E-H) following incubations at pH 7.7 (A-B, E-F) and pH 7.3 (C-D, G-H). Note: all specimens are still complete with no obvious signs of wall dissolution or breakage.

H. germanica and *E. williamsoni* may require exposure to low pH conditions for a period longer than six weeks for extreme dissolution to occur. Structural and morphological analyses were not undertaken as part of this chapter but are presented in detail in chapters 5 and 6. In these chapters, both short- and long-term exposure to ocean acidification are incorporated.

3.4.1.4 Missing specimens

At the start of each experimental incubation an average of 46 foraminiferal specimens were housed in each culture chamber. Following the experimental incubations, some specimens were missing from the culture chambers. It is possible that some specimens were lost during the process of sampling (Haynert et al., 2011). Alternatively, emigration from the culture chambers may have occurred. In a previous study involving *E. williamsoni*, individuals that migrated up the sides of the culture chambers became trapped in the menisci of air bubbles and were transported out of the culture chambers (Allison et al., 2010).

3.4.2 Foraminiferal growth

All specimens were incubated in calcein prior to the experimental incubations to allow assessment of new chamber growth within each of the pH treatments. Similarly to results observed by Haynert et al. (2011) some foraminiferal specimens contained calcein in newly precipitated calcite even though it had not grown in calcein-stained seawater. This means we cannot assess accurately the numbers of new chambers added for individual specimens during the experiment. Calcein may have been absorbed to the organic lining during calcein-staining or incorporated and stored within vacuoles filled with seawater (Haynert et al., 2011). The calcein may therefore

have been remobilised during the formation of new chambers leading to newly formed chambers that also fluoresced (Haynert et al., 2011).

The average population sizes were calculated for each of the culture chambers before and after the experimental incubations. pH treatment had no significant effect on the average numbers of chambers or average maximum diameter in *H. germanica*. *Haynesina* therefore shows signs of resilience to low pH. This species may require longer exposure periods to reduced pH to observe any significant effects on their morphology.

Previous experiments on *E. williamsoni* observed no significant difference in the number of chambers deposited in a culture system with manipulated pH (Allison et al., 2010). In this experiment, however, pH treatment had a significant effect on the average maximum diameter and number of chambers in *E. williamsoni*. Significant differences in average maximum diameter were observed between those cultured at pH 7.7 and those in both of the reduced pH treatments. There was also a significant difference in average number of chambers between the highest and lowest pH treatments. The differences in the responses observed between this study and Allison et al. (2010) could be attributed to the manipulation method employed. In Allison et al. (2010), the addition of acid (HCl), or base (NaOH) were used to manipulate pH. Acid addition does not reproduce the increase in bicarbonate concentration generated by natural ocean acidification, whereas gas bubbling does (Gattuso et al., 2010). The data here indicate that *E. williamsoni* continued to grow in the low pH treatment. Given that it is an intertidal species, *E. williamsoni* is likely adapted to variable physicochemical conditions and low pH.

Keul et al. (2013) observed high variability in final shell diameter in specimens of *Ammonia* spp. They concluded that the variability they found was a natural characteristic among *Ammonia* spp and was not induced by their experimental treatments (Keul et al., 2013).

Size frequency distribution shifts suggest there was growth in some of the foraminiferal specimens. The average, mean and mode measurements for maximum diameter did however decrease in some cases. Size reductions have been attributed to the loss of the outer shell wall and partial collapse of the inner organic lining (Haynert et al., 2011). Since all specimens at the end of the experiment were intact this cannot explain the reductions in average maximum diameter measurements. As only live specimens were used to construct the frequency distributions it is possible that the larger representatives of the initial cultures were part of the dead assemblage or missing at the end of the experiment.

3.4.3 Future work

From this chapter we were able to determine that two dominant intertidal species of foraminifera are able to survive, albeit in lower numbers, under reduced pH conditions. The experiment was however carried out over the short-term and did not incorporate possible temperature effects. Temperature has been shown to be the most important factor determining experimental foraminiferal assemblages (Goldstein & Alve, 2011). In the following mesocosm experiment (Chapters 4 - 7), we introduce a seasonal component in terms of temperature variation and multiple species to investigate community level responses of foraminifera to ocean acidification. This will help to improve our understanding of the possible effects of ocean acidification on benthic foraminiferal community structure.

4. The effects of long-term exposure to ocean acidification on benthic foraminiferal diversity and community composition.

4.1 Introduction

Foraminifera are often used as indicators of environmental conditions because they are abundant and have diverse assemblages specific to varied contextual settings (Scott et al., 2001). Therefore, with sufficient knowledge of their ecology foraminiferal community structures can provide information on ecological conditions and the general characteristics of the environment. The composition of benthic foraminiferal assemblages reflects the complex interaction between biotic and abiotic parameters and their variation in space and time (Debenay et al., 2000).

The comparatively high species diversity of benthic foraminiferal populations renders local assemblages responsive to a broad range of environmental change (Scott et al., 2001). Some foraminifera exhibit species-specific sensitivities to environmental variables (Debenay et al., 2000; Geslin et al., 2000). The response of an organism to environmental change depends on a number of factors including their physiological capabilities, and whether they have a short or long turnover time in relation to the change and whether the change is potentially favourable or unfavourable (e.g. an increase in stress) (Murray, 2000).

The simplest types of foraminiferal proxies are based on abundances of ecologically significant (indicator) species (Kucera, 2007). The preserved remains of foraminiferal assemblages give a time-averaged record of successive living assemblages (Murray & Alve, 1999a; Debenay et al., 2000) while living assemblages record the conditions at the time of sampling. Studies investigating varying impacts on foraminiferal communities often use numerical characterisations of assemblage structure such as

diversity index and the dominance index (Murray. 1973). Although these indices allow detection of changes in the assemblages they do not take into account the role of the species in the community or their potential as bioindicators (Debenay et al., 2000). Analysis of changes in abundance of species (including introduction of a new species or serious loss of an existing species), in addition to documenting changes in species diversity, dominance and abundance make it possible to examine the extent of environmental changes that have, or are, taking place (Murray, 2000).

Increasing levels of environmental stress have been considered to decrease diversity and species richness while increasing dominance (Clarke & Warwick, 2001; Magurran, 2004). It is at intermediate levels of disturbance that diversity is highest (Clarke & Warwick, 2001, and references therein). Some ecological factors are limiting in the sense that there is a threshold for specific species. Beyond this threshold, the organism is incapable of reproduction or, at the extreme, may die (Murray, 2000).

There may be time lags of longer than one year between a disturbance and an ecological response (Murray, 2000). The duration of response will depend on the frequency of reproduction of the organisms involved, which is commonly only a few months in the case of foraminifera (Murray, 2000). For many benthic foraminifera the generation time is less than 3 to 4 months (Murray, 2000).

In studies where authors have used multivariate and statistical measures to determine relationships between abiotic variables and species there has always been a great deal of co-variance and interdependence between variables (Murray, 2006). Culturing provides an opportunity to test the impacts of specific environmental variables under carefully controlled laboratory conditions to better understand the responses of target organisms and assemblages.

The aim of this study is to establish the relationship between the community structure of foraminiferal populations under possible ocean acidification scenarios. To-date, very few studies have investigated the community level impact of ocean acidification on benthic foraminifera although many have focussed on single species and short-term (days to weeks) responses. In this chapter, the foraminiferal community level response to ocean acidification is examined in the longest running experimental exposure that we know of to-date (64 weeks). In this novel time-series, both short-term (potential shock) and long-term (possible acclimation) responses of benthic foraminifera were recorded. Seasonal temperature regimes were also incorporated under each ocean acidification scenario as sensitivities in other organisms (e.g. intertidal polychaete *Alitta virens*) have been shown to be enhanced at different times of the year (Godbold & Solan, 2013). Diversity measures (species richness & evenness) were combined with community analyses to examine the effects of ocean acidification on foraminiferal population responses and identify species-specific sensitivities.

4.2 Materials and Methods

4.2.1 Sediment collection

Sediment was collected from mesocosms maintained as part of a long-term time-series culturing experiment (Chapter 2.2). This chapter is based on surface sediment samples collected from mesocosms maintained at either 10°C constant (average local conditions) or seasonally varying ambient temperature. Seawater pH was controlled by bubbling air with a known concentration of CO_2 (380 ppm, 750 ppm or 1000 ppm) into each mesocosm (Godbold & Solan, 2013). Throughout a 64 week exposure period, surface scrapes of 1 - 2 mm depth were collected from each of the treatments for the isolation of living benthic foraminifera. Samples were taken from the constant temperature regime at week 0 to provide a baseline and enable assessment of the foraminiferal assemblages prior to the onset of CO_2 bubbling. Experimental samples were then exposed for a period of 4, 8, 24, 30, 36, 50 or 64 weeks. Sampling dates, regimes and methodology are provided in detail in Chapter 2.2. All seawater chemistry measurements are summarised in Appendix I (Table S1).

4.2.2 Foraminiferal isolation and identification

Foraminifera were extracted and stored following the methods outlined in Chapter 2.2. Species were identified based on the classification of Loeblich & Tappan (1988) and Haynes (1973), and species abundances were tabulated. Specimens of each newly identified species were prepared and imaged using scanning electron microscopy (methodology outlined in Chapter 2.3). Additional live specimens from the mesocosms were sent for genetic analyses to verify species identifications as belonging to a single genotype. Total assemblage data was collected and included the number living (based on Rose Bengal staining) and dead specimens at the time

of sampling in addition to documentation of wall structures (agglutinated, calcareous hyaline or calcareous porcelaneous). From the live assemblages, data were extracted on the total abundance of living individuals and the number of living species (species richness) per sample in addition to species-specific abundance data.

4.2.3 Statistical analyses

To determine possible associations between foraminiferal assemblages and specific experimental treatments statistical analyses were performed using multivariate community analysis (Primer). A species matrix was constructed tabulating the live foraminiferal assemblage data together with the experimental factors. Experimental factors were labelled as follows: CO_2 Treatment: L (380 ppm), M (750 ppm) and H (1000 ppm); Temperature Regime: C (constant) and A (ambient); Week: 1 (week 0), 2 (week 4), 3 (week 8), 4 (week 24), 5 (week 30), 6 (week 36), 7 (week 50) and 8 (week 64). A Bray-Curtis species resemblance matrix was constructed for patterns of occurrences of each of the species across each set of samples. Rare species (never constituting > 3% of assemblage) were omitted from the data matrix before computing species similarities (Clarke & Warwick, 2001).

Cluster analysis was performed on the species similarity matrix to define species assemblages and to identify groupings (species abundances that tend to co-occur across sites). Samples were linked using a hierarchical method and a visual representation of the clusters was constructed in the form of a dendrogram. A ‘similarity profile’ (SIMPROF) permutation test was incorporated to identify statistically significant clusters in the samples.

Nonmetric multidimensional scaling (MDS) ordination plots in two dimensions were used to identify samples that were similar in community compositions (points that lie close together on an MDS plot) and to distinguish samples that were dissimilar (points that were more distant on the MDS plot). Clusters identified by SIMPROF analysis were plotted on each MDS plot to identify factors controlling cluster relatedness. Bubble plots were constructed to track behaviour of single species under different experimental regimes.

To assess diversity and equitability of the assemblages, the Shannon diversity index (H') and Pielou's evenness index were calculated for each sample. Analyses of live abundance and species richness data was performed using the statistical programming software 'R' (R Development Core Team, 2012), and the additional 'nlme' (linear and non-linear mixed effects models package (version 3.1-110, Pinheiro et al., 2012). A basic linear regression model (containing all explanatory variables and their interactions) was applied to both the live abundance and species richness data. Data was assessed for normality (Q-Q plots) and homogeneity of variance by plotting the standardised residuals versus fitted values and the standardised residuals versus each individual explanatory variable (Zurr et al., 2007; Zurr et al., 2009). To avoid data transformations and in order to account for heterogeneity of variance, a generalised least squares (GLS) mixed modelling approach was adopted (West et al., 2007; Zurr et al., 2007; Zurr et al., 2009). The most appropriate variance-covariance structure for each model was determined using AIC scores and the examination of plots of fitted values versus residuals based on a full model specification using restricted maximum likelihood (REML, West et al., 2007). Once the optimal random component structure was found, the optimal fixed component structure was determined. This was established through a manual

backwards stepwise selection approach where explanatory variables that were not significant were dropped using maximum likelihood (ML) estimation. The influence of each independent term was assessed using a likelihood ratio test between the minimum adequate model and reduced models, with all terms involving the relevant independent factor removed (including interactions).

4.3 Results

4.3.1. Total assemblage data

A total of 135 sediment samples were analysed for benthic foraminifera and their abundances were recorded. Approximately 42,000 individual benthic foraminiferal specimens were counted and identified across the time-series. Many samples had higher numbers of dead specimens in relation to living at the time of sampling (Table 4.1). A total of 63 species representing 33 genera were identified from the total assemblages (Table 4.2). Based on wall structure, 10 agglutinated, 47 hyaline and 6 porcelaneous species were identified (Figs. 4.1 – 4.6).

Not all species identified in the total assemblages were represented by living specimens. Living assemblages are described in detail in section 4.3.2. The dead assemblages were dominated by calcareous hyaline forms after exposure to all treatments (Table 4.3). Overall, specimens from all treatments showed good preservation with little evidence of fragmentation observed in the sample material.

Table 4.1 Average numbers of live and dead benthic foraminifera in each sample (values represent mean ± SD, n = 3). CO_2 concentrations are 380 ppm, 750 ppm or 1000 ppm. Temperature regimes are represented as 10°C (constant regime) or Amb (seasonally varying ambient regime).

Sample		380 ppm		750 ppm		1000 ppm	
Week	**Temperature**	**Live**	**Dead**	**Live**	**Dead**	**Live**	**Dead**
0	**10°C**	9.7 ± 1.5	304.7 ± 11.0	15.7 ± 2.1	285.3 ± 4.2	21.3 ± 3.1	277.7 ± 10.7
4	**10°C**	41.7 ± 17.2	266.0 ± 20.2	34.7 ± 10. 8	271.7 ± 10.5	46.3 ± 5.1	256.3 ± 7.2
4	**Amb**	45.0 ± 9.5	259.7 ± 8.1	60.7 ± 12.7	241.7 ± 13.6	61.0 ± 20.2	245.3 ± 17.4
8	**10°C**	47.3 ± 11.0	256.3 ± 7.5	65.7 ± 29.1	245.3 ± 20.6	62.7 ± 29.5	241.3 ± 30.6
8	**Amb**	25.7 ± 9.1	275.0 ± 8.9	58.7 ± 20.8	242.7 ± 21.5	28.7 ± 5.5	271.7 ± 5.5
24	**10°C**	70.3 ± 25.1	231.3 ± 27.0	50.3 ± 17.2	258.0 ± 21.6	29.7 ± 20.2	272.7 ± 20.6
24	**Amb**	58.3 ± 25.0	251.0 ± 18.2	57.3 ± 12.0	246.0 ± 12.1	44.0 ± 18.2	259.7 ± 20.3
30	**10°C**	55.7 ± 31.5	246.7 ± 30.5	54.7 ± 10.8	250.0 ± 10.8	30.0 ± 3.6	274.0 ± 4.6
30	**Amb**	16.0 ± 10.0	287.0 ± 12.5	21.7 ± 6.4	281.3 ± 6.4	25.3 ± 7.1	276.7 ± 6.5
36	**10°C**	29.0 ± 12.0	275.7 ± 14.5	26.0 ± 7.5	275.7 ± 8.5	7.7 ± 3.1	294.0 ± 4.6
36	**Amb**	15.0 ± 6.6	290.0 ± 6.6	14.3 ± 8.7	287.7 ± 10.1	19.0 ± 11.5	287.0 ± 12.5
50	**10°C**	50.7 ± 18.5	256.7 ± 20.6	63.3 ± 21.0	240.0 ± 21.9	19.3 ± 13.2	285.0 ± 18.0
50	**Amb**	22.7 ± 4.2	283.3 ± 4.6	20.0 ± 2.6	282.3 ± 4.7	19.0 ± 4.4	285.7 ± 5.9
64	**10°C**	41.7 ± 11.6	261.7 ± 10.1	45.3 ± 11.4	259.0 ± 9.8	14.0 ± 6.1	289.3 ± 5.5
64	**Amb**	28.0 ± 10.5	277.0 ± 15.4	27.0 ± 8.0	276.7 ± 7.4	30.3 ± 6.7	274.3 ± 7.2

Chapter 4 – Foraminiferal Diversity & Community Composition

Table 4.2 Species identified in total populations from across the time-series (full taxonomic list detailed in Appendix II). Species are listed according to the nature of the test and grouped into the three suborders to which they belong (* indicates no specimens of this species were identified as living).

Textulariina (agglutinated tests)	**Rotaliina (calcareous hyaline tests)**	**Miliolina (calcareous porcelaneous tests)**
Agglut, sp. A	*Ammonia falsobeccarii*	*Cyclogyra involvens**
Eggerella scabra	*Amphicoryna scalaris**	*Quinqueloculina bicornis*
Biserial agglutinate sp. A	*Bolivina difformis*	*Quinqueloculina* sp. A
Biserial agglutinate sp. B	*Bolivina pseudoplicata*	*Quinqueloculina* sp. B*
Miliammina fusca	*Bolivina pseudopunctata*	*Quinqueloculina* sp. cf. *Q. lata*
Miliammina sp. A	*Bolivina skagerrakensis*	*Quinqueloculina seminulum**
Reophax sp.	*Bulimina elongata*	
Textularia earlandi	*Bulimina gibba*	
Trochammina sp. A	*Bulimina marginata*	
Trochammina sp. aff. *T. ochracea*	*Bulimina* sp. A	
	Bulimina sp. B	
	*Cassidulina obtusa**	
	Cassidulina reniforme	
	Cassidulinoides bradyi	
	Cibicides lobatulus	
	Elphidium albiumbilicatum	
	Elphidium excavatum	
	Elphidium oceanensis	
	Elphidium williamsoni	
	Epistomonella exigua	
	*Fissurina elliptica**	
	Fissurina lucida	
	*Fissurina serrata**	
	Fissurina sp. cf. *F. lucida*	
	Fissurina sp. A	
	Haynesina germanica	
	Heterohelix striata (reworked)*	
	Hyaline sp. A	
	Hyaline sp. B	
	Hyaline sp. C	
	Hyaline sp. D	
	*Lagena gracillima**	
	*Lagena mollis**	
	*Lagena setigera**	
	Lagena sp. cf. *L. laevis**	
	Lagena sulcata var. *torquiformis**	
	Lamarckina haliotidea	
	Nonion sp. A*	
	Nonion sp. B*	
	Nonionella turgida	
	Nonionella sp. aff. *N. turgida**	
	*Oolina hexagona**	
	Rosalina anomala	
	Rosalina sp. A	
	Rosalina sp. B	
	Rosalina sp. C	
	Stainforthia fusiformis	

Table 4.3 Average percentages of total (live + dead) benthic foraminifera in each sample grouped based on the nature of their test (A = agglutinated tests, H = calcareous hyaline tests, P = porcelaneous tests) Values represent mean ± SD (n = 3). CO_2 concentrations are 380 ppm, 750 ppm or 1000 ppm. Temperature regimes are represented as 10°C (constant regime) or Amb (seasonally varying ambient regime).

Sample		380 ppm			750 ppm			1000 ppm		
Week	**Temp.**	**A (%)**	**H (%)**	**P (%)**	**A (%)**	**H (%)**	**P (%)**	**A (%)**	**H (%)**	**P (%)**
0	**10°C**	1.6 ± 0.4	98.3 ± 0.2	0.1 ± 0.2	2.1 ± 0.5	97.7 ± 0.5	0.2 ± 0.2	1.8 ± 1.4	98.1 ± 1.2	0.1 ± 0.2
4	**10°C**	2.2 ± 0.7	97.6 ± 0.5	0.2 ± 0.4	4.8 ± 1.2	94.8 ± 1.6	0.4 ± 0.5	2.5 ± 0.8	97.1 ± 0.8	0.3 ± 0.0
4	**Amb**	3.5 ± 0.2	96.2 ± 0.3	0.3 ± 0.3	2.5 ± 1.5	97.1 ± 1.5	0.3 ± 0.0	2.8 ± 0.8	97.0 ± 0.7	0.2 ± 0.2
8	**10°C**	4.2 ± 1.9	95.5 ± 1.8	0.3 ± 0.6	3.2 ± 1.6	96.8 ± 1.6	0.0	2.8 ± 1.0	97.1 ± 1.2	0.1 ± 0.2
8	**Amb**	2.3 ± 0.3	97.6 ± 0.2	0.1 ± 0.2	2.0 ± 0.6	97.8 ± 0.5	0.2 ± 0.2	2.3 ± 0.9	97.7 ± 0.9	0.0
24	**10°C**	2.2 ± 1.1	97.4 ± 1.1	0.4 ± 0.2	2.0 ± 1.0	97.9 ± 0.8	0.1 ± 0.2	6.3 ± 4.3	93.4 ± 4.4	0.3 ± 0.3
24	**Amb**	1.6 ± 1.4	98.0 ± 1.5	0.4 ± 0.2	2.9 ± 1.0	96.9 ± 1.3	0.2 ± 0.4	2.3 ± 0.4	96.9 ± 0.9	0.8 ± 0.5
30	**10°C**	2.5 ± 0.2	97.1 ± 0.7	0.3 ± 0.6	2.5 ± 0.5	97.4 ± 0.6	0.1 ± 0.2	5.0 ± 0.7	94.8 ± 0.8	0.1 ± 0.2
30	**Amb**	2.5 ± 0.2	97.5 ± 0.2	0.0	4.4 ± 0.7	95.2 ± 0.8	0.4 ± 0.2	1.9 ± 0.2	97.8 ± 0.5	0.3 ± 0.3
36	**10°C**	2.2 ± 0.5	97.7 ± 0.6	0.1 ± 0.2	1.7 ± 0.3	97.9 ± 0.5	0.4 ± 0.8	3.2 ± 1.4	96.7 ± 1.4	0.1 ± 0.2
36	**Amb**	4.6 ± 1.2	94.8 ± 0.9	0.7 ± 0.6	3.5 ± 1.0	96.2 ± 0.8	0.2 ± 0.2	6.6 ± 4.7	93.3 ± 4.9	0.1 ± 0.2
50	**10°C**	4.2 ± 2.4	92.3 ± 5.4	3.5 ± 6.0	2.9 ± 1.1	87.8 ± 8.6	9.4 ± 9.4	4.8 ± 1.2	94.5 ± 2.2	0.7 ± 1.2
50	**Amb**	3.3 ± 0.9	96.6 ± 1.1	0.1 ± 0.2	6.3 ± 0.4	93.5 ± 0.2	0.2 ± 0.4	5.7 ± 5.1	93.9 ± 5.1	0.4 ± 0.4
64	**10°C**	3.5 ± 2.0	90.3 ± 10.0	6.2 ± 8.2	2.3 ± 0.7	86.8 ± 18.6	10.9 ± 18.6	5.3 ± 1.4	93.8 ± 2.2	0.9 ± 1.0
64	**Amb**	5.6 ± 1.2	93.6 ± 1.4	0.8 ± 0.8	2.9 ± 0.7	97.0 ± 0.9	0.1 ± 0.2	5.9 ± 1.5	93.9 ± 1.7	0.2 ± 0.2

Figure 4.1

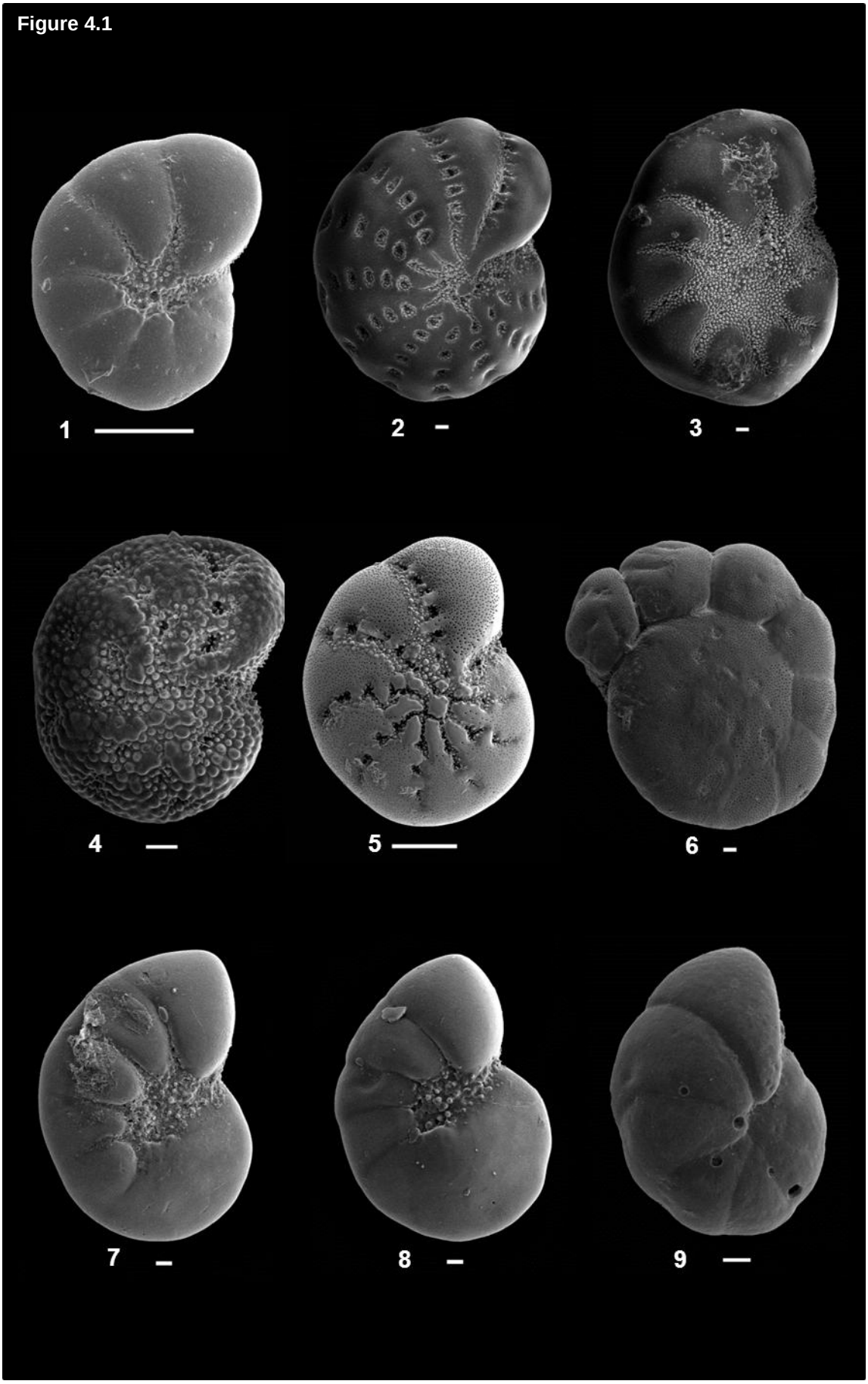

Figure 4.1 Scanning electron micrographs of foraminiferal species (scale bars = 10 µm unless otherwise noted)

1. *Haynesina germanica* (Ehrenberg) (scale bar = 100 µm)

2. *Elphidium williamsoni* (Haynes)

3. *Elphidium albiumbilicatum* (Weiss)

4. *Elphidium oceanensis* (d'Orbigny)

5. *Elphidium excavatum* (Terquem) (scale bar = 100 µm)

6. *Ammonia falsobeccarii* (Rouvillois)

7. *Nonion* sp. A

8. *Nonion* sp. B

9. *Cibicides lobatulus* (Walker and Jacob)

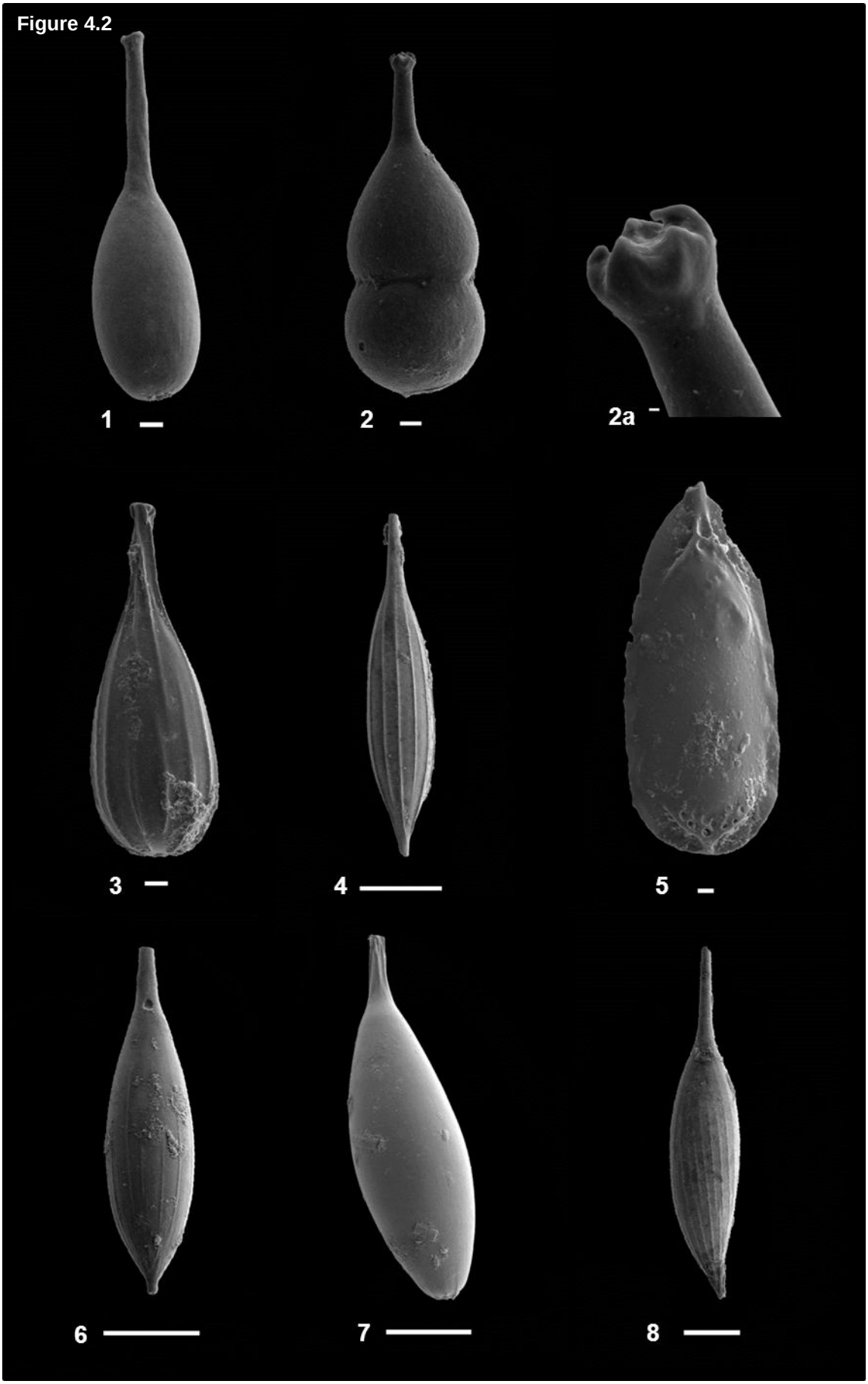
Figure 4.2
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Figure 4.2 Scanning electron micrographs of foraminiferal species (scale bars = 10 µm unless otherwise noted)

1. *Lagena* sp. cf. *L. laevis* (Montagu)

2. *Amphicoryna scalaris* (Batsch)

2a. Detail of *Amphicoryna scalaris* (scale bar = 1 µm)

3. *Lagena sulcata* var. *torquiformis* (Haynes)

4. *Lagena mollis* (scale bar = 100 µm)

5. *Fissurina serrata* (Schlumberger)

6. *Lagena gracillima* (Seguenza) (scale bar = 100 µm)

7. *Lagena setigera* (Millet) (scale bar = 100 µm)

8. *Lagena mollis* (Cushman) (scale bar = 100 µm)

Figure 4.3

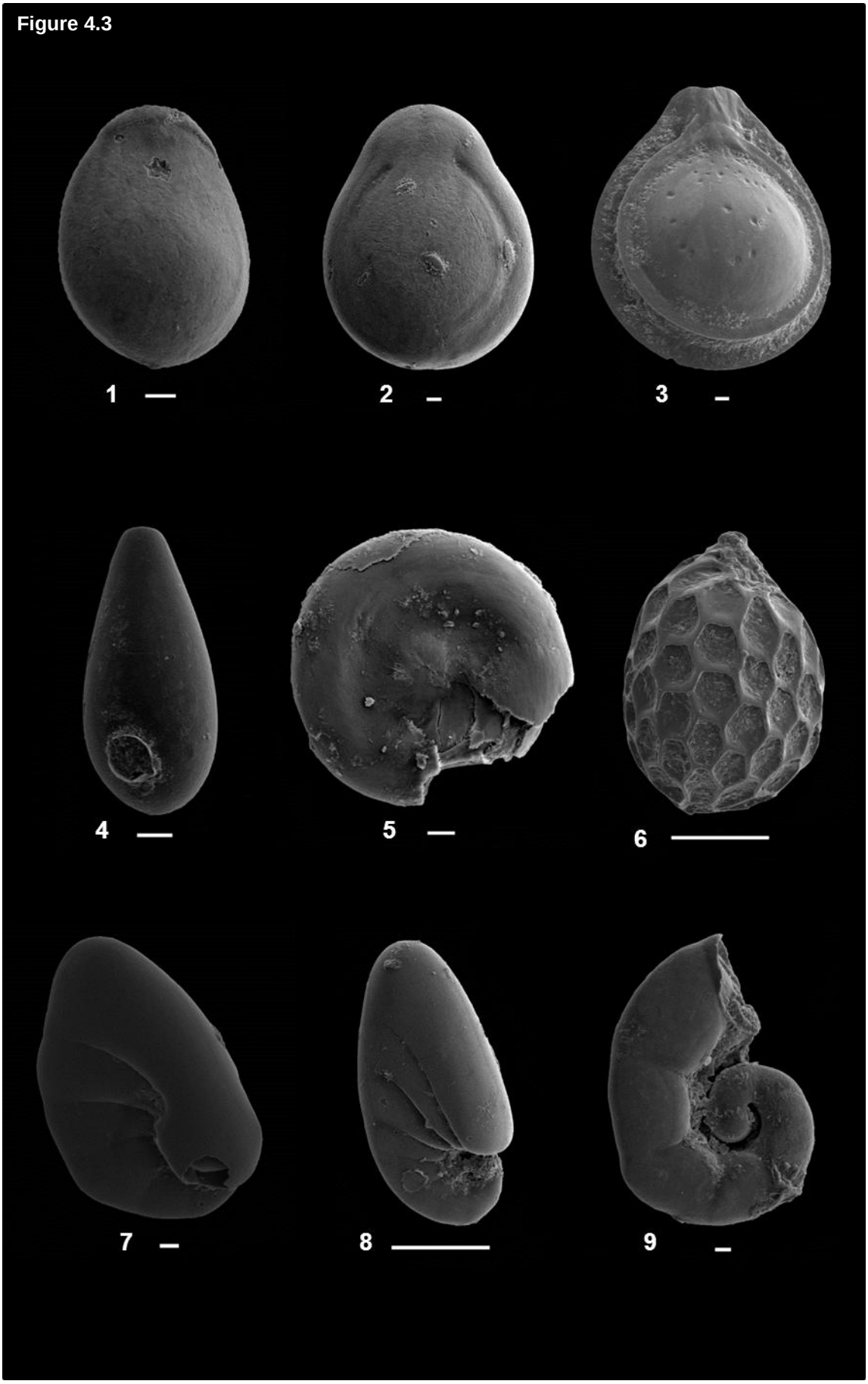

Figure 4.3 Scanning electron micrographs of foraminiferal species (scale bars = 10 µm unless otherwise noted)

1. *Fissurina lucida* (Williamson)

2. *Fissurina* sp. A

3. *Fissurina elliptica* (Cushman)

4. *Fissurina* sp. cf. *F. lucida*

5. *Cyclogyra involvens* (Reuss)

6. *Oolina hexagona* (scale bar = 100 µm)

7. *Nonionella turgida* (Williamson)

8. *Nonionella* sp. aff. *N. turgida* (scale bar = 100 µm)

9. *Lamarckina haliotidea* (Heron-Allen and Earland)

Figure 4.4

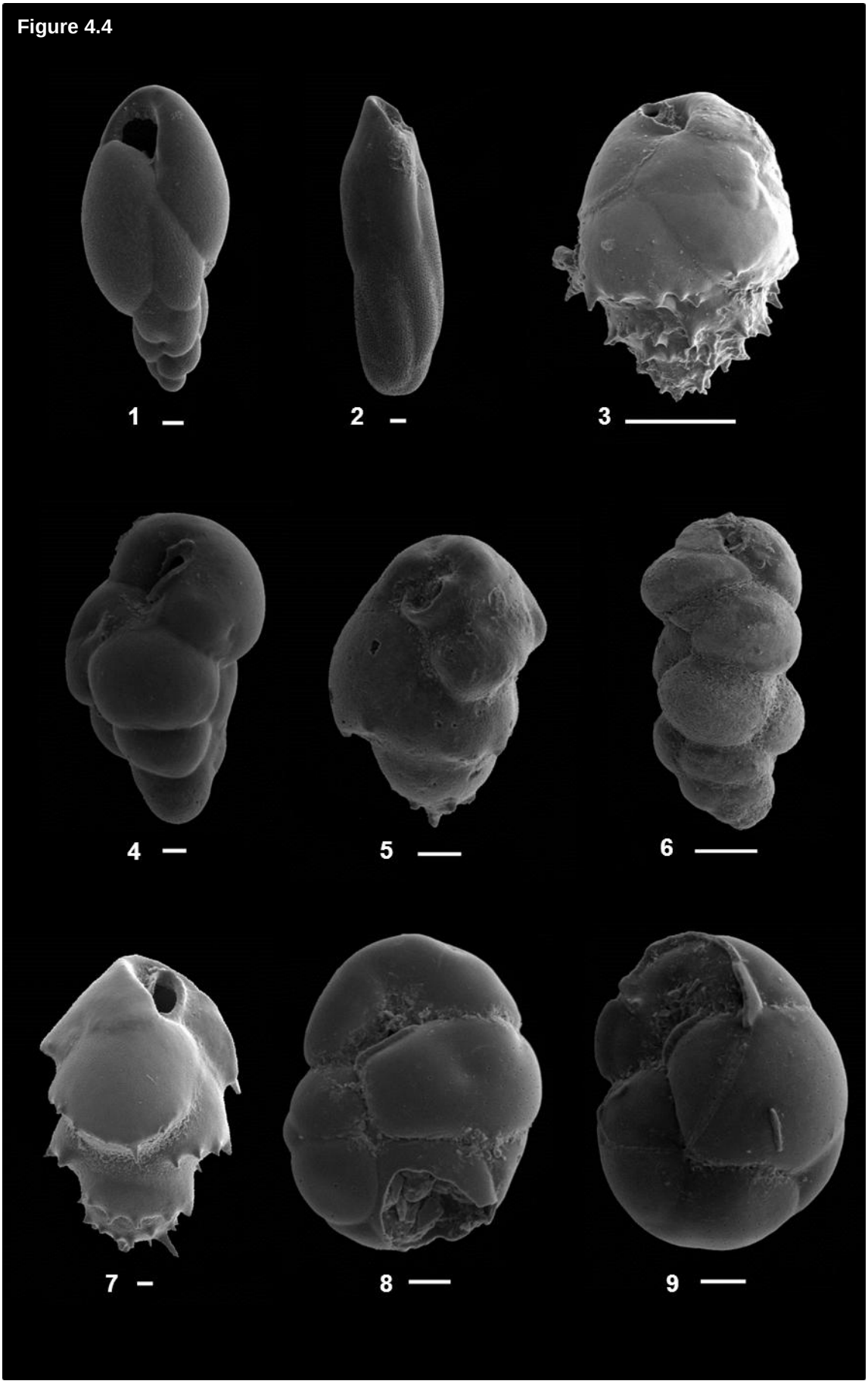

Figure 4.4 Scanning electron micrographs of foraminiferal species (scale bars = 10 µm unless otherwise noted)

1. *Stainforthia fusiformis* (Williamson)

2. *Cassidulinoides bradyi* (Norman)

3. *Bulimina marginata* (d'Orbigny) (scale bar = 100 µm)

4. *Bulimina elongata* (d'Orbigny)

5. *Bulimina gibba* (Fornasini)

6. *Bulimina* sp. A (scale bar = 100 µm)

7. *Bulimina marginata* (d'Orbigny)

8. *Cassidulina obtusa* (Williamson)

9. *Cassidulina reniforme* (Nørvangi)

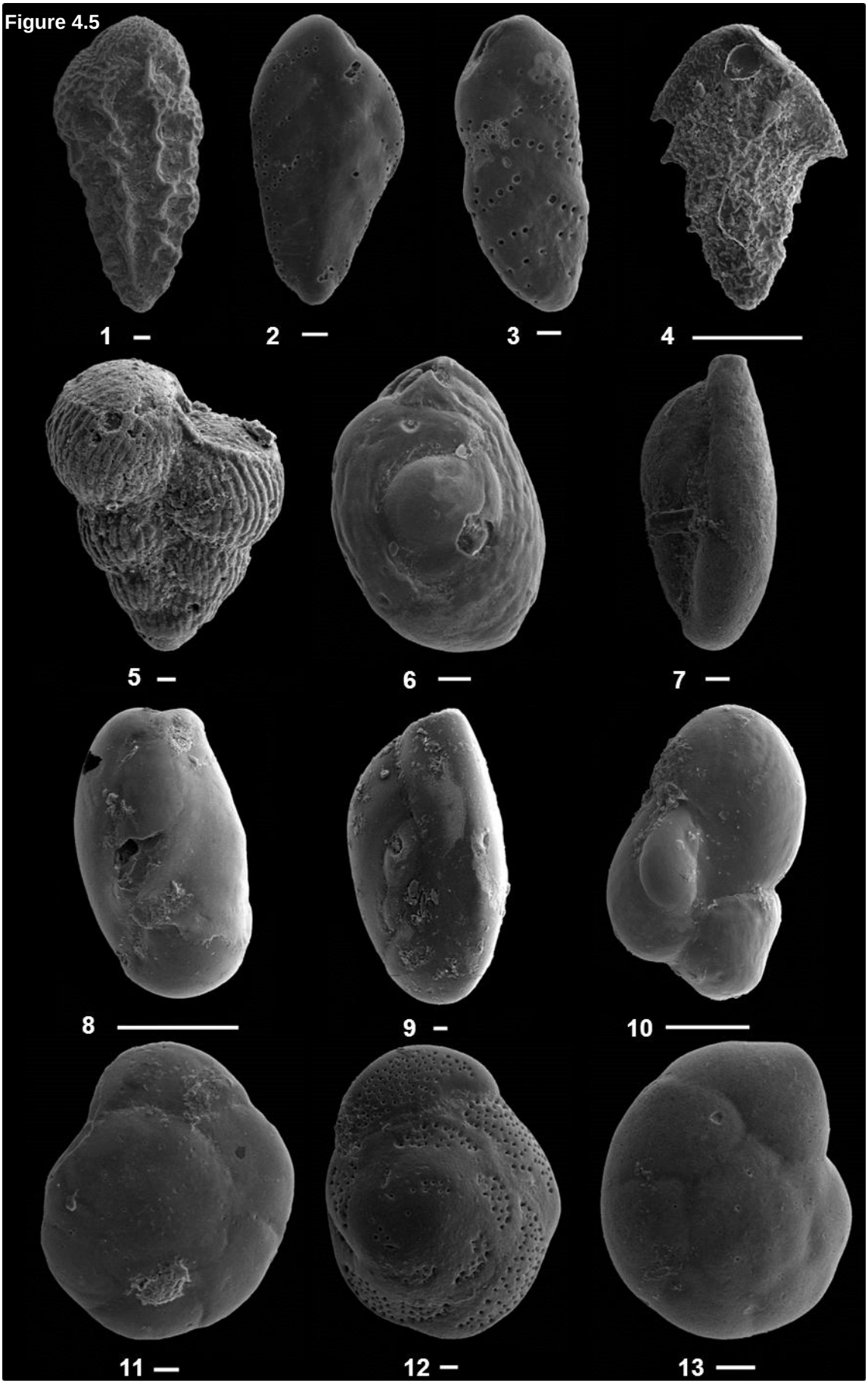
Figure 4.5
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Figure 4.5 Scanning electron micrographs of foraminiferal species (scale bars = 10 µm unless otherwise noted)

1. *Bolivina pseudoplicata* (Heron-Allen and Earland)

2. *Bolivina skagerrakensis* (Qvale and Nigam)

3. *Bolivina pseudopunctata* (Höglund)

4. *Bolivina difformis* (Williamson) (scale bar = 100 µm)

5. *Heterohelix striata* (Ehrenberg)

6. *Quinqueloculina bicornis* (Walker and Jacob)

7. *Quinqueloculina* sp. cf. *Q. lata* (Terquem)

8. *Quinqueloculina* sp. A (scale bar = 100 µm)

9. *Quinqueloculina seminulum* (Linnaeus)

10. *Quinqueloculina* sp. B (scale bar = 100 µm)

11. *Rosalina* sp. A

12. *Rosalina anomala* (Terquem)

13. *Epistominella exigua* (Brady)

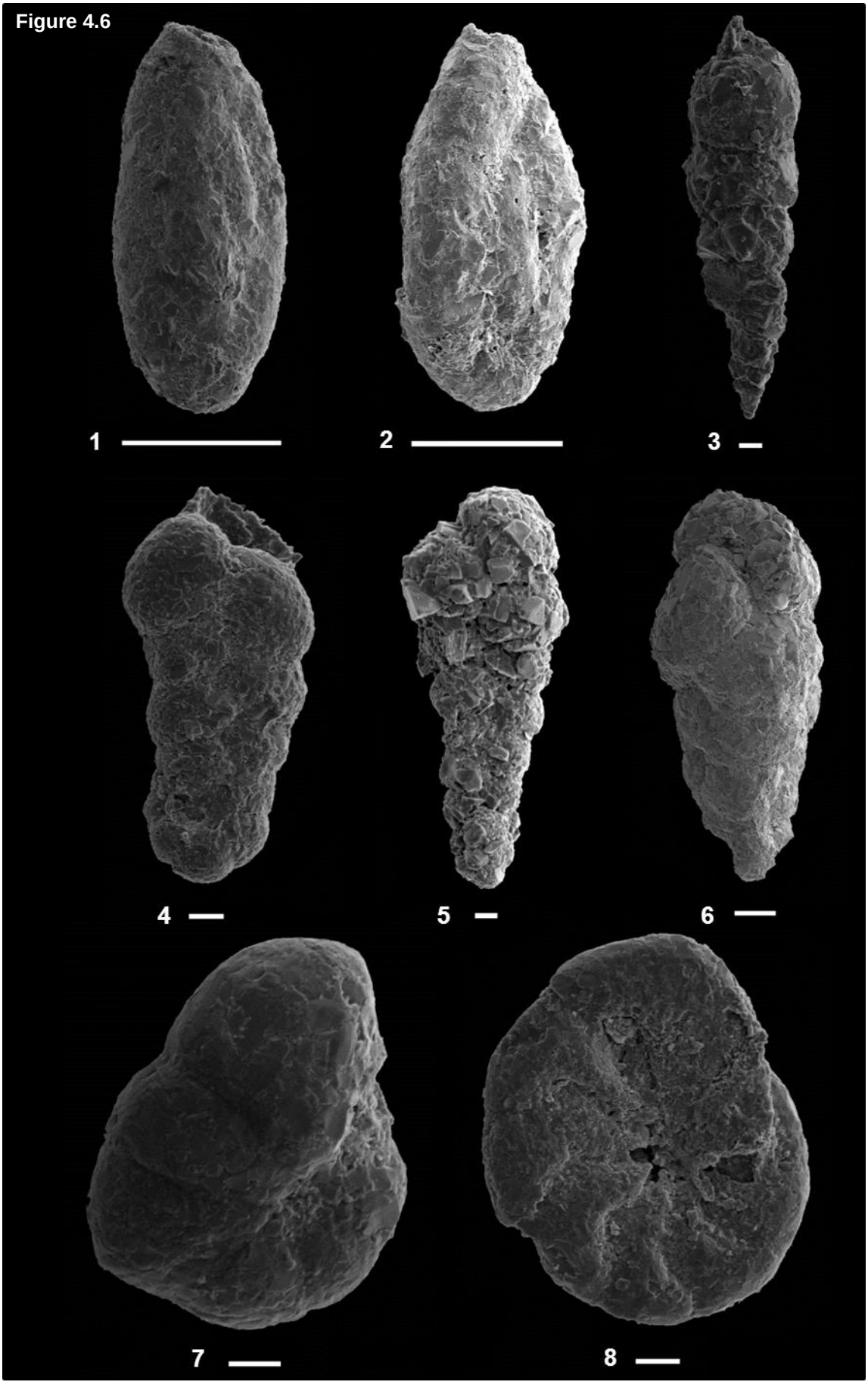
Figure 4.6
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Figure 4.6 Scanning electron micrographs of foraminiferal species (scale bars = 10 μm unless otherwise noted)

1. *Miliammina fusca* (Brady) (scale bar = 100 μm)

2. *Miliammina* sp. A (scale bar = 100 μm)

3. *Reophax* sp.

4. *Textularia earlandi* (Parker)

5. *Eggerella scabra* (Williamson)

6. Agglut. sp. A (scale bar = 100 μm)

7. *Trochammina* sp. A

8. *Trochammina* cf. aff. *T. ochracea* (Williamson)

4.3.2 Live assemblage data

4.3.2.1 Wall structure

44 representatives of the 63 total species present in the time-series were identified as living. These species were grouped based on the nature of their test (agglutinated, porcelaneous or hyaline) and the percentage data for each assemblage plotted on ternary diagrams (Fig. 4.7 - 4.9). Many of the assemblages fall along the agglutinated-hyaline side of the ternary diagrams (Fig. 4.7 & 4.8), which was to be expected given the environmental area sampled (Murray, 1973). By weeks 50 and 64, these assemblages still largely contain mostly agglutinated-hyaline species but also have varying amounts of porcelaneous walls (Fig. 4.9). Both *Quinqueloculina bicornis* and *Quinqueloculina* sp. A increased in numbers during weeks 50 and 64.

The optimal model with number of agglutinated specimens as the dependant variable consisted of a three-way interaction (CO_2 x Temperature Regime x Week: L-ratio = 25.295, d.f. = 12, $p < 0.015$). There was a larger CO_2 effect over time under ambient temperature conditions in comparison with the constant temperature regime (Fig. 4.10). Under the constant temperature regime (Fig. 4.10A), the highest numbers of agglutinated individuals were observed in the higher CO_2 treatments (750 ppm and 1000 ppm). The one exception to this is at week 36. Under ambient temperature conditions (Fig. 4.10B) there was an increased CO_2 effect with length of exposure. No CO_2 effect was observed under ambient temperature conditions until week 24. From this point in the time-series the highest numbers of agglutinated individuals were found in the highest CO_2 treatments (750 ppm and 1000 ppm). The highest numbers of agglutinated individuals were observed after 36 weeks of

exposure under ambient temperature conditions and the highest CO_2 treatment (1000 ppm). Overall, the most distinctive CO_2 effect was observed after 64 weeks.

4.3.2.2 Diversity measures

Throughout the experimental exposure period the number of live taxa varied (Fig. 4.11). The highest number of taxa was recorded after 64 weeks of exposure under the lowest CO_2 concentration (380 ppm) and under constant temperature conditions (Fig 4.11: A1). Indeed, in the majority of samples the highest number of taxa were observed under the constant temperature regime (Fig. 4.11: A1) when compared to ambient (Fig. 4.11: B1). Under the constant temperature regime, the number of live taxa varied for the first 8 weeks of exposure. After 24 weeks of exposure under the constant temperature regime the highest number of taxa were consistently observed at the lowest CO_2 concentration (380 ppm) (Fig. 4.11: A1). Under ambient temperature conditions the number of taxa varied with CO_2 concentration throughout the time-series (Fig. 4.11: B1). In all CO_2 treatments, under ambient temperature, the number of taxa decreased during weeks 24 and 30 before increasing from week 36.

To assess diversity and equitability, the Shannon diversity index (H') and Pielou's evenness index were calculated for each sample. During the first 30 weeks of exposure, diversity was highest under the constant temperature regime (Fig. 4.11: A2) when compared to ambient (Fig. 4.11: B2). After 36 weeks of exposure under the constant temperature regime the highest diversity was observed in the lowest CO_2 treatment (380 ppm) with the lowest diversity in the highest CO_2 treatment (1000 ppm). During weeks 50 and 64, diversity increases in the highest CO_2 treatment (1000 ppm) while remaining low under medium CO_2 conditions (750 ppm). Under ambient temperature conditions (Fig. 4.11: B2), diversity remains low for the first 30 weeks of exposure. An increase in diversity was observed in all CO_2

treatments from week 36, with the highest diversity and variability in the medium CO_2 treatment (750 ppm).

Under constant temperature conditions evenness was generally lower in the medium CO_2 treatment (750 ppm) (Fig. 4.11: A3). For the majority of time points, evenness was highest in the highest CO_2 treatment (1000 ppm). Two exceptions to this are at weeks 4 and 36 where the highest evenness was at the lowest CO_2 treatment (380 ppm). Under the highest CO_2 treatment, evenness increases with length of exposure from week 30 for the duration of the experiment. Under ambient temperature conditions, evenness decreases in all CO_2 treatments between weeks 8 and 24 (Fig. 4.11: B3). Evenness increases in all CO_2 treatments between weeks 36 and 64 and remains similar for the duration of the experiment.

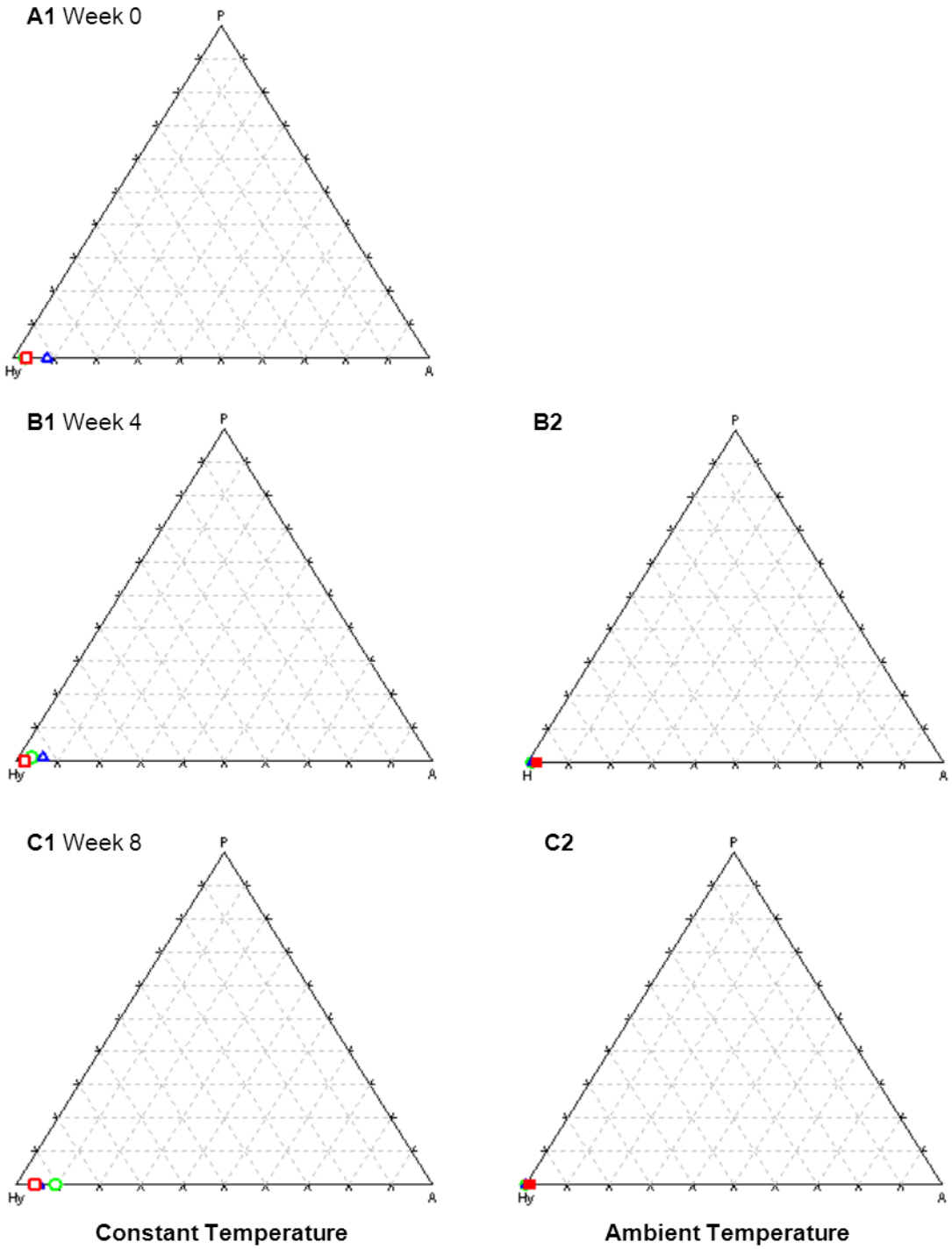

Figure 4.7 Ternary plots of wall-structure groups for live benthic foraminiferal assemblages (triangle corners represent 100% of the labelled component: A = agglutinated, P = porcelaneous, Hy = hyaline). Weeks 0 (A1 & A2), 4 (B1 & B2) & 8 (C1 & C2) are displayed for both constant (open symbols) and ambient (closed symbols) temperature regimes. CO_2 treatments are 380 ppm (green), 750 ppm (blue) and 1000 ppm (red).

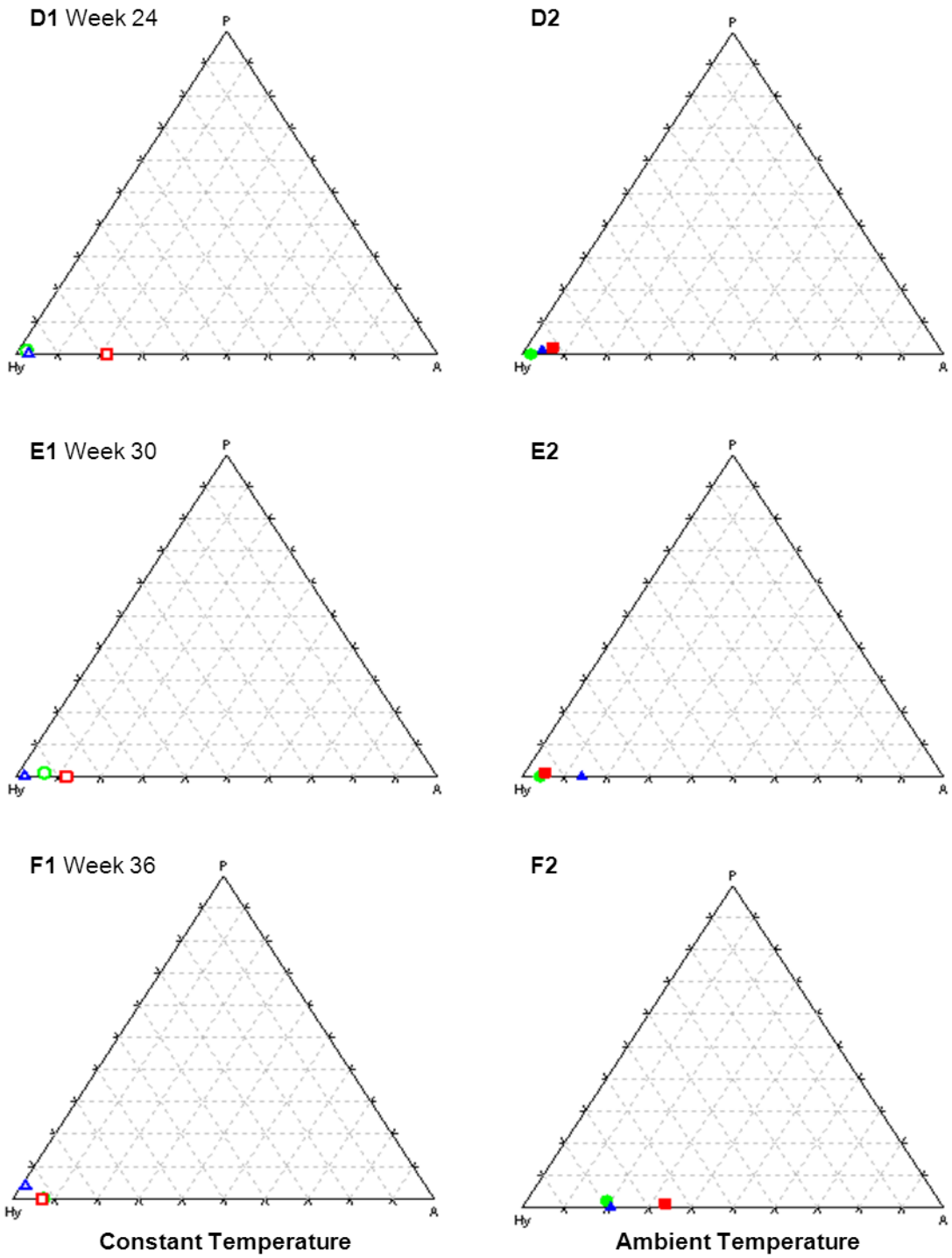

Figure 4.8 Ternary plots of wall-structure groups for live benthic foraminiferal assemblages (triangle corners represent 100 % of the labelled component: A = agglutinated, P = porcelaneous, Hy = hyaline). Weeks 24 (D1 & D2), 30 (E1 & E2) & 36 (F1 & F2) are displayed for both constant (open symbols) and ambient (closed symbols) temperature regimes. CO_2 treatments are 380 ppm (green), 750 ppm (blue) and 1000 ppm (red).

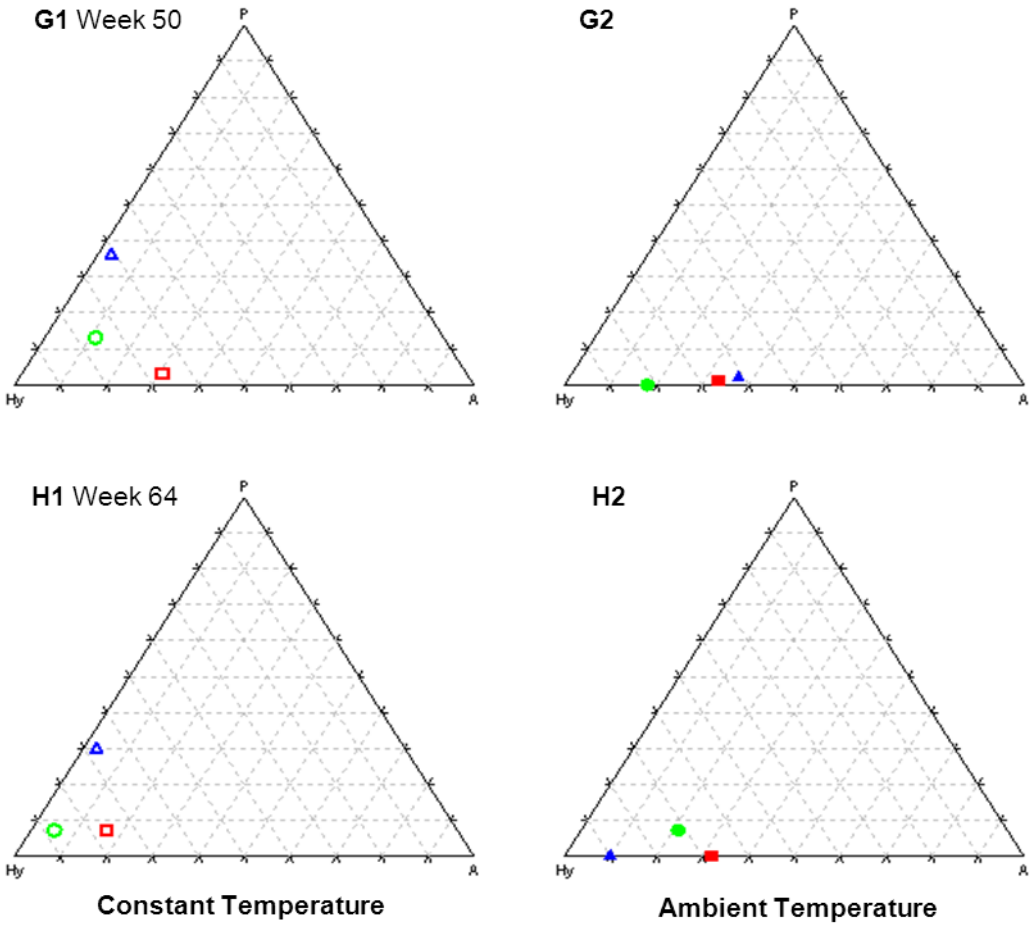

Figure 4.9 Ternary plots of wall-structure groups for live benthic foraminiferal assemblages (triangle corners represent 100 % of the labelled component: A = agglutinated, P = porcelaneous, Hy = hyaline). Weeks 50 (G1 & G2) and 64 (H1 & H2) are displayed for both constant (open symbols) and ambient (closed symbols) temperature regimes. CO_2 treatments are 380 ppm (green), 750 ppm (blue) and 1000 ppm (red).

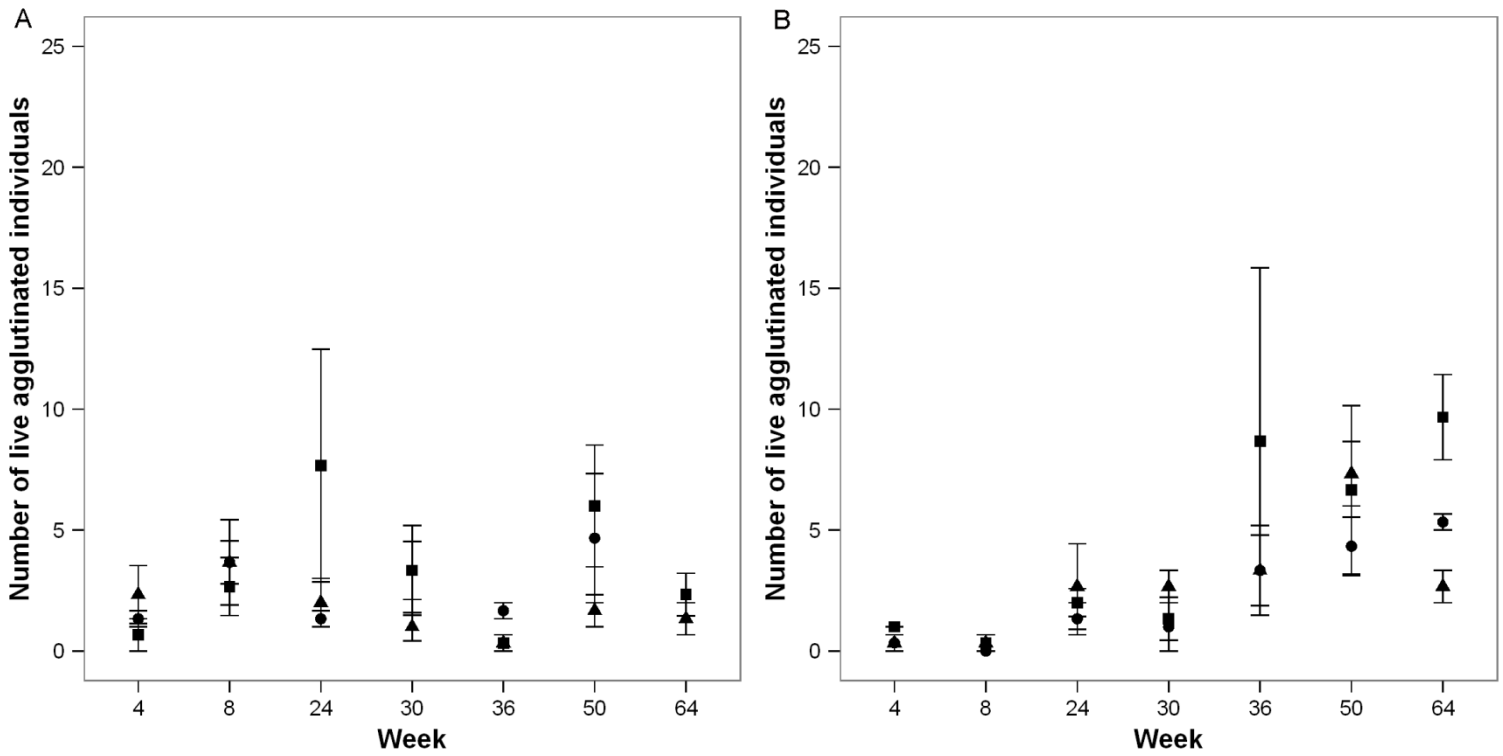

Figure 4.10 Model visualisation of three-way interaction (CO_2 x Temperature Regime x Week) on the number of live agglutinated individuals (y-axis) across each CO_2 treatment at each time point (week 4, 8, 24, 30, 36, 50, 64). **A)** Constant temperature regime, **B)** Ambient temperature regime. The visualisation shows time point along the x-axis. The different CO_2 treatments are represented by circles (380 ppm), triangles (750 ppm) and squares (1000 ppm).

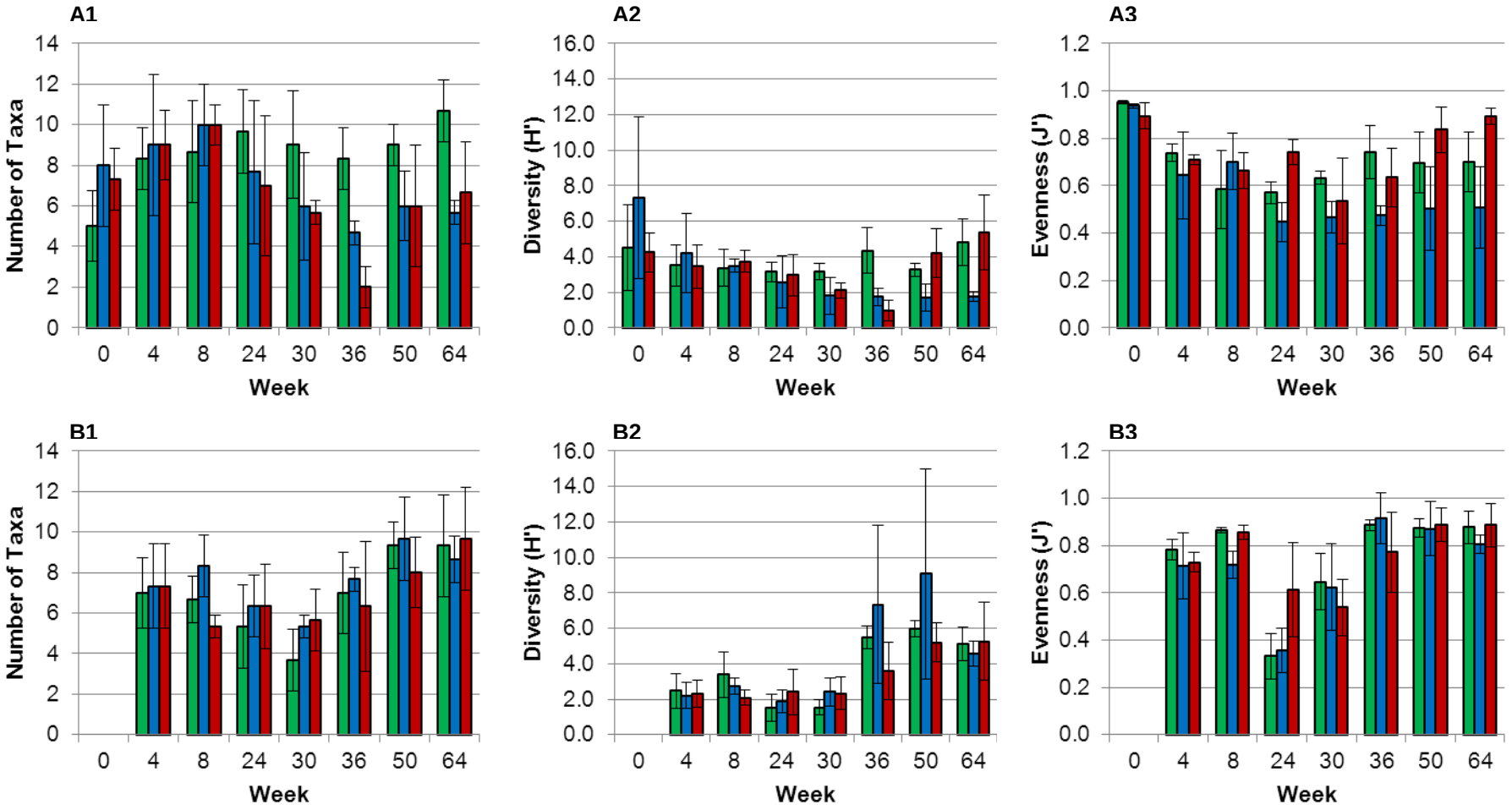

Figure 4.11 Foraminiferal assemblage data. 1) Total number of taxa, 2) Diversity (H') and 3) Evenness (J') based on foraminiferal species count data throughout the time-series (Weeks 0, 4, 8, 24, 30, 36, 50 & 64) for A) constant temperature and B) ambient temperature regimes. CO_2 treatments are represented by green (380 ppm), blue (750 ppm) or red (1000 ppm).

To compare assemblages, both species richness and abundances of live individuals were analysed through mixed effects modelling. The optimal model with species richness as the dependant variable consisted of two two-way interactions (Temperature Regime x Week: d.f. = 6, F = 5.12, $p < 0.00015$, CO_2Treatment x Week: d.f. = 2, F = 6.17 $p < 0.003$).

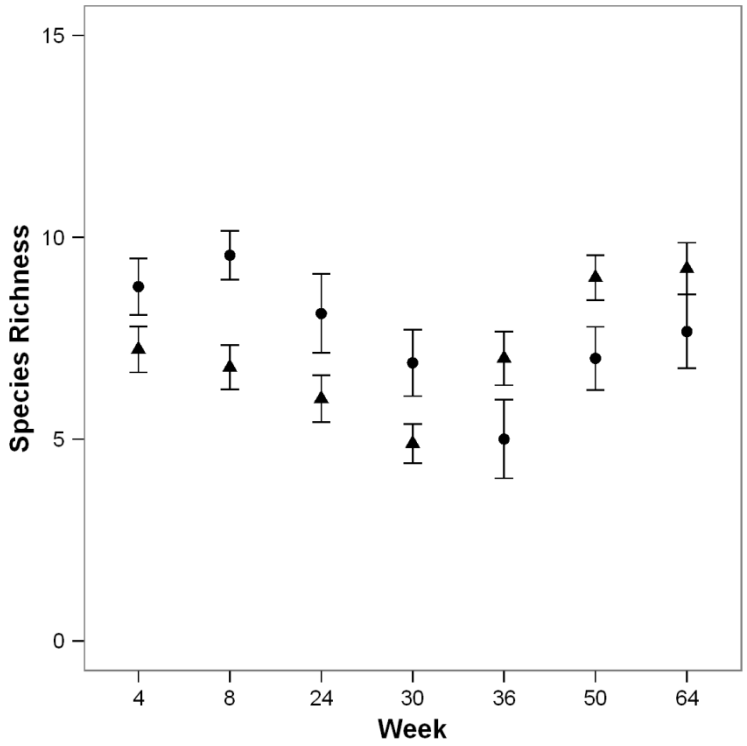

Figure 4.12 Model visualisation of the two-way interaction Temperature x Week on species richness (y-axis) across the two temperature regimes for each time point (week 4, 8, 24, 30, 36, 50, 64). The visualisation shows the time point (week) along the x-axis. The temperature regimes are represented by circles (constant temperature) and triangles (ambient temperature).

A temperature effect on species richness (number of taxa) was observed throughout the entire time-series (Fig. 4.12). Species richness was highest under constant temperature conditions for the first 30 weeks of exposure. From week 36, the highest

species richness was observed under ambient temperature conditions in relation to constant. Seasonal fluctuations in species richness were documented under both the constant and ambient temperature regimes.

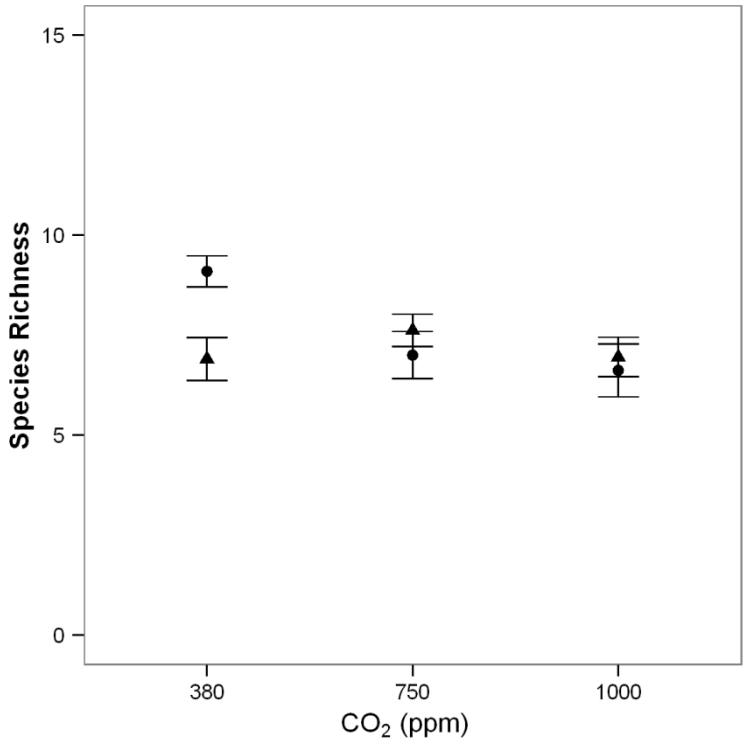

Figure 4.13 Model visualisation of the two-way interaction CO_2 x Temperature on species richness (y-axis) across the two temperature regimes for each CO_2 concentration (380, 750 & 1000 ppm). The visualisation shows the CO_2 concentration along the x-axis and at each concentration the different temperatures are represented by circles (constant temperature) and triangles (ambient temperature).

The optimal model with total number of live individuals as the dependant variable consisted of three two-way interactions (CO_2Treatment x Temperature Regime: L-ratio = 23.402, d.f. = 2, $p < 0.0001$, Temperature Regime x Week: L-ratio = 25.732, d.f. = 6, $p < 0.0002$, CO_2 Treatment x Week: L-ratio = 23.277, d.f. = 12, $p < 0.03$).

At the lowest CO_2 treatment (380 ppm), there was a clear temperature effect (Fig. 4.14). At 380 ppm and 750 ppm, the highest abundances of living individuals occur under the constant temperature regime. At the highest CO_2 concentration (1000 ppm), the temperature effect seen at the lowest CO_2 levels breaks down. At higher CO_2 levels the highest abundances of living individuals occur under the ambient temperature regime.

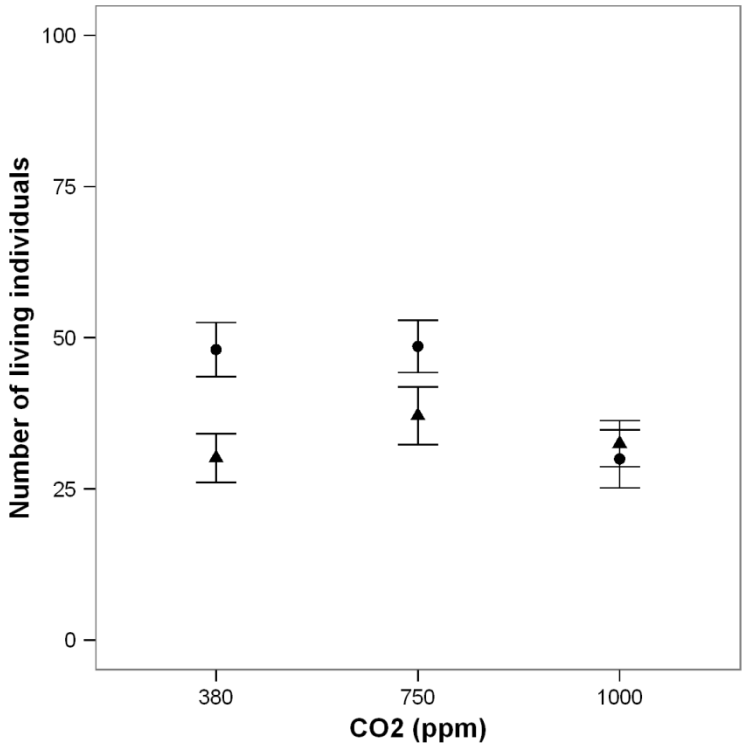

Figure 4.14 Model visualisation of the two-way interaction CO_2 x Temperature on the total number of living individuals (y-axis) across the two temperature regimes for each CO_2 concentration (380, 750 & 1000 ppm). The visualisation shows the CO_2 concentration along the x-axis and at each concentration the different temperatures are represented by circles (constant temperature) and triangles (ambient temperature).

A temperature effect was also observed with length of exposure (Fig, 4.15). This temperature effect breaks down during weeks 24, 36 and 64. The highest abundances of living individuals varied between the constant and ambient temperature regimes for the first 24 weeks of exposure. Following 24 weeks, the highest abundances of living individuals were observed under the constant temperature regime.

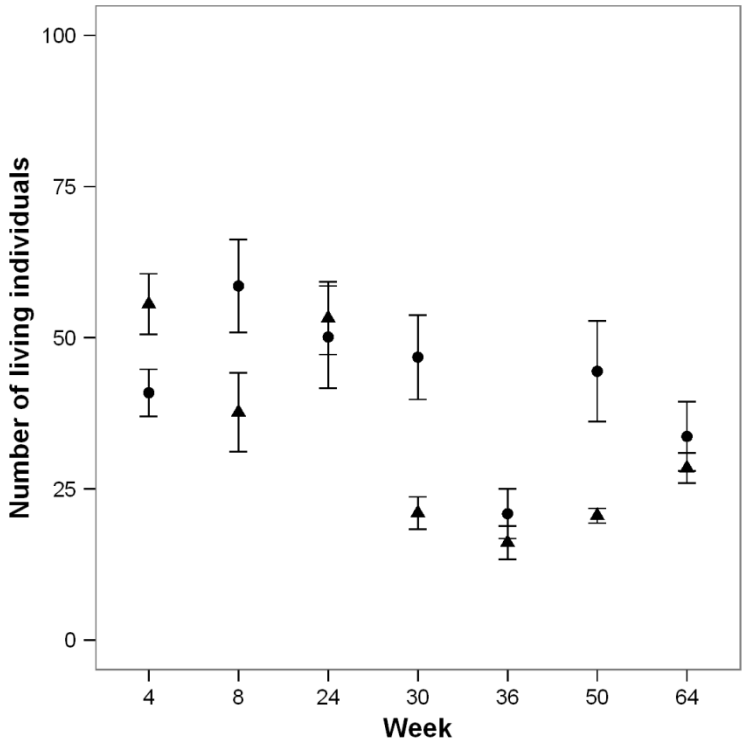

Figure 4.15 Model visualisation of the two-way interaction Temperature x Week on the total number of living individuals (y-axis) across the two temperature regimes for each time point (week 4, 8, 24, 30, 36, 50, 64). The visualisation shows the time point (week) along the x-axis. The temperature regimes are represented by circles (constant temperature) and triangles (ambient temperature).

A significant effect of CO_2 treatment was also observed with length of exposure (Fig. 4.16). The CO_2 effect is larger after 24, 50 and 64 weeks of exposure. The highest abundance of living individuals was observed after 24 weeks under the lowest CO_2

treatment (380 ppm). A reduction in live abundances was documented in all CO_2 treatments between weeks 24 and 36. After 24 weeks of exposure the lowest abundances of living individuals were consistently observed in the highest CO_2 treatment (1000 ppm).

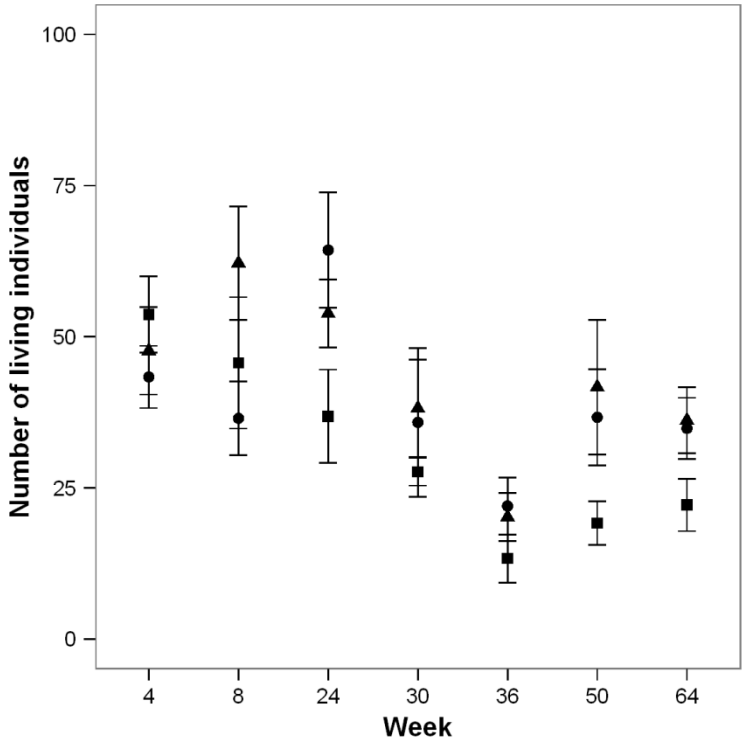

Figure 4.16 Model visualisation of the two-way interaction CO_2 x Week on the total number of living individuals (y-axis) across the three CO_2 treatments for each time point (week 4, 8, 24, 30, 36, 50, 64). The visualisation shows the time point (week) along the x-axis. The different CO_2 treatments are represented by circles (380 ppm), triangles (750 ppm) and squares (1000 ppm).

The above results on abundances of living individuals incorporate all live specimens from all identified species. The community compositions within these samples vary across the time-series. The community compositions and individual species responses of the top-ranked species are analysed below.

4.3.2.3 Community compositions

Cluster analysis was performed on 135 samples and 43 taxa matrix after elimination of rare taxa which never constitute > 3% of any assemblage (e.g. *Eggerella scabra*). The resulting dendrogram (Fig. 4.17) indicated that the foraminiferal assemblages could be significantly differentiated by the SIMPROF test. Black lines indicate structuring between samples (samples connected by red dashed lines cannot be significantly differentiated). Samples within a group are more similar to each other, generally, than samples in different groups (Clarke & Warwick, 2001).

At 36.5% similarity there is an initial division into two groups ($\pi = 1.0$, $p < 3.5\%$). This was followed by two further divisions, one in the right-hand group at 41.2% similarity ($\pi = 1.6$, $p < 2.5\%$) and one in the left hand group at 41.5% similarity ($\pi = 1.8$, $p < 3\%$). At 41.2% similarity the majority (~80%) of the right hand group consisted of samples from the constant temperature regime.

The initial left hand group split into two further groups at 47 % similarity ($\pi = 1.6$, $p < 4\%$). All samples from time point zero (week 1) group together at this percentage threshold. The other group from this split comprised mainly (> 80%) of samples from the ambient temperature regime.

The initial right hand group had two further successive divisions. The first was at 59.6% similarity ($\pi = 3.4\%$, $p < 1\%$) and the second at 77.2% similarity ($\pi = 2.9$, $p < 2\%$). All samples grouped at 59.6% similarity were from the ambient temperature regime and from either week 2 or 3.

The data matrix was plotted on a non-metric multi-dimensional scaling (MDS) ordination plot and labelled by CO_2 treatment, temperature regime and length of exposure (Fig. 4.18). A low stress value 0.14 gives confidence that the two

dimensional plots are an accurate representation of the sample relationships (Clarke & Warwick, 2001). Points that are close together represent samples that are very similar in community composition (Clarke & Warwick, 2001). The percentage thresholds identified from the SIMPROF analyses were overlaid on each MDS plot to cluster samples at 41, 47, 60 and 77% similarity.

4.3.2.4 Species-specific variability

The abundances of live benthic foraminiferal species varied with exposure to the CO_2 treatments and temperature regimes throughout the time-series (Table 4.4 – 4.11). Quantitatively, four species *Haynesina germanica* (47.6%), *Elphidium williamsoni* (11%), *Elphidium albiumbilicatum* (9%) and *Stainforthia fusiformis* (7.5%) made up the majority (> 75%) of the living foraminifera identified.

Table 4.4 Percentages of live benthic foraminiferal species in each treatment at Week 0 (values represent mean ± SD, n = 3).“–“ indicates no live individuals of this species were present in any of the samples. CO_2 concentrations were 380 ppm, 750 ppm and 1000 ppm. All samples at Week 0 were sampled at 10°C.

Species	380 ppm	750 ppm	1000 ppm
Agglut. sp. A	-	-	-
Ammonia falsobeccarii	-	-	-
Biserial agglutinate sp. A	-	-	-
Biserial agglutinate sp. B	-	-	-
Bolivina difformis	-	-	-
Bolivina pseudoplicata	1.0 ± 1.0	0.7 ± 0.6	1.0 ± 1.7
Bolivina pseudopunctata	-	0.7 ± 0.6	-
Bolivina skagerrakensis	-	0.3 ± 0.6	-
Bulimina elongata	-	-	-
Bulimina gibba	-	-	-
Bulimina marginata	-	-	-
Bulimina sp. A	-	-	-
Bulimina sp. B	-	-	-
Cassidulina sp.	-	0.3 ± 0.6	0.7 ± 1.2
Cassidulinoides bradyi	-	-	0.3 ± 0.6
Cibicides lobatulus	-	0.7 ± 1.2	0.7 ± 0.6
Eggerella scabra	-	-	-
Elphidium albiumbilicatum	1.3 ± 0.6	2.3 ± 1.2	6.3 ± 1.2
Elphidium excavatum	-	-	-
Elphidium oceanensis	-	-	-
Elphidium williamsoni	1.3 ± 1.2	2.0 ± 0.0	1.7 ± 2.1
Epistomonella exigua	-	-	-
Fissurina lucida	0.3 ± 0.6	-	0.3 ± 0.6
Fissurina sp. cf. *F. lucida*	-	-	-
Fissurina sp. A	-	-	-
Haynesina germanica	2.3 ± 0.6	3.7 ± 0.6	3.7 ± 0.6
Hyaline sp. A	-	-	-
Hyaline sp. B	-	-	-
Hyaline sp. C	-	-	0.3 ± 0.6
Indeterminate juvenille	-	0.3 ± 0.6	1.3 ± 1.5
Miliammina fusca	-	-	-
Miliammina sp. A	-	-	-
Nonionella turgida	-	-	-
Quinqueloculina bicornis	-	-	-
Quinqueloculina sp. cf. *Q. lata*	-	-	-
Quinqueloculina sp. A	-	-	-
Reophax sp.	0.3 ± 0.6	-	-
Rosalina anomala	-	-	-
Rosalina sp. A	-	0.3 ± 0.6	1.7 ± 0.6
Rosalina sp. B	-	-	-
Rosalina sp. C	-	0.3 ± 0.6	-
Stainforthia fusiformis	3.0 ± 1.0	2.7 ± 1.5	2.7 ± 1.5
Trochammina sp. A	-	1.0 ± 1.0	0.7 ± 1.2
Trochammina sp. aff. *T. ochracea*	-	0.3 ± 0.6	-

Table 4.5 Percentages of live benthic foraminiferal species in each treatment at Week 4 (values represent mean ± SD, n = 3). "–" indicates no live individuals of this species were present in any of the samples. CO_2 concentrations were 380 ppm, 750 ppm and 1000 ppm. Temperature Regimes are represented as 10°C (constant regime) or Amb (seasonally varying ambient regime).

Species	380 ppm		750 ppm		1000 ppm	
	10°C	Amb	10°C	Amb	10°C	Amb
Agglut. sp. A	-	-	-	-	-	-
Ammonia falsobeccarii	-	-	-	-	-	-
Biserial agglutinate sp. A	0.3 ± 0.6	-	-	-	-	-
Biserial agglutinate sp. B	0.3 ± 0.6	-	1.7 ± 1.5	-	-	0.3 ± 0.6
Bolivina difformis	-	-	-	-	-	-
Bolivina pseudoplicata	0.7 ± 1.2	1.0 ± 1.0	0.7 ± 0.6	0.3 ± 0.6	0.7 ± 0.6	-
Bolivina pseudopunctata	0.3 ± 0.6	-	0.7 ± 0.6	0.3 ± 0.6	0.7 ± 0.6	-
Bolivina skagerrakensis	-	-	-	-	0.3 ± 0.6	-
Bulimina elongata	-	-	-	-	-	-
Bulimina gibba	-	-	-	-	-	-
Bulimina marginata	-	-	-	-	-	-
Bulimina sp. A	-	-	-	-	-	-
Bulimina sp. B	-	-	-	-	-	-
Cassidulina sp.	-	-	-	0.3 ± 0.6	-	-
Cassidulinoides bradyi	0.3 ± 0.6	-	0.3 ± 0.6	-	-	-
Cibicides lobatulus	0.7 ± 0.6	0.3 ± 0.6	0.3 ± 0.6	-	1.0 ± 1.0	0.3 ± 0.6
Eggerella scabra	-	-	-	-	-	-
Elphidium albiumbilicatum	4.7 ± 3.2	10.0 ± 2.0	4.3 ± 5.8	28.7 ± 10.4	11.0 ± 3.5	17.7 ± 6.7
Elphidium excavatum	-	-	-	-	-	0.3 ± 0.6
Elphidium oceanensis	0.3 ± 0.6	1.7 ± 2.1	1.0 ± 1.7	1.7 ± 1.2	1.0 ± 1.7	0.7 ± 0.6
Elphidium williamsoni	7.7 ± 5.7	16.7 ± 7.6	1.0 ± 1.7	14.3 ± 1.5	9.3 ± 5.5	15.0 ± 6.6
Epistomonella exigua	-	-	-	-	-	-
Fissurina lucida	0.3 ± 0.6	-	0.3 ± 0.6	-	0.3 ± 0.6	-
Fissurina sp. cf. *F. lucida*	-	-	-	-	-	-
Fissurina sp. A	-	-	-	-	-	-
Haynesina germanica	19.0 ± 7.0	11.7 ± 5.8	18.0 ± 10.6	10.7 ± 3.1	19.0 ± 3.5	16.3 ± 1.5

Table 4.5 (continued)	**380 ppm**		**750 ppm**		**1000 ppm**	
	10°C	**Amb**	**10°C**	**Amb**	**10°C**	**Amb**
Hyaline sp. A	0.3 ± 0.6	1.0 ± 1.0	0.7 ± 1.2	0.7 ± 1.2	-	0.3 ± 0.6
Hyaline sp. B	-	-	0.3 ± 0.6	-	-	-
Hyaline sp. C	-	-	-	-	-	-
Indeterminate juvenille	1.3 ± 2.3	-	-	0.7 ± 1.2	-	6.7 ± 7.6
Miliammina fusca	0.3 ± 0.6	-	-	0.3 ± 0.6	-	-
Miliammina sp. A	0	-	-	-	-	-
Nonionella turgida	-	-	-	0.3 ± 0.6	-	-
Quinqueloculina bicornis	0.3 ± 0.6	-	-	-	-	-
Quinqueloculina sp. cf. *Q. lata*	-	-	0.3 ± 0.6	-	-	-
Quinqueloculina sp. A	-	-	-	-	-	-
Reophax sp.	0.3 ± 0.6	0.3 ± 0.6	-	-	-	-
Rosalina anomala	-	-	-	-	-	0.3 ± 0.6
Rosalina sp. A	-	0.3 ± 0.6	0.7 ± 0.6	-	0.3 ± 0.6	0.7 ± 0.6
Rosalina sp. B	-	-	-	-	0.3 ± 0.6	-
Rosalina sp. C	-	-	-	-	0.3 ± 0.6	-
Stainforthia fusiformis	4.3 ± 2.5	2.0 ± 1.0	3.7 ± 2.9	2.0 ± 1.0	1.0 ± 0.0	1.7 ± 1.5
Trochammina sp. A	-	-	0.3 ± 0.6	-	0.3 ± 0.6	0.3 ± 0.6
Trochammina sp. aff. *T. ochracea*	-	-	0.3 ± 0.6	-	0.3 ± 0.6	0.3 ± 0.6

Table 4.6 Percentages of live benthic foraminiferal species in each treatment at Week 8 (values represent mean ± SD, n = 3). "–" indicates no live individuals of this species were present in any of the samples. CO_2 concentrations were 380 ppm, 750 ppm and 1000 ppm. Temperature Regimes are represented as 10°C (constant regime) or Amb (seasonally varying ambient regime).

Species	380 ppm		750 ppm		1000 ppm	
	10°C	Amb	10°C	Amb	10°C	Amb
Agglut. sp. A	-	-	-	-	-	-
Ammonia falsobeccarii	-	-	-	-	-	-
Biserial agglutinate sp. A	1.0 ± 1.0	-	-	-	-	-
Biserial agglutinate sp. B	1.7 ± 2.1	-	2.0 ± 2.6	-	0.7 ± 0.6	-
Bolivina difformis	-	-	-	-	-	-
Bolivina pseudoplicata	2.0 ± 1.0	-	2.3 ± 0.6	1.3 ± 0.6	1.7 ± 0.6	0.7 ± 1.2
Bolivina pseudopunctata	0.3 ± 0.6	-	-	-	0.3 ± 0.6	-
Bolivina skagerrakensis	-	-	-	-	-	-
Bulimina elongata	-	-	-	-	-	-
Bulimina gibba	-	-	-	-	-	-
Bulimina marginata	-	-	-	-	-	-
Bulimina sp. A	-	-	-	-	-	0.3 ± 0.6
Bulimina sp. B	-	-	-	-	-	-
Cassidulina sp.	-	-	-	0.3 ± 0.6	0.3 ± 0.6	-
Cassidulinoides bradyi	0.3 ± 0.6	-	0.7 ± 2.1	-	-	-
Cibicides lobatulus	0.7 ± 1.2	-	0.7 ± 1.2	1.3 ± 1.5	1.0 ± 1.0	-
Eggerella scabra	-	-	-	-	-	-
Elphidium albiumbilicatum	1.3 ± 2.3	6.3 ± 3.2	5.7 ± 3.5	17.0 ± 5.2	0.3 ± 0.6	8.3 ± 5.1
Elphidium excavatum	-	-	0.3 ± 0.6	0.3 ± 0.6	-	-
Elphidium oceanensis	0.3 ± 0.6	0.3 ± 0.6	1.0 ± 1.0	1.7 ± 1.5	1.3 ± 1.2	0.3 ± 0.6
Elphidium williamsoni	2.3 ± 1.5	5.3 ± 1.2	6.0 ± 1.0	23.3 ± 11.6	17.3 ± 10.0	8.0 ± 1.0
Epistomonella exigua	0.3 ± 0.6	0.3 ± 0.6	-	-	0.3 ± 0.6	-
Fissurina lucida	0.3 ± 0.6	0.3 ± 0.6	0.3 ± 0.6	-	-	-
Fissurina sp. cf. *F. lucida*	-	-	-	-	-	-
Fissurina sp. A	-	0.3 ± 0.6	-	-	-	-
Haynesina germanica	26.0 ± 1.0	7.0 ± 3.6	33.3 ± 18.6	8.0 ± 4.4	25.7 ± 12.6	6.0 ± 3.6

Table 4.6 (continued)	**380 ppm**		**750 ppm**		**1000 ppm**	
	10°C	**Amb**	**10°C**	**Amb**	**10°C**	**Amb**
Hyaline sp. A	-	1.0 ± 1.0	-	0.7 ± 0.6	0.7 ± 0.6	-
Hyaline sp. B	-	-	-	-	-	-
Hyaline sp. C	0.3 ± 0.6	0.3 ± 0.6	1.3 ± 1.5	-	0.3 ± 0.6	-
Indeterminate juvenille	6.3 ± 0.6	2.0 ± 1.0	5.3 ± 5.9	1.3 ± 2.3	6.7 ± 6.0	2.3 ± 2.1
Miliammina fusca	-	-	-	0.3 ± 0.6	0.3 ± 0.6	-
Miliammina sp. A	-	-	-	-	-	-
Nonionella turgida	-	-	-	-	-	-
Quinqueloculina bicornis	-	-	-	0.3 ± 0.6	-	-
Quinqueloculina sp. cf. *Q. lata*	-	-	-	-	-	-
Quinqueloculina sp. A	-	-	-	-	0.3 ± 0.6	-
Reophax sp.	1.0 ± 1.7	-	1.7 ± 0.6	-	1.7 ± 2.1	0.3 ± 0.6
Rosalina anomala	0.3 ± 0.6	-	0.3 ± 0.6	-	-	-
Rosalina sp. A	-	0.3 ± 0.6	-	-	0.3 ± 0.6	-
Rosalina sp. B	-	-	-	-	-	-
Rosalina sp. C	-	-	0.3 ± 0.6	-	-	-
Stainforthia fusiformis	2.7 ± 1.5	2.0 ± 1.0	4.3 ± 2.1	2.7 ± 1.5	3.3 ± 2.5	2.3 ± 0.6
Trochammina sp. A	-	-	-	-	-	-
Trochammina sp. aff. *T. ochracea*	-	-	-	-	-	-

Table 4.7 Percentages of live benthic foraminiferal species in each treatment at Week 24 (values represent mean ± SD, n = 3). "–" indicates no live individuals of this species were present in any of the samples. CO_2 concentrations were 380 ppm, 750 ppm and 1000 ppm. Temperature Regimes are represented as 10°C (constant regime) or Amb (seasonally varying ambient regime).

Species	380 ppm		750 ppm		1000 ppm	
	10°C	Amb	10°C	Amb	10°C	Amb
Agglut. sp. A	-	-	-	-	0.3 ± 0.6	-
Ammonia falsobeccarii	0.3 ± 0.6	-	-	-	0.3 ± 0.6	-
Biserial agglutinate sp. A	-	-	-	0.3 ± 0.6	-	-
Biserial agglutinate sp. B	0.7 ± 1.2	0.7 ± 0.6	0.7 ± 0.6	1.3 ± 1.5	2.7 ± 1.5	-
Bolivina difformis	-	-	-	-	-	-
Bolivina pseudoplicata	2.0 ± 1.0	-	1.0 ± 1.7	0.3 ± 0.6	1.3 ± 1.5	0.3 ± 0.6
Bolivina pseudopunctata	-	-	1.0 ± 1.0	-	-	-
Bolivina skagerrakensis	-	-	-	-	-	-
Bulimina elongata	0.7 ± 0.6	-	0.7 ± 0.6	-	-	0.3 ± 0.6
Bulimina gibba	-	-	-	-	-	-
Bulimina marginata	-	-	-	-	-	-
Bulimina sp. A	-	-	-	-	-	-
Bulimina sp. B	1.0 ± 1.7	-	-	-	-	-
Cassidulina sp.	-	-	-	-	-	-
Cassidulinoides bradyi	1.3 ± 1.5	-	0.3 ± 0.6	-	0.3 ± 0.6	0.3 ± 0.6
Cibicides lobatulus	-	-	0.3 ± 0.6	0.7 ± 0.6	-	0.7 ± 1.2
Eggerella scabra	-	-	-	-	-	-
Elphidium albiumbilicatum	0.7 ± 0.6	-	-	0.3 ± 0.6	-	-
Elphidium excavatum	-	-	-	-	-	-
Elphidium oceanensis	1.3 ± 2.3	-	-	0.3 ± 0.6	0.3 ± 0.6	-
Elphidium williamsoni	8.0 ± 6.0	1.7 ± 1.2	1.3 ± 0.6	2.0 ± 1.7	3.3 ± 1.5	5.7 ± 2.1
Epistomonella exigua	-	-	-	-	-	-
Fissurina lucida	-	-	0.3 ± 0.6	-	-	0.3 ± 0.6
Fissurina sp. cf. *F. lucida*	-	-	-	-	-	-
Fissurina sp. A	-	-	-	-	-	-
Haynesina germanica	44.0 ± 18.0	51.7 ± 24.6	39.0 ± 13.0	46.7 ± 12.4	14.0 ± 7.0	29.7 ± 22.0

Table 4.7 (continued)	**380 ppm**		**750 ppm**		**1000 ppm**	
	10°C	**Amb**	**10°C**	**Amb**	**10°C**	**Amb**
Hyaline sp. A	-	0.3 ± 0.6	-	-	-	-
Hyaline sp. B	-	-	-	-	0.3 ± 0.6	-
Hyaline sp. C	1.0 ± 1.7	-	-	-	-	-
Indeterminate juvenille	2.3 ± 2.5	-	-	2.3 ± 3.2	0.7 ± 1.2	1.7 ± 2.9
Miliammina fusca	-	0.3 ± 0.6	-	0.3 ± 0.6	-	0.3 ± 0.6
Miliammina sp. A	-	-	-	-	-	-
Nonionella turgida	-	0.3 ± 0.6	-	-	-	-
Quinqueloculina bicornis	0.3 ± 0.6	-	-	0.3 ± 0.6	-	0.7 ± 1.2
Quinqueloculina sp. cf. *Q. lata*	-	-	-	-	-	-
Quinqueloculina sp. A	-	-	-	-	-	-
Reophax sp.	0.7 ± 0.6	0.3 ± 0.6	1.0 ± 1.0	0.3 ± 0.6	4.7 ± 8.1	1.7 ± 1.5
Rosalina anomala	1.0 ± 0.0	0.3 ± 0.6	-	-	0.3 ± 0.6	0.3 ± 0.6
Rosalina sp. A	-	0.3 ± 0.6	-	-	-	0.3 ± 0.6
Rosalina sp. B	-	-	0.3 ± 0.6	-	-	-
Rosalina sp. C	-	-	-	-	-	-
Stainforthia fusiformis	5.0 ± 1.7	2.3 ± 2.1	4.0 ± 1.7	1.7 ± 2.1	1.0 ± 0.0	1.7 ± 1.5
Trochammina sp. A	-	-	0.3 ± 0.6	0.3 ± 0.6	-	-
Trochammina sp. aff. *T. ochracea*	-	-	-	-	-	-

Table 4.8 Percentages of live benthic foraminiferal species in each treatment at Week 30 (values represent mean ± SD, n = 3). "–" indicates no live individuals of this species were present in any of the samples. CO_2 concentrations were 380 ppm, 750 ppm and 1000 ppm. Temperature Regimes are represented as 10°C (constant regime) or Amb (seasonally varying ambient regime).

Species	380 ppm		750 ppm		1000 ppm	
	10°C	Amb	10°C	Amb	10°C	Amb
Agglut. sp. A	-	-	-	0.7 ± 0.6	-	-
Ammonia falsobeccarii	-	-	-	-	-	-
Biserial agglutinate sp. A	0.3 ± 0.6	-	-	-	-	-
Biserial agglutinate sp. B	1.0 ± 1.7	0.7 ± 1.2	0.3 ± 0.6	0.3 ± 0.6	2.3 ± 2.3	-
Bolivina difformis	-	-	-	-	-	-
Bolivina pseudoplicata	1.7 ± 2.9	-	-	-	0.3 ± 0.6	0.3 ± 0.6
Bolivina pseudopunctata	0.7 ± 0.6	-	-	-	-	-
Bolivina skagerrakensis	-	-	0.3 ± 0.6	-	-	-
Bulimina elongata	0.7 ± 1.2	-	-	-	0.3 ± 0.6	-
Bulimina gibba	-	-	-	-	-	-
Bulimina marginata	-	-	-	-	-	-
Bulimina sp. A	-	-	-	-	-	-
Bulimina sp. B	0.7 ± 1.2	-	1.3 ± 1.5	-	-	-
Cassidulina sp.	-	-	-	-	-	-
Cassidulinoides bradyi	0.3 ± 0.6	-	0.3 ± 0.6	-	0.3 ± 0.6	-
Cibicides lobatulus	-	0.7 ± 1.2	-	-	-	-
Eggerella scabra	-	-	-	-	-	-
Elphidium albiumbilicatum	1.0 ± 1.0	2.3 ± 3.2	1.7 ± 1.5	1.3 ± 1.5	1.7 ± 2.1	1.0 ± 1.0
Elphidium excavatum	-	-	-	-	-	-
Elphidium oceanensis	1.0 ± 1.0	-	-	0.3 ± 0.6	-	-
Elphidium williamsoni	5.3 ± 4.6	-	4.3 ± 3.5	0.3 ± 0.6	0.3 ± 0.6	0.7 ± 0.6
Epistomonella exigua	-	-	-	-	-	-
Fissurina lucida	-	-	-	-	-	-
Fissurina sp. cf. *F. lucida*	-	-	-	-	-	-
Fissurina sp. A	-	-	-	-	-	-
Haynesina germanica	32.7 ± 18.1	11.0 ± 7.9	43.3 ± 10.7	15.0 ± 7.0	22.0 ± 7.0	19.0 ± 6.9

Table 4.8 (continued)	**380 ppm**		**750 ppm**		**1000 ppm**	
	10°C	**Amb**	**10°C**	**Amb**	**10°C**	**Amb**
Hyaline sp. A	-	-	-	-	-	-
Hyaline sp. B	-	-	-	-	-	-
Hyaline sp. C	0.3 ± 0.6	0.3 ± 0.6	-	-	-	-
Indeterminate juvenille	0.3 ± 0.6	-	-	-	-	-
Miliammina fusca	0.3 ± 0.6	0.3 ± 0.6	0.7 ± 0.6	1.3 ± 1.2	1.0 ± 1.0	0.3 ± 0.6
Miliammina sp. A	-	-	-	-	-	-
Nonionella turgida	-	0.3 ± 0.6	-	-	-	-
Quinqueloculina bicornis	0.7 ± 1.2	-	-	-	-	0.3 ± 0.6
Quinqueloculina sp. cf. *Q. lata*	-	-	-	-	-	-
Quinqueloculina sp. A	-	-	-	-	-	-
Reophax sp.	1.7 ± 2.1	-	-	0.3 ± 0.6	-	1.0 ± 1.0
Rosalina anomala	-	-	-	-	-	-
Rosalina sp. A	0.3 ± 0.6	-	-	0.3 ± 0.6	-	0.7 ± 0.6
Rosalina sp. B	-	-	-	-	-	0.3 ± 0.6
Rosalina sp. C	2.3 ± 4.0	-	-	-	-	-
Stainforthia fusiformis	4.3 ± 3.2	0.3 ± 0.6	2.3 ± 1.5	1.7 ± 1.5	1.7 ± 1.2	1.7 ± 1.5
Trochammina sp. A	-	-	-	-	-	-
Trochammina sp. aff. *T. ochracea*	-	-	-	-	-	-

Table 4.9 Percentages of live benthic foraminiferal species in each treatment at Week 36 (values represent mean ± SD, n = 3). "–" indicates no live individuals of this species were present in any of the samples. CO_2 concentrations were 380 ppm, 750 ppm and 1000 ppm. Temperature Regimes are represented as 10°C (constant regime) or Amb (seasonally varying ambient regime).

Species	380 ppm		750 ppm		1000 ppm	
	10°C	Amb	10°C	Amb	10°C	Amb
Agglut. sp. A	-	-	-	-	-	-
Ammonia falsobeccarii	-	-	-	-	-	-
Biserial agglutinate sp. A	-	-	0.3 ± 0.6	-	-	-
Biserial agglutinate sp. B	-	2.0 ± 2.6	-	2.7 ± 2.5	-	6.7 ± 10.7
Bolivina difformis	-	-	-	-	-	-
Bolivina pseudoplicata	1.3 ± 2.3	2.3 ± 2.1	-	0.7 ± 0.6	-	0.3 ± 0.6
Bolivina pseudopunctata	0.3 ± 0.6	-	-	-	-	0.3 ± 0.6
Bolivina skagerrakensis	-	-	-	0.3 ± 0.6	-	-
Bulimina elongata	0.3 ± 0.6	-	-	-	-	-
Bulimina gibba	-	-	0.3 ± 0.6	-	-	-
Bulimina marginata	-	-	-	-	-	-
Bulimina sp. A	-	-	-	0.3 ± 0.6	-	-
Bulimina sp. B	-	-	-	-	-	-
Cassidulina sp.	-	-	-	-	-	-
Cassidulinoides bradyi	0.7 ± 1.2	-	-	0.3 ± 0.6	0.3 ± 0.6	-
Cibicides lobatulus	-	-	0.3 ± 0.6	0.3 ± 0.6	-	-
Eggerella scabra	-	-	-	-	-	-
Elphidium albiumbilicatum	1.7 ± 2.1	0.3 ± 0.6	0.7 ± 0.6	1.0 ± 1.0	-	0.3 ± 0.6
Elphidium excavatum	-	-	0.3 ± 0.6	-	-	-
Elphidium oceanensis	-	-	-	-	-	-
Elphidium williamsoni	3.7 ± 5.5	1.0 ± 0.0	0.3 ± 0.6	0.7 ± 0.6	-	0.7 ± 1.2
Epistomonella exigua	-	-	-	-	-	0.3 ± 0.6
Fissurina lucida	-	0.3 ± 0.6	-	-	-	0.3 ± 0.6
Fissurina sp. cf. *F. lucida*	-	0.7 ± 1.2	-	0.3 ± 0.6	-	-
Fissurina sp. A	-	-	-	-	-	-
Haynesina germanica	14.3 ± 8.0	4.7 ± 0.6	20.7 ± 6.5	4.0 ± 5.2	6.7 ± 2.5	4.7 ± 4.7

Table 4.9 (continued)	**380 ppm**		**750 ppm**		**1000 ppm**	
	10°C	**Amb**	**10°C**	**Amb**	**10°C**	**Amb**
Hyaline sp. A	-	-	-	-	-	-
Hyaline sp. B	-	-	-	-	-	-
Hyaline sp. C	0.3 ± 0.6	0.3 ± 0.6	-	-	-	-
Indeterminate juvenille	0.7 ± 0.6	-	0.3 ± 0.6	-	-	-
Miliammina fusca	0.3 ± 0.6	1.0 ± 1.0	-	0.7 ± 0.6	-	1.7 ± 1.5
Miliammina sp. A	-	-	-	-	-	0.3 ± 0.6
Nonionella turgida	-	-	-	-	-	-
Quinqueloculina bicornis	-	0.3 ± 0.6	1.3 ± 2.3	-	-	0.3 ± 0.6
Quinqueloculina sp. cf. *Q. lata*	-	-	-	-	-	-
Quinqueloculina sp. A	-	-	-	-	-	-
Reophax sp.	0.7 ± 0.6	0.3 ± 0.6	-	-	-	-
Rosalina anomala	-	-	-	-	-	-
Rosalina sp. A	0.7 ± 0.6	-	0.3 ± 0.6	0.3 ± 0.6	0.3 ± 0.6	0.7 ± 1.2
Rosalina sp. B	0.3 ± 0.6	-	-	-	-	-
Rosalina sp. C	0.7 ± 0.6	-	0.3 ± 0.6	0.3 ± 0.6	-	-
Stainforthia fusiformis	2.3 ± 0.6	1.7 ± 1.2	0.7 ± 1.2	2.3 ± 1.5	-	2.3 ± 0.6
Trochammina sp. A	0.7 ± 0.6	-	-	-	0.3 ± 0.6	-
Trochammina sp. aff. *T. ochracea*	-	-	-	-	-	-

Table 4.10 Percentages of live benthic foraminiferal species in each treatment at Week 50 (values represent mean ± SD, n = 3). "–" indicates no live individuals of this species were present in any of the samples. CO_2 concentrations were 380 ppm, 750 ppm and 1000 ppm. Temperature Regimes are represented as 10°C (constant regime) or Amb (seasonally varying ambient regime).

Species	380 ppm		750 ppm		1000 ppm	
	10°C	Amb	10°C	Amb	10°C	Amb
Agglut. sp. A	0.3 ± 0.6	0.3 ± 0.6	-	0.3 ± 0.6	-	0.3 ± 0.6
Ammonia falsobeccarii	0.7 ± 1.2	-	-	-	-	-
Biserial agglutinate sp. A	-	0.3 ± 0.6	-	-	1.3 ± 1.5	0.3 ± 0.6
Biserial agglutinate sp. B	1.0 ± 0.0	2.3 ± 0.6	0.3 ± 0.6	4.7 ± 3.8	2.7 ± 2.1	3.7 ± 3.1
Bolivina difformis	-	0.3 ± 0.6	-	-	-	-
Bolivina pseudoplicata	3.0 ± 5.2	5.0 ± 2.6	1.3 ± 0.6	1.3 ± 1.2	-	1.0 ± 1.0
Bolivina pseudopunctata	-	0.7 ± 0.6	-	0.3 ± 0.6	-	0.7 ± 1.2
Bolivina skagerrakensis	-	-	-	-	-	-
Bulimina elongata	0.3 ± 0.6	0.7 ± 0.6	-	-	-	-
Bulimina gibba	0.3 ± 0.6	-	-	-	-	0.7 ± 1.2
Bulimina marginata	-	-	-	-	-	-
Bulimina sp. A	-	-	-	-	-	-
Bulimina sp. B	-	-	-	-	-	-
Cassidulina sp.	-	0.3 ± 0.6	-	-	-	-
Cassidulinoides bradyi	0.3 ± 0.6	-	-	0.3 ± 0.6	0.3 ± 0.6	0.3 ± 0.6
Cibicides lobatulus	0.7 ± 1.2	0.3 ± 0.6	0.3 ± 0.6	0.3 ± 0.6	-	0.7 ± 1.2
Eggerella scabra	-	-	-	-	-	-
Elphidium albiumbilicatum	2.3 ± 1.5	-	0.3 ± 0.6	-	-	0.3 ± 0.6
Elphidium excavatum	-	-	-	-	-	-
Elphidium oceanensis	-	-	-	-	-	-
Elphidium williamsoni	1.0 ± 1.0	-	-	0.7 ± 0.6	-	1.3 ± 1.5
Epistomonella exigua	-	-	-	-	-	-
Fissurina lucida	-	0.7 ± 0.6	0.3 ± 0.6	0.3 ± 0.6	0.3 ± 0.6	-
Fissurina sp. cf. *F. lucida*	-	-	-	-	-	-
Fissurina sp. A	-	-	-	-	-	-
Haynesina germanica	21.7 ± 10.1	6.7 ± 0.6	29.7 ± 11.6	5.3 ± 3.1	9.0 ± 6.1	4.7 ± 3.1

Table 4.10 (continued)	**380 ppm**		**750 ppm**		**1000 ppm**	
	10°C	**Amb**	**10°C**	**Amb**	**10°C**	**Amb**
Hyaline sp. A	-	-	-	-	-	-
Hyaline sp. B	-	-	-	-	-	-
Hyaline sp. C	-	-	-	0.7 ± 0.6	-	-
Indeterminate juvenille	-	-	0.3 ± 0.6	0.3 ± 0.6	0.7 ± 0.6	-
Miliammina fusca	-	-	0.3 ± 0.6	1.0 ± 0.0	-	0.3 ± 0.6
Miliammina sp. A	-	-	-	-	-	-
Nonionella turgida	-	-	-	-	-	-
Quinqueloculina bicornis	9.7 ± 16.7	-	26.3 ± 26.0	0.3 ± 0.6	0.3 ±0.6	0.3 ± 0.6
Quinqueloculina sp. cf. *Q. lata*	-	-	-	-	-	-
Quinqueloculina sp. A	-	-	-	-	0.7 ± 1.2	-
Reophax sp.	3.3 ± 4.0	1.3 ± 1.2	1.0 ± 1.0	1.3 ± 1.2	1.7 ± 1.5	2.0 ± 2.0
Rosalina anomala	1.3 ± 2.3	-	-	0.3 ± 0.6	-	-
Rosalina sp. A	0.3 ± 0.6	1.0 ± 1.0	-	0.7 ± 0.6	0.3 ± 0.6	-
Rosalina sp. B	-	-	-	-	-	-
Rosalina sp. C	-	0.3 ± 0.6	-	-	-	-
Stainforthia fusiformis	4.3 ± 3.2	2.3 ± 0.6	3.0 ± 1.0	1.7 ± 0.6	1.7 ± 2.1	2.3 ± 0.6
Trochammina sp. A	-	-	-	-	0.3 ± 0.6	-
Trochammina sp. aff. *T. ochracea*	-	-	-	-	-	-

Table 4.11 Percentages of live benthic foraminiferal species in each treatment at Week 64 (values represent mean ± SD, n = 3). "–" indicates no live individuals of this species were present in any of the samples. CO_2 concentrations were 380 ppm, 750 ppm and 1000 ppm. Temperature Regimes are represented as 10°C (constant regime) or Amb (seasonally varying ambient regime).

Species	380 ppm		750 ppm		1000 ppm	
	10°C	Amb	10°C	Amb	10°C	Amb
Agglut. sp. A	0.3 ± 0.6	-	0.7 ± 1.2	-	0.3 ± 0.6	1.3 ± 2.3
Ammonia falsobeccarii	-	0.3 ± 0.6	-	-	0.3 ± 0.6	-
Biserial agglutinate sp. A	-	0.3 ± 0.6	-	0.7 ± 0.6	0.3 ± 0.6	-
Biserial agglutinate sp. B	0.3 ± 0.6	3.3 ± 1.5	-	0.7 ± 0.6	1.3 ± 1.5	3.0 ± 3.0
Bolivina difformis	-	-	-	-	-	0.3 ± 0.6
Bolivina pseudoplicata	0.3 ± 0.6	2.3 ± 0.6	0.7 ± 0.6	1.7 ± 2.1	-	0.7 ± 1.2
Bolivina pseudopunctata	0.7 ± 0.6	0.3 ± 0.6	0.7 ± 0.6	0.3 ± 0.6	-	1.0 ± 1.0
Bolivina skagerrakensis	-	-	-	-	-	0.7 ± 1.2
Bulimina elongata	0.7 ± 1.2	0.3 ± 0.6	4.7 ± 7.2	0.3 ± 0.6	1.0 ± 1.0	-
Bulimina gibba	-	-	-	-	-	-
Bulimina marginata	0.3 ± 0.6	-	-	-	0.3 ± 0.6	-
Bulimina sp. A	-	-	-	-	-	-
Bulimina sp. B	-	-	-	-	-	-
Cassidulina sp.	0.3 ± 0.6	-	-	-	-	0.3 ± 0.6
Cassidulinoides bradyi	0.3 ± 0.6	-	-	0.3 ± 0.6	-	0.3 ± 0.6
Cibicides lobatulus	-	0.7 ± 0.6	0.3 ± 0.6	0.3 ± 0.6	-	-
Eggerella scabra	-	-	-	-	-	-
Elphidium albiumbilicatum	1.3 ± 1.5	1.0 ± 1.0	-	0.7 ± 0.6	0.7 ± 0.6	0.7 ± 1.2
Elphidium excavatum	-	-	-	-	-	-
Elphidium oceanensis	0.7 ± 1.2	-	-	-	-	-
Elphidium williamsoni	1.7 ± 0.6	1.0 ± 1.0	-	4.3 ± 4.2	-	2.7 ± 0.6
Epistomonella exigua	-	-	-	-	-	-
Fissurina lucida	-	-	0.3 ± 0.6	-	0.3 ± 0.6	0.7 ± 1.2
Fissurina sp. cf. *F. lucida*	-	-	-	-	-	-
Fissurina sp. A	-	-	-	-	-	-
Haynesina germanica	21.3 ± 11.9	7.3 ± 2.1	17.7 ± 12.1	6.7 ± 2.1	4.3 ± 1.5	8.7 ± 5.7

Table 4.11 (continued)	**380 ppm**		**750 ppm**		**1000 ppm**	
	10°C	**Amb**	**10°C**	**Amb**	**10°C**	**Amb**
Hyaline sp. A	0.7 ± 0.6	0.3 ± 0.6	-	0.3 ± 0.6	-	-
Hyaline sp. B	-	-	-	-	-	-
Hyaline sp. C	-	-	-	-	0.3 ± 0.6	-
Indeterminate juvenille	-	1.0 ± 1.0	0.7 ± 1.2	-	0.3 ± 0.6	-
Miliammina fusca	0.7 ± 0.6	-	-	-	-	1.0 ± 1.0
Miliammina sp. A	-	-	-	-	-	-
Nonionella turgida	-	-	-	-	-	-
Quinqueloculina bicornis	2.7 ± 2.9	1.7 ± 2.1	16.7 ± 28.9	-	1.3 ± 2.3	-
Quinqueloculina sp. cf. *Q. lata*	-	-	-	-	-	-
Quinqueloculina sp. A	-	-	-	-	-	-
Reophax sp.	1.0 ± 1.0	1.7 ± 1.5	0.7 ± 1.2	1.0 ± 1.7	0.3 ± 0.6	3.7 ± 2.1
Rosalina anomala	-	-	-	-	0.3 ± 0.6	-
Rosalina sp. A	0.7 ± 1.2	0.3 ± 0.6	0.3 ± 0.6	1.3 ± 1.5	-	-
Rosalina sp. B	-	-	-	-	-	0.3 ± 0.6
Rosalina sp. C	0.3 ± 0.6	-	-	-	-	-
Stainforthia fusiformis	7.3 ± 2.1	6.0 ± 5.3	2.0 ± 2.0	8.0 ± 4.0	2.3 ± 2.5	4.3 ± 2.1
Trochammina sp. A	-	-	-	0.3 ± 0.6	-	-
Trochammina sp. aff. *T. ochracea*	-	-	-	-	-	0.7 ± 0.6

The MDS ordination plots of Bray-Curtis similarities from the data were modified to include superimposed circles of increasing numbers of each of these four species (Fig. 4.19 - 4.22). The similarity clusters as identified by SIMPROF analysis were overlaid on each of the MDS plots. With each of these species superimposed in turn as bubble plots the following patterns emerge.

Live specimens of *Haynesina germanica* were recorded throughout the time-series and across each CO_2 treatment and temperature regime (Fig. 4.19). Numbers of living specimens of this species varied depending on the treatment. The lowest live numbers were generally present in samples from the beginning (week 0) and towards the end of the time-series (weeks 36 - 64). Live specimens of *Elphidium williamsoni* and *Elphidium albiumbilicatum* did not occur across all treatments (Fig. 4.20 – 4.21). The highest numbers of live individuals of *E. williamsoni* and *E. albiumbilicatum* were documented in weeks 4 and 8 and generally under ambient temperature conditions (Fig. 4.20 – 4.21). Live *E. williamsoni* were recorded in each CO_2treatment with the highest numbers occurring at 750 ppm (Fig. 4.20). Fewer live individuals of *E. albiumbilicatum* were documented when compared to *E. williamsoni* but they also occurred across all CO_2 treatments (Fig, 4.21).*Stainforthia fusiformis* occurred in the majority of samples although in low live numbers (Fig. 4.22). This species was present in similar amounts across the time-series and in each of the CO_2 and temperature regimes.

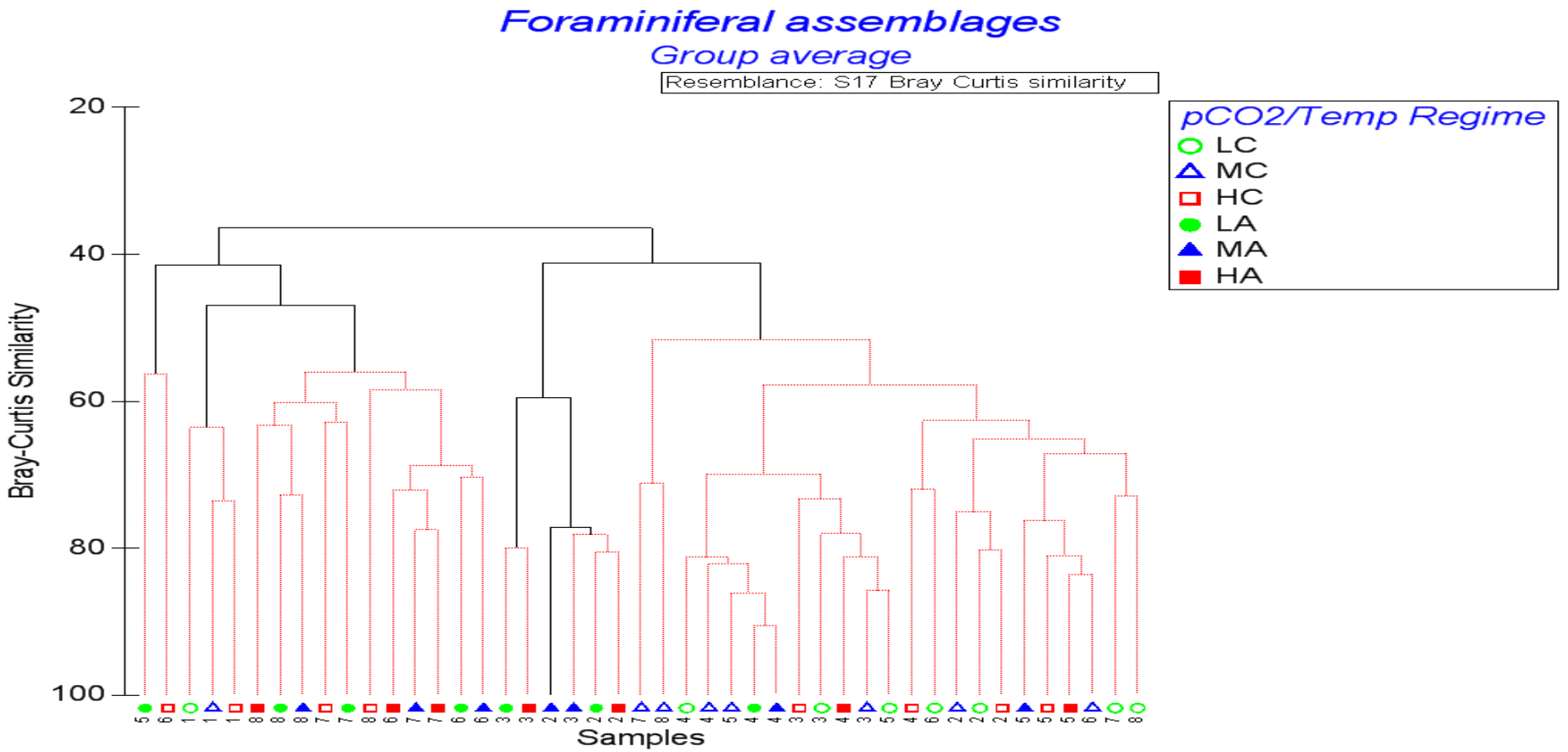

Figure 4.17 Dendrogram resulting from cluster analysis performed on foraminiferal assemblage data on a 45 sample x 42 taxa matrix using the S17 Bray-Curtis similarity index (5% level of significance) at each time point (1 = week 0, 2 = week 4, 3 = week 8, 4 = week 24, 5 = week 30, 6 = week 36, 7 = week 50 and 8 = week 64). CO_2 treatments are represented by green (380 ppm = L), blue (750 ppm = M) and red (1000 ppm = H) symbols. Temperature regimes are represented by open (constant = C) and closed (ambient = A) symbols. Solid lines (black) represent statistically similar group structure by SIMPROF test. Red (dashed) lines indicate that analysis by SIMPROF showed no statistical evidence for any sub-structure within those branches.

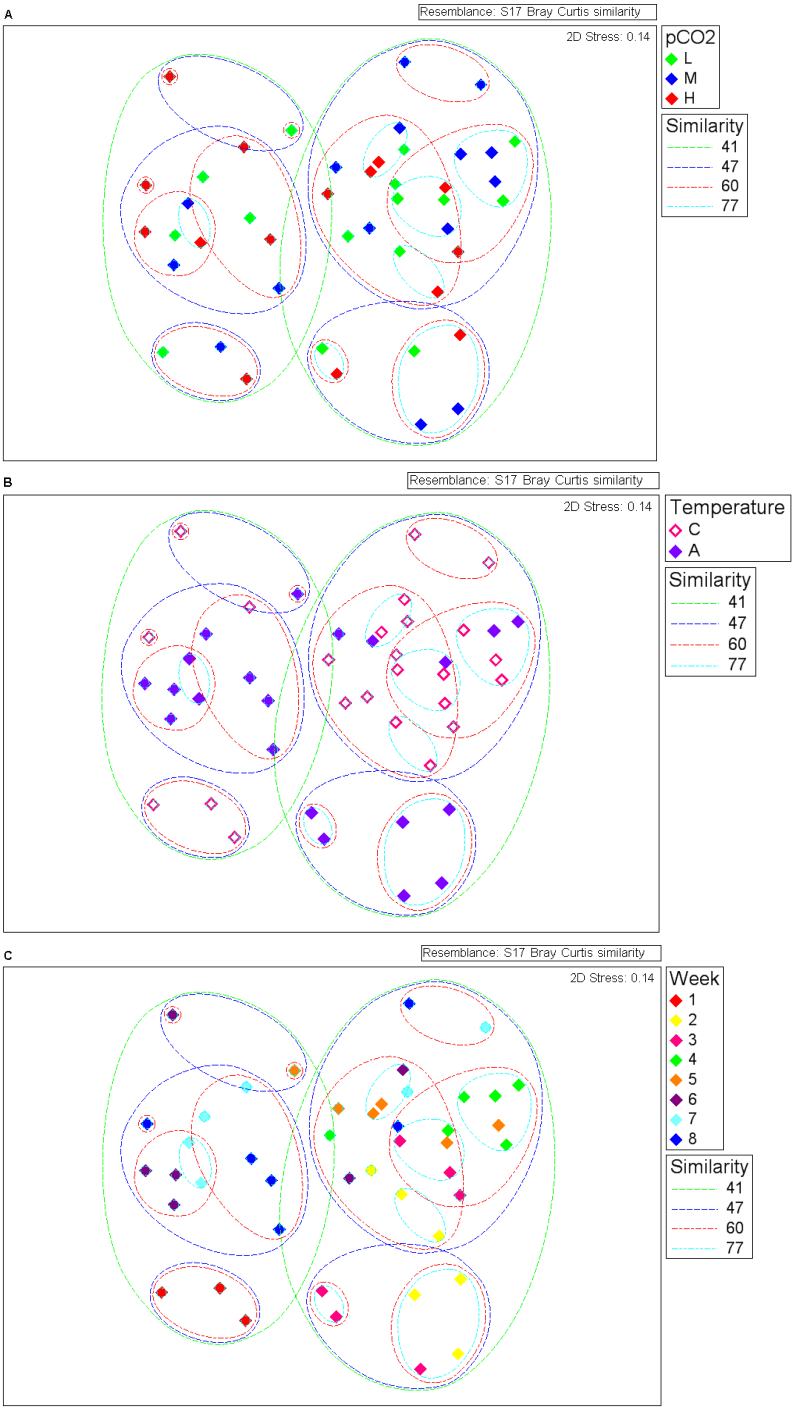

Figure 4.18 2D nMDS (minimum stress: 0.14), with overlays of Bray Curtis similarity of 41% (green dashed line), 47% (dark blue dashed line), 60% (red dashed line) and 77% (light blue dashed line) identified by SIMPROF analysis and displayed in the dendrogram (slack = 70%). A) CO_2treatment (L = 380 ppm, M = 750 ppm, H = 1000 ppm). B) Temperature Regime (C = constant, A = ambient). C) Week (1 = week 0, 2 = week 4, 3 = week 8, 4 = week 24, 5 = week 30, 6 = week 36, 7 = week 50, 8 = week 64).

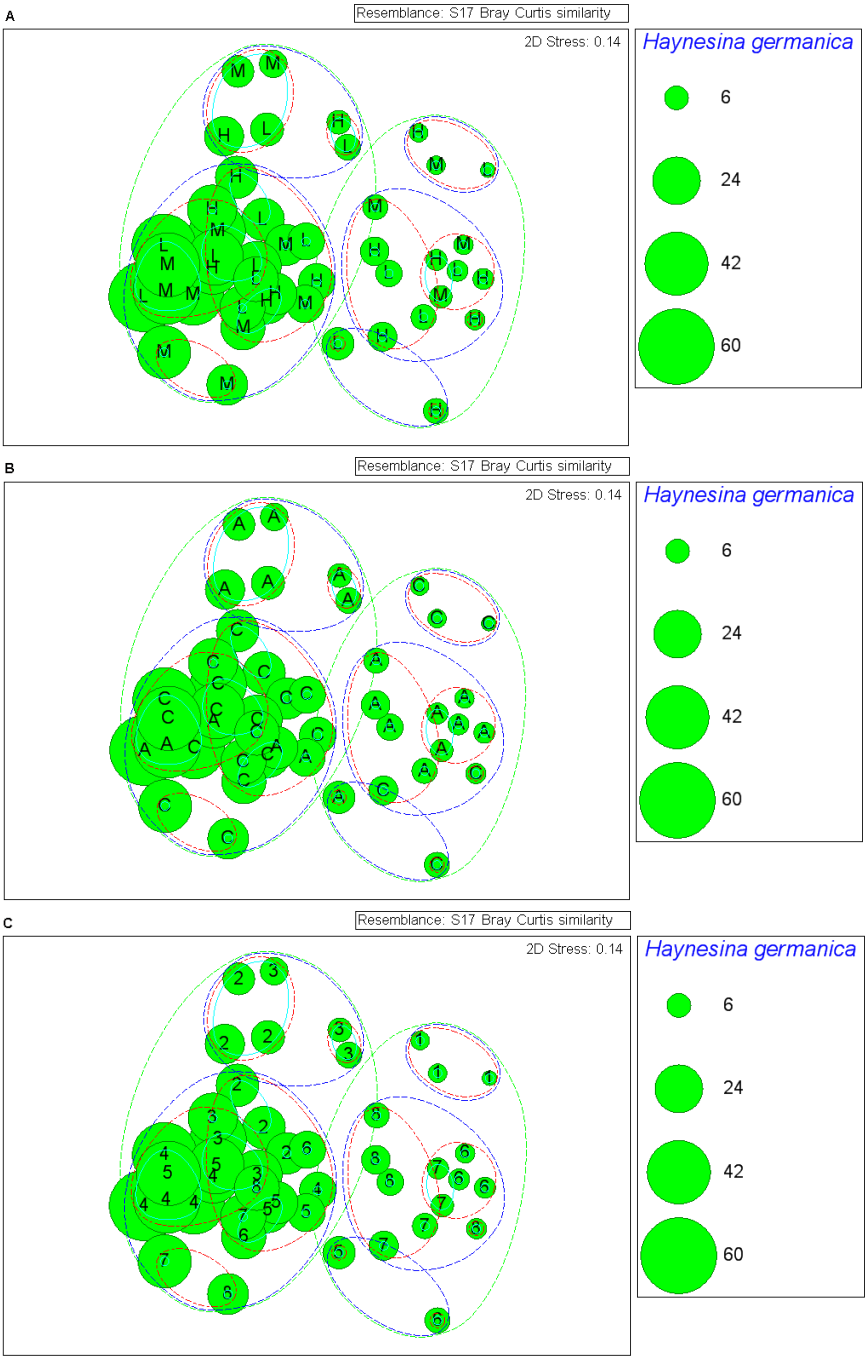

Figure 4.19 Bubble plot species 'overlays' of *Haynesina germanica* labelled by A) CO_2 treatment (L = 380 ppm, M = 750 ppm, H = 1000 ppm), B) Temperature Regime (C = constant, A = ambient), and C) Week (1 = week 0, 2 = week 4, 3 = week 8, 4 = week 24, 5 = week 30, 6 = week 36, 7 = week 50, 8 = week 64).The bubble size represents the scale for the average number of individuals within each sample. Each MDS plot (minimum stress: 0.14), has overlays of Bray Curtis similarity of 41% (green dashed line), 47% (dark blue dashed line), 60% (red dashed line) and 80% (light blue line) identified by SIMPROF analysis (slack = 70%).

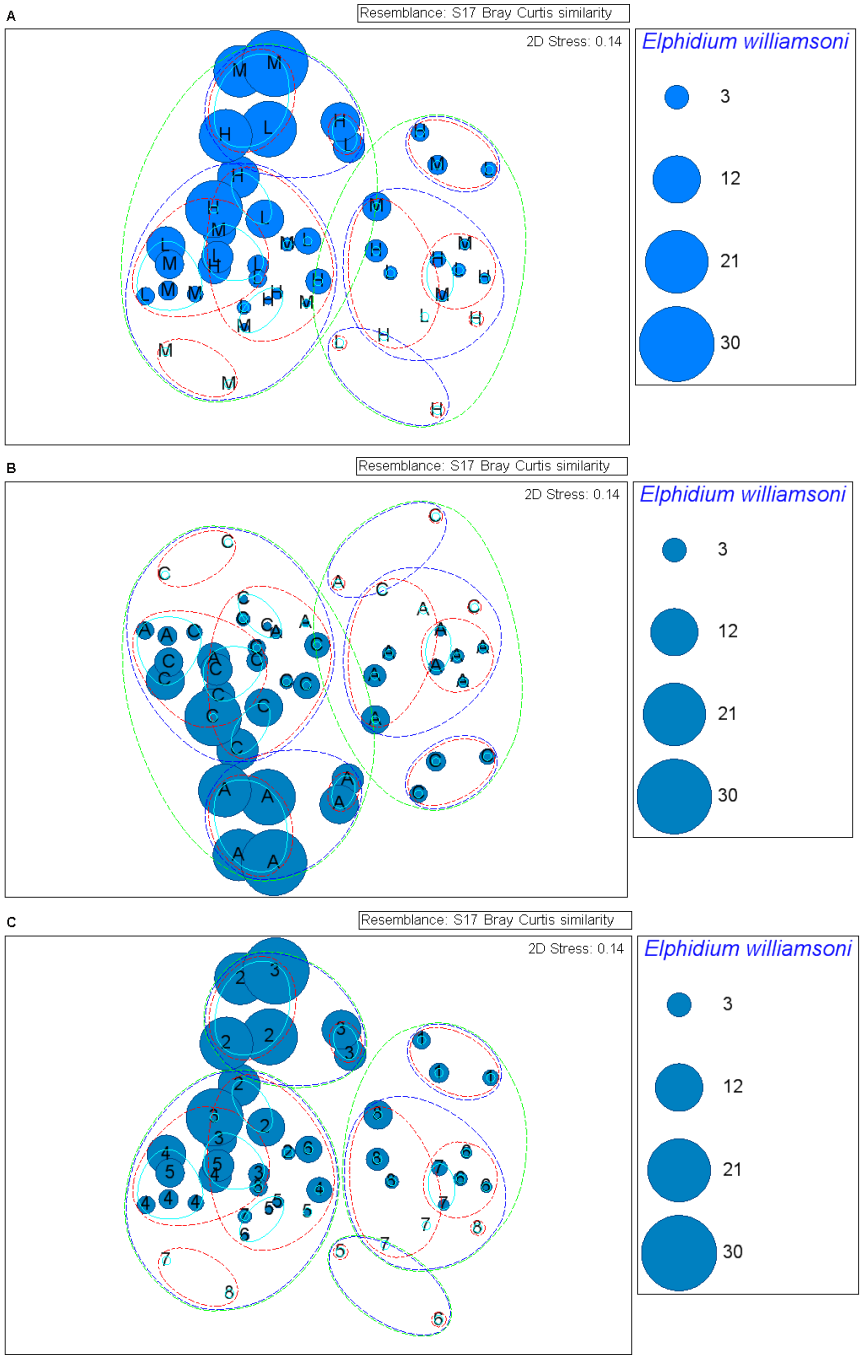

Figure 4.20 Bubble plot species 'overlays' of *Elphidium williamsoni* labelled by A) CO_2 treatment (L = 380 ppm, M = 750 ppm, H = 1000 ppm), B) Temperature Regime (C = constant, A = ambient), and C) Week (1 = week 0, 2 = week 4, 3 = week 8, 4 = week 24, 5 = week 30, 6 = week 36, 7 = week 50, 8 = week 64). The bubble size represents the scale for the average number of individuals within each sample. Each MDS plot (minimum stress: 0.14), has overlays of Bray Curtis similarity of 41% (green dashed line), 47% (dark blue dashed line), 60% (red dashed line) and 77% (light blue line) identified by SIMPROF analysis (slack = 70%).

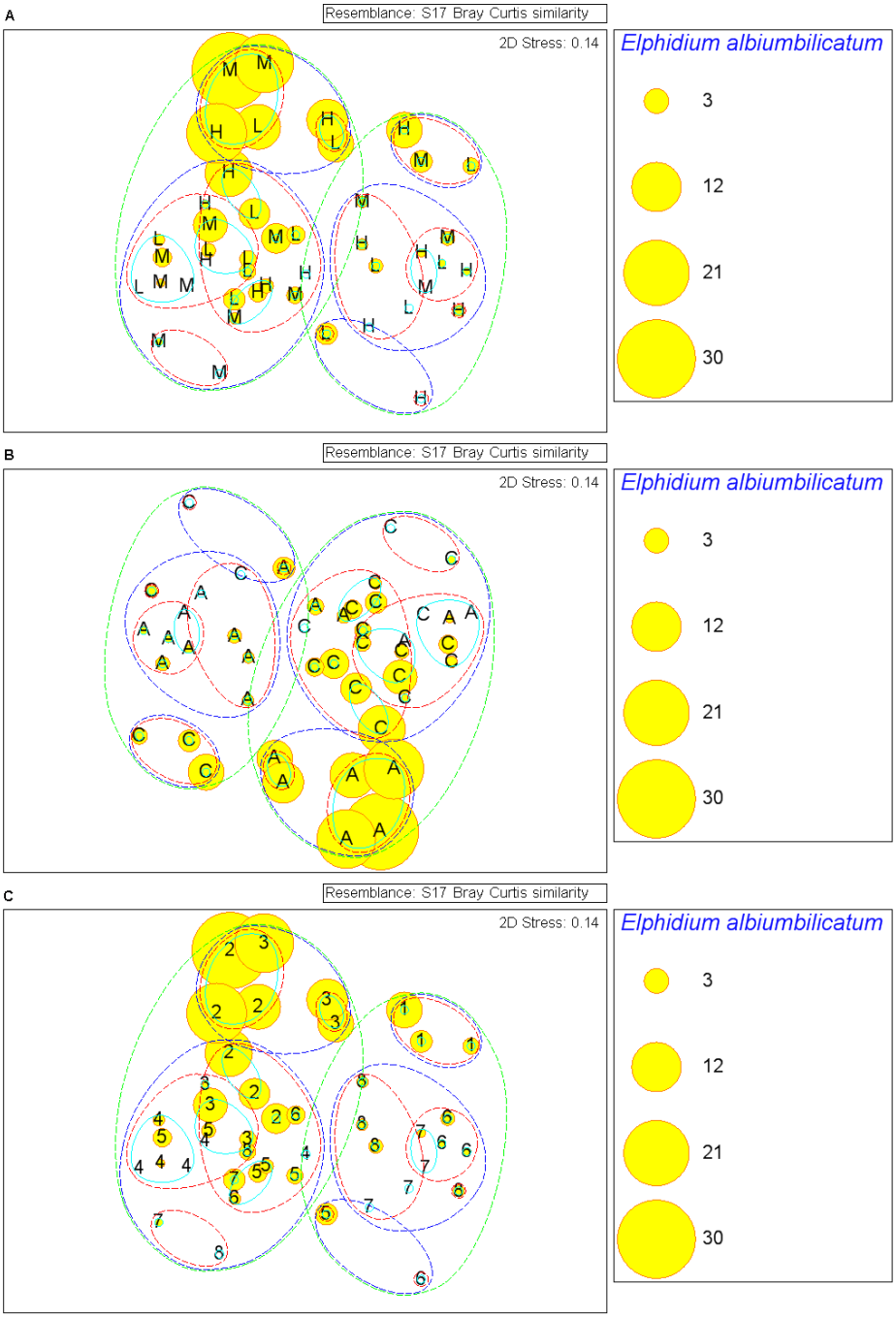

Figure 4.21 Bubble plot species 'overlays' of *Elphidium albiumbilicatum* labelled by A) CO_2 treatment (L = 380 ppm, M = 750 ppm, H = 1000 ppm), B) Temperature Regime (C = constant, A = ambient), and C) Week (1 = week 0, 2 = week 4, 3 = week 8, 4 = week 24, 5 = week 30, 6 = week 36, 7 = week 50, 8 = week 64). The bubble size represents the scale for the average number of individuals within each sample. Each MDS plot (minimum stress: 0.14), has overlays of Bray Curtis similarity of 41% (green dashed line), 47% (dark blue dashed line), 60% (red dashed line) and 77% (light blue line) identified by SIMPROF analysis (slack = 70%).

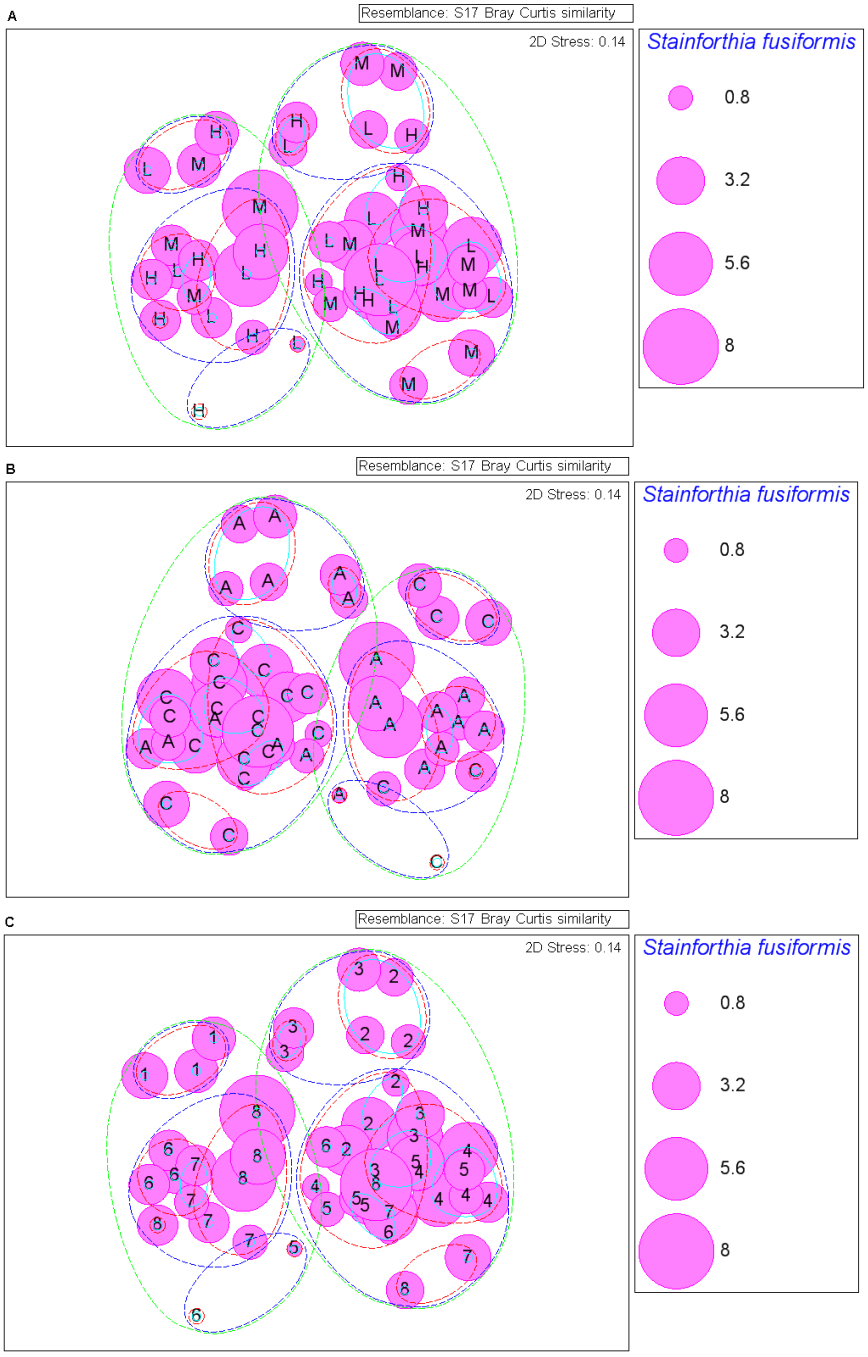

Figure 4.22 Bubble plot species 'overlays' of *Stainforthia fusiformis* labelled by A) CO_2 treatment (L = 380 ppm, M = 750 ppm, H = 1000 ppm), B) Temperature Regime (C = constant, A = ambient), and C) Week (1 = week 0, 2 = week 4, 3 = week 8, 4 = week 24, 5 = week 30, 6 = week 36, 7 = week 50, 8 = week 64). The bubble size represents the scale for the average number of individuals within each sample. Each MDS plot (minimum stress: 0.14), has overlays of Bray Curtis similarity of 41% (green dashed line), 47% (dark blue dashed line), 60% (red dashed line) and 77% (light blue line) identified by SIMPROF analysis (slack = 70%).

4.4 Discussion

4.4.1 Wall structure

Plotting the relative proportions of the three most common benthic groups, the agglutinate Textulariida, the porcelaneous Miliolida, and the perforate Rotaliida, on ternary diagrams, provide an indication as to the environments which the organisms are best adapted (Murray, 1973). The majority of foraminiferal assemblages from the time-series plotted along the agglutinate-hyaline side of the ternary diagrams. This is typical of the distribution expected from estuaries that have only a small porcelaneous component (Murray, 2006). The Miliolida secrete imperforate shells of high-magnesium calcite, which are most easily produced where $CaCO_3$ saturation is high. High Mg-calcite is the most soluble form of carbonate and organisms that produce high Mg-calcite are considered particularly vulnerable to ocean acidification (Fujita et al., 2011; Uthicke et al., 2013). Very few live porcelaneous specimens were observed across all treatments therefore the effects of ocean acidification on these forms could not be assessed. The porcelaneous component did however show an increase in weeks 50 and 64, with both Q*uinqueloculina bicornis* and *Quinqueloculina* sp increasing in numbers. It is feasible that both of these species were present in the original sample material as propagules (Goldstein & Alve, 2011). Fine-fractions of depositional sediments can contain a bank of abundant and diverse foraminiferal propagules that grow to maturity only when exposed to appropriate environmental conditions (Alve & Goldstein, 2003). The increase in species and numbers of live individuals after 50 weeks exposure highlight the importance of long-term experimental incubations.

Foraminifera with complex rotalid shells have adapted to a range of environments (Hallock, 2000a). For example, at CO_2 vent sites in the northern Gulf of California

living calcareous forms of foraminifera were found alive (in low numbers) under low pH conditions (7.55) (Pettit et al., 2013). Calcareous individuals dominated in both the total and live assemblages throughout the time-series. Even after 64 weeks, the dead assemblages were still dominated by calcareous forms. These results differ to those found at some natural volcanic vent sites (e.g. Papua New Guinea) where calcareous biota, including foraminifera, were almost completely absent in surface sediments under high pCO_2conditions (Fabricus et al., 2011). In an additional vent site, both calcifying and non-calcifying taxa were absent under low pH (< 7.9) samples (Uthicke et al., 2013). As the dead assemblage is a time-averaged record of the living assemblage, it is likely that live specimens of other species (agglutinated and porcelaneous forms) were not present in high enough numbers to significantly contribute or impact on the total populations. The sediment may also have provided buffering capacity (Leclercq et al., 2002; Andersson et al., 2003). Foraminiferal migrations within the sediments could have prevented complete dissolution. Alternatively, calcareous foraminifera may require more than 18 months exposure under high CO_2treatments to fully dissolve and no longer contribute to the surface sediments.

Agglutinated forms generally dominate environments where seawater is undersaturated with respect to $CaCO_3$ and can survive in sediments where no carbonate is available (Scott et al., 2001). For example, in the deep sea, dissolution of calcareous tests is common and the dead assemblages are dominated by agglutinated tests (Alve & Murray, 1997). Low pH conditions in previous studies have recorded changes in assemblage composition through dissolution of calcareous tests (Murray, 1989; Murray & Alve, 1999a), with agglutinated forms being able to survive long exposures to low pH conditions (Schafer, 2000). For example, in the

fossil record, where dissolution of calcareous tests is clearly active, dead assemblages are often enriched in agglutinated tests (Murray & Alve, 1999b). In normal estuaries, the increasing proportion of agglutinated species landward may be related to decreasing pH with increasing continental runoff that affects calcareous species (Debenay et al., 2000).

The hypothesis that agglutinated taxa, which do not rely on calcification, may replace calcifying species following exposure to ocean acidification should be considered. For example, assemblage shifts to ones dominated by agglutinated taxa have been documented at volcanic vent sites in Ischia, Italy (Dias et al., 2010). In this experiment, the highest numbers of agglutinated foraminifera were recorded under the higher CO_2 treatments. Although these numbers varied through the time-series they did not dominate the living assemblages. The most likely explanation is that the agglutinated taxa were too rare in the initial sediments sampled for the experiment. For example, at a natural vent site in Papua New Guinea, agglutinated taxa were too rare to fill the niches vacated by the calcifying taxa (Uthicke et al., 2013). It may also be possible that the exposure periods, although long-term, were not long enough for the agglutinated taxa to dominate (e.g. the calcifying foraminifera were still contributing significantly to the living populations). For example, responses in other benthic invertebrates, e.g. an intertidal polychaete (*Alitta virens*), did not appear until several months had elapsed in longer-term experiments (Godbold & Solan, 2013). A time-lag was observed in the response of the agglutinated individuals with no CO_2 effect present until after 24 weeks exposure. There was a significant impact of CO_2 and temperature on the numbers of agglutinated individuals and this was most distinctive after 64 weeks exposure.

4.4.2 Diversity measures

Typically, species diversity is low in extreme environments e.g. estuaries (Murray, 2006).In this study, a higher diversity was recorded than expected for foraminifera from intertidal sediments. For example, in the Eden Estuary, NE Scotland, a total of 15 species were recorded (Austin, 2003) compared to the 63 species identified here in samples from the Ythan Estuary.

However, not all species identified were represented by live individuals. Dead specimens may have been introduced into the experimental material at the time of sampling. For example, *Heterohelix striata* and *Cassidulina* spp. could have been introduced into the mesocosms from reworked sediments at or adjacent to the intertidal area sampled.

The highest numbers of live species were observed after 64 weeks exposure at the lowest CO_2 concentration (380 ppm) under the constant temperature regime. Species richness was therefore highest under the least 'stressed' conditions. For the first eight weeks of the experiment the number of species recorded under the constant temperature regime varied. Following this initial period, the highest number of species were consistently recorded under the lowest CO_2 treatment (380 ppm). It is possible that for the first eight weeks that a "shock" response was a factor and it is only after this point that we begin to observe the true experimental response. There was a significant effect of temperature on species richness with seasonal fluctuations observed under both the constant and ambient temperature regimes.

Under the ambient temperature regimes the number of species decreased over weeks 24 and 30. The decrease coincides with the maximum temperatures seen during the time-series. At weeks 24 and 30, temperatures in the ambient regime

increased to 15.2°C and 14.9°C, respectively. Species richness only began to increase in the ambient temperature regime from week 36 when the temperature fell to 13.4°C. Temperature has also played a key role in response of other organisms. For example, in the benthic intertidal polychaete *Alitta virens*, effects on growth and behaviour were mostly driven by absolute temperature, rather than enhanced levels of CO_2 (Godbold & Solan, 2013).

The lowest foraminiferal species diversity was recorded under the highest CO_2 treatments. This is a predictable response to environmental change where diversity decreases as stress increases (Murray, 2000). Results similar to this have been documented at natural CO_2 vents e.g. reduced benthic foraminiferal diversity and abundance was recorded as markedly reduced at low pH sites (Dias et al., 2010; Uthicke et al., 2013). In Ischia, diversity fell from 24 species in normal marine areas to just 4 species in areas with high CO_2 levels (Dias et al., 2010). A similar reduction in diversity was also recorded in Papua New Guinea (Uthicke et al., 2013). At this shallow volcanic CO_2 seep, foraminifera were almost completely absent at sites with pH < 7.9 (Uthicke et al., 2013). In the present study, live individuals were still found under the high CO_2 conditions (1000 ppm). A complex response was observed with 3 x 2-way interactions acting upon the live abundances. A significant temperature effect was observed at lower CO_2 concentrations (380 ppm) which then broke down under high CO_2 conditions (1000 ppm). At a vent site in the northern Gulf of California live foraminifera were also observed under low pH conditions. At this site, foraminiferal species assemblages were impoverished under low pH conditions and showed evidence of post-mortem test dissolution (Pettit et al., 2013). Test dissolution, damage and deformation are discussed further in Chapters 5-7.

4.4.3 Community composition and species-specific responses

Community changes are caused by different tolerances of benthic organisms to high pCO_2 (Widdicombe et al., 2009). The numbers of live individuals of each species showed a complex response. The bubble plots (Figs. 4.18 – 4.20) show the limitations in relating the community structure to a single environmental variable at a time (Clarke & Warwick, 2001).

Shifts in competitive interactions between taxa have been observed under low pH conditions at natural volcanic seeps (Fabricus et al., 2011). Typically, assemblages affected by dissolution are enriched in species resistant to dissolution (Kucera, 2007). In the case of foraminifera, some species are more prone to dissolution due to the different morphology of their shells (Kucera, 2007, and references therein). Extraordinary adaptations have been demonstrated in a few populations of calcareous species (Sen Gupta, 2002). In the current study, four species persisted in the foraminiferal communities: *Haynesina germanica*, *Elphidium williamsoni*, *Elphidium albiumbilicatum* and *Stainforthia fusiformis*. Combined, these four species (all of which are calcareous) account for > 75% of the total living specimens present throughout the time-series.

The rank order of species may be consistent or change from month to month (Murray, 2000). This has been recorded in the Eden Estuary, NE Scotland, where the top ranked species varied between *Haynesina germanica* and *Elphidium williamsoni* over a seasonal cycle (Austin, 2003). Even with species-specific fluctuations, *Haynesina germanica* continued to be the top ranked species in terms of numbers of live specimens throughout the time-series. *Elphidium williamsoni* is a calcareous species characteristic of intertidal communities (Sen Gupta, 2002) and is adapted to living in highly variable physicochemical conditions (Allison et al., 2010).

For example, some species of *Elphidium* are well-known for their tolerance to salinity fluctuations (Sen Gupta, 2002). Schafer (1970) reported specimens of *Elphidium* surviving at pH 6.7.*Stainforthia* is a known opportunist species (Murray, 2000). *Stainforthia fusiformis* were able to survive and tolerate the high pCO_2 conditions but were present in low numbers throughout the experiment.

It was hypothesised that the agglutinated species would benefit from reduced competition. Nine agglutinated species were identified, none of which dominated the live counts throughout the time-series. As discussed above, it is likely that these species were not present in high enough numbers in the initial sample material to allow for them to significantly contribute to the live populations.

Finally, although *Haynesina germanica* dominated in terms of live numbers throughout the time series it is not considered here as a resilient species. Numbers of *H. germanica* were often lower under high CO_2 conditions (1000 ppm) with individuals showing signs of dissolution. In the following chapters therefore, the focus will be on *H. germanica* as the dominant species from the time-series and present data on test dissolution, deformation and possible compromised feeding function.

5. The effects of long-term exposure to ocean acidification on benthic foraminiferal morphology – deformation and damage.

5.1 Introduction

Benthic foraminifera provide one of the most sensitive and readily available markers of environmental stress in both naturally and anthropogenically stressed aquatic environments (Coccioni, 2000). Their small tests are generally numerically abundant, readily preserved in most marine sediments and are able to record evidence of environmental stresses through time (Yanko et al., 2002). Deformation or abnormality of foraminiferal tests can yield information on the environmental characteristics of the area where they lived (Le Cadre et al., 2003). In addition to foraminiferal abundance and taxonomic composition data (described in Chapter 4) abnormal tests are often considered as useful bioindicators of environmental stress (Stouff et al., 1999a). Environmental changes may be reconstructed based on the proportions of abnormal tests and the types of morphological abnormality present in a population (Geslin et al., 2002). Furthermore, it has been suggested that investigation into connections between modes of deformation and environmental properties could help to distinguish between ecological stress responses and diagenetic processes (Coccioni, 2000).

Abnormalities have been reported in recent (Alve, 1991; Yanko et al., 1994, 1998) as well as fossil foraminiferal specimens (Cann & De Deckker, 1981; Caralp, 1989; Yanko et al., 2002; and references therein). For example, abnormalities in the number, shape, and especially the disposition of shell chambers have been detected and described (Boltovskoy & Wright, 1976; Haynes, 1981; Boltovskoy et al., 1991;

Stubbles, 1993; Yanko et al., 1994, 1995, 1998). Abnormal tests are induced by various causes (discussed in Section 5.1.1) characterizing different environmental stress (Geslin et al., 2002). However, it is widely acknowledged that more studies on the relation between morphological abnormalities and different environmental stresses need to be carried out in order to refine this proxy (Geslin et al., 2002).

5.1.1 Causes of deformation and damage

Morphological abnormalities of fossil and recent foraminiferal tests have long been reported and may be caused either by physical or ecological influences (Geslin et al., 2002, and references therein). Investigators have previously noted that deformities might also be the result of mechanical damage, in addition to environmental stress. Mechanical, ecological and genetic factors can all be responsible for the development of abnormal tests in benthic foraminifera (Almogi-Labin et al., 1992). Some biological causes may be genetic or due to disruption of normal metabolic processes such as calcification, leading to weak, damage-prone test walls (Yanko et al., 2002).

Stouff et al. (1999a) believe that environmental conditions may influence the formation of abnormal tests by acting at a cellular level to slow down some essential metabolic processes. Because the cytoskeleton controls both the shape of the cell and the transport of organelles or vesicular structures, injury to the cytoskeleton may alter the shape and arrangement of chambers and the transport of proteins and calcium to the shell wall (Bresler & Yanko-Hombach, 2000).

Severe malformations may also follow the processes of reproduction (Geslin et al., 2000). Specimens may survive after asexual reproduction and construct new chambers with abnormal sizes, shapes and dispositions (Geslin et al., 2000).

5.1.2 Deformation

There have been a number of detailed reviews of foraminiferal deformities and their probable reasons (Boltovskoy et al., 1991; Alve, 1995; Yanko et al., 1998). However, there are certain parameters that are measured much more frequently than others and as a result their importance may have been overemphasized in the available literature.

It is likely that changes in test morphology are controlled by a combination of factors (Debenay et al., 2000). Based on a substantial literature review, Boltovskoy et al. (1991) indicated that morphological test variations in benthic foraminifera might be related to several environmental variables (e.g. temperature, salinity, carbonate solubility, depth, nutrition, substratum, dissolved oxygen, illumination, pollution, water motion, trace elements, and rapid environmental fluctuation), suggesting that a combination of these factors probably determine test morphologies. Deformities that have quite different appearances may also have the same underlying physiological cause (Yanko et al., 2002). Possible causes of abnormality, both natural and anthropogenic, are discussed further below.

5.1.3 Natural causes of deformation

Abnormal shapes, sizes or disposition of chamber(s) are not always induced by environmental stress or contamination effects (Alve, 1991). In coastal environments test deformations may have natural as well as anthropogenic origins (Le Cadre et al., 2003) and most papers published before 1980 relate abnormalities to natural environmental stress.

Natural environmental stresses including extreme environmental conditions (Almogi-Labin et al., 1992) or those arising from very rapid changes in ecological conditions

(Debenay et al., 2000) which can result in high proportions of abnormal tests. The process of decalcification under acidic conditions and recalcification under favourable conditions can also take place in natural environments (Geslin et al., 2002).In a non-polluted estuary, the highest percentage of abnormal tests was caused by decalcification/recalcification processes resulting from natural periodical acidification. In this particular example, higher percentages of abnormal tests occurred in non-polluted areas than in polluted areas (Geslin et al., 2002).

5.1.4 Pollution

In recent literature, test abnormalities are considered to result from various anthropogenic activities (review by Yanko et al., 1999). Most papers relating abnormalities to pollution have been published since 1980. High frequencies of deformed tests of living benthic foraminifera are generally regarded as powerful *in situ* bioindicators of heavy metal pollution (Debenay et al., 2000; Yanko et al., 2002; Le Cadre et al., 2003). For example, abnormal wall structures and test deformation were recorded in *Ammonia* spp. due to heavy metal contamination. Frequency of deformity also increased in other benthic foraminifera in sites polluted by toxic metals or aromatic hydrocarbons (Bresler & Yanko-Hombach, 2000). The tremendous taxonomic diversity of foraminifera give them the potential for diverse biological responses to various pollutants, which in turn adds to their potential as index species for monitoring pollution from diverse sources (Yanko et al., 2002).

It is important to highlight that the proportion of abnormal tests is sometimes very low and cannot be considered as significantly higher than in most natural non-polluted environments (Geslin et al., 2000). This observation highlights the importance of determining the limit above which the percentage of deformed specimens is significant (Geslin et al., 2002).

Environmental factors (unrelated to pollution) can also produce morphologic abnormalities.

5.1.5 Salinity

Environmental stress such as extremes in salinity (hyposalinity, hypersalinity, or variations in salinity) can also cause morphological abnormality in foraminiferal tests (Geslin et al., 2000, 2002; Debenay et al., 2000). Debenay et al. (2001) report a high percentage of aberrant tests from a lagoon that has been hypersaline since at least the 16th century and conclude that the aberrations were associated with high and variable salinity. Strong salinity variations seem to perturb the test construction of foraminifera (Geslin et al., 2002).

Increased frequencies of species-specific deformity have also been linked to low salinity, e.g. *Adelosina cliarensis* (Yanko et al., 2002). High numbers of abnormal tests of *Ammonia beccarii tepida* have been related to stress conditions resulting from high temperatures, salinities and peculiar ion composition (Almogi-Labin et al., 1992). Double test formation in some species has also been attributed to high salinity conditions and changes in salinity (Stouff et al., 1999a).

5.1.6 pH

There is no doubt that a relationship exists between pH and the physiology of most marine organisms, including foraminifera (Boltovskoy & Wright, 1976). For foraminifera that live in changing pH conditions, test deformation may also be induced (Le Cadre et al., 2003).

Morphological abnormalities have also been linked to pH in cultures of *Ammonia beccarii* (Le Cadre et al., 2003). Upon reintroduction to normal pH seawater (following partial decalcification at lower pH) foraminiferal specimens recalcified in a

different way to their original calcification state, and this was accompanied in many case by morphological abnormalities (e.g. abnormal expansions, irregular chamber sizes, wall with concave form) (Le Cadre et al., 2003). The mechanisms of decalcification-recalcification studied in cultures of *A. beccarii* may occur in coastal environments as these environments are subjected to natural pH variations such as daily changes due to modifications in photosynthetic activity or seasonal changes resulting from variable inputs of low pH freshwater (Le Cadre et al., 2003). For example, Geslin (1999) reported high percentages of abnormalities in an estuary as a consequence of recalcification after decalcification caused by temporary acidic (pH 4) conditions during the rainy season.

5.1.7 Damage

Severe abnormalities may also be brought about by mechanical trauma (Bé & Spero, 1981). Mechanical breaks can result from strong hydrodynamic forcing or from the action of predators (Geslin et al., 2000; Debenay et al., 2001). Magaeu & Walker (1976) noted gouges and scratches occurring frequently on tests ingested by polychaetes, crustaceans, gastropods, echinoderms, and other macroinvertebrates.

Following mechanical damage, subsequent regeneration of damaged tests can lead to morphological abnormality (Geslin et al., 2000, 2002; Debenay et al., 2001, and references therein). They are often easy to distinguish by SEM because they are often characterised by presence of scars, irregular contours of crushed or repaired chambers, or by the construction of new chambers in a coiling plane different from the original (Geslin et al., 2000). Detection of such damaged tests is important for environmental studies and should be systematically ignored when using test abnormalities as indicators of non-mechanical stress (Geslin et al., 2000).

5.1.8 Types of deformation

Foraminifera often show species-specific responses to ecological conditions (Fursenko, 1978). Deformities are therefore extremely variable and may be different from species to species in both calcareous and agglutinated foraminifera (Alve, 1991; Yanko et al., 1994). Within living assemblages, several different types of morphological abnormality have been observed according to the affected part of the test and the nature of deformation (Debenay et al., 2000). However, it is sometimes complicated to determine exactly what constitutes an abnormality because of the subjective determination of the limit between normal and abnormal forms (Geslin et al., 2000). Care must also be taken to distinguish between test abnormalities and intraspecific variation (Schafer, 2000).

Various workers have catalogued lists of a wide variety of morphological test abnormalities and a number of classifications have been proposed (Caralp, 1989; Alve, 1991; Sharifi et al., 1991; Almogi-Labin et al., 1992; Yanko et al., 1994, 1998; Geslin et al., 1998, 2000). From each of these classifications several different modes of abnormality have been recognised.

Firstly, reduced or abnormal chamber size (in one or more chambers) has been recognised as a mode of deformation. Very small deformation can be observed in tests where a chamber may be reduced or overdeveloped (Geslin et al., 2000). This mode of deformation is different to deformities caused by reduced feeding activity (Murray, 1963), reduced growth in cold season (Cedhagen, 1991) or salinity stress (Stouff et al., 1999b), as in these cases the shape of the chambers is abnormal but the surface of the walls has a normal aspect (Le Cadre et al., 2003). A reduced or overdeveloped chamber may correspond to momentary changes in local environmental conditions as the construction of a new chamber needs only about an

hour (Geslin et al., 2000). Small abnormalities may therefore be used to detect short-term environmental impacts and should not be considered as characterising strong widespread environmental stress (Geslin et al., 2000).

Abnormal or aberrant chamber shapes may be seen in addition to abnormal chamber sizes. During the regeneration process the inner organic lining acts as a template and is often folded or irregular resulting in the irregular shape of the new wall and deformation of the recalcified test (Le Cadre et al., 2003).

An additional mode of abnormality includes a change in coiling or distorted chamber arrangements. This can include abnormal arrangement of the first chambers (Geslin et al., 2000) and modification of the coiling plane of the first chamber (Stouff et al., 1999b). Deformities following repair and overgrowth by subsequent whorls can result in distortion of normal shell geometry, such as twisted keels and distortion of the equatorial plane (Yanko et al., 2002). Following laboratory experiments, Saraswat et al. (2011) observed abnormalities that included exceptionally large or small chambers and the addition of chambers in a plane other than the normal plane of growth with altered salinity.

Specimens may also exhibit abnormal additional and/or abnormal protruding chambers (Geslin et al., 2000). An abnormally protruding proloculus or protruding chamber originates from a perturbation during ontogenetic development, similar to those leading to the formation of some double tests (Stouff et al., 1999a).

Complex abnormalities can also exist in the form of double, siamese or triple tests. These forms probably result from the fusion or attachment of two or more tests of embryos or juveniles and leads to the development of complex abnormal forms (Geslin et al., 2000). The attachment may occur in different planes either by the

sides of the two specimens, or between one umbilical and one spiral side, or even between two spiral sides (Almogi-Labin et al., 1992).

Stouff et al. (1999a) were the first to report detailed observations of embryonic and juvenile stages of benthic foraminifera with abnormalities and enabled a better understanding of the formation of double or multiple tests. Environmental conditions seem to influence the formation of double tests. They probably act at the cellular level by slowing down some metabolic processes essential to a normal ontogenetic development (Stouff et al., 1999a). Stouff et al. (1999a, b) showed that high proportions of double tests can arise from hypersalinity. Multiple apertures or twinning may also be attributable to differing responses to different toxins (Yanko et al., 2002).

Several types of abnormality may occur on the same test resulting in extremely complex forms where the perturbation cannot be precisely determined. Some abnormalities are so severe that it is not only impossible to determine which part of the test is affected but also impossible to determine the genus of the organism (Geslin et al., 2000). Classification of deformity rely on the kind, degree, frequency, and species-specificity of deformity, but the frequency is the easiest measure to quantify (Yanko et al., 2002).

5.1.9 Culturing

Foraminiferal culturing is an essential tool for understanding deformities since morphological abnormalities can result from a combination of factors, many of which co-occur in most natural environments. Given that there is no general consensus as to the underlying cause of most deformities, controlled laboratory experiments allow the study of possible interactions between species and environmental variables.

Such experiments should help us to resolve the mechanisms that control test deformity as a morphological reaction to key environmental stressors. In this study, the appearance, frequency and mode of foraminiferal deformation and damage is documented following long-term exposure to an experimental system that simulates conditions of ocean acidification.

5.2 Materials and Methods

5.2.1. Experimental regime and sampling

This chapter is based on the study of 135 experimental surface sediment samples collected during the long-term culturing experiment. Details of the experimental setup, sampling regime and environmental data are provided in Chapter 2.2. Samples were taken from systems exposed to three CO_2 concentrations (380, 750, 1000 ppm) and two temperature regimes (constant or ambient) Eight time points were sampled (weeks 0, 4, 8, 30, 36, 50 and 64). For each sample interval, three replicate cores were processed.

5.2.2. Foraminiferal extraction and categorisation

Foraminifera were extracted from processed samples using methods described in Chapter 2.2. For each sample, at least 300 specimens were separated and counted for faunal analysis.

Live and dead, normal, abnormal and broken specimens were identified. Specimens were categorised as LN (living normal), LA (living abnormal) or LD (living damaged). All tests were counted to determine the percentage (relative abundance) of both abnormal and damaged tests. All quantitative analyses were conducted using relative abundance data. Only living specimens were taken into account when evaluating the possible correlation between ocean acidification and the percentage of morphological abnormalities.

5.2.3 Scanning electron microscopy

Once categorised, all modes of shell abnormality were imaged using scanning electron microscopy (SEM). All specimens were prepared for SEM and imaged following the protocol outlined in Chapter 2.3.

5.2.4. Statistical analyses

A basic linear regression model (containing all explanatory variables and their interactions) was applied to each of the foraminiferal measures (abnormal and damaged specimens). Data was assessed for normality (Q-Q plots) and homogeneity of variance by plotting the standardised residuals versus fitted values and the standardised residuals versus each individual explanatory variable (Zurr et al., 2007; Zurr et al., 2009). To avoid data transformations and in order to account for heterogeneity of variance, a generalised least squares (GLS) mixed modelling approach was adopted (West et al., 2007; Zurr et al., 2007; Zurr et al., 2009). The most appropriate variance-covariance structure for each model was determined using AIC scores and the examination of plots of fitted values versus residuals based on a full model specification using restricted maximum likelihood (REML, West et al., 2007). Once the optimal random component structure was found, the optimal fixed component structure was determined. This was established through a manual backwards stepwise selection approach where explanatory variables that were not significant were dropped using maximum likelihood (ML) estimation. The influence of each independent term was assessed using a likelihood ratio test between the minimum adequate model and reduced models, with all terms involving the relevant independent factor removed (including interactions). All analyses were performed using the statistical programming software ‘R’ (R Development Core Team, 2012), and the additional ‘nlme’ (linear and non-linear mixed effects models package (version 3.1-110, Pinheiro et al., 2012).

5.3 Results

A total of 41,060 individual foraminiferal specimens were picked and recorded. Throughout the sampling period, 4890 individuals were recorded as living at the time of sampling. Of these, 183 and 596 specimens showed signs of abnormality and damage, respectively. The percentages of abnormal and damaged specimens found in the living populations of each sample are displayed in Table 5.1.

5.3.1 Deformed specimens

A total of six modes of abnormality were observed (Fig. 5.1):

1 – Distorted chamber arrangement or change in coiling direction

2 – Stunted shell or reduction in size of at least one chamber

3 – Twisted shell

4 – Abnormally protruding or enlarged chamber (s)

5 – Abnormal additional chamber (s)

6 – Double tests with normal shaped chamber (s)

In some cases, a number of morphological abnormalities co-occurred in the same specimen. For example, a number of specimens had a twisted or distorted chamber arrangement in addition to either an aberrant or reduced sized chamber.

Test morphological abnormalities were observed over all time periods but did not occur under every treatment condition (Table 5.2). The most common modes of abnormality observed were stunted shell or reduction in the size of at least one chamber, twisted tests, and abnormally protruding or enlarged chambers.

Table 5.1 Percentages of Total Living (TL), Living Abnormal (LA), and Living Damaged (LD) benthic foraminifera for each sample (values represent mean ± SD, n = 3). Living Abnormal and Living Damaged values were calculated from live populations only. CO_2 concentrations are 380 ppm, 750 ppm or 1000 ppm. Temperature regimes were represented as 10°C (constant regime) or Amb (seasonally varying ambient regime).

Sample		380 ppm			750 ppm			1000 ppm		
Week	**Temp**	**TL (%)**	**LA (%)**	**LD (%)**	**TL (%)**	**LA (%)**	**LD (%)**	**TL (%)**	**LA (%)**	**LD (%)**
0	**10°C**	3 ± 0.6	9 ± 15.7	21 ± 9.4	5 ± 0.6	4 ± 3.6	15 ± 6.7	7 ± 1.2	0	7 ± 9.5
4	**10°C**	14 ± 5.7	3 ± 3.0	5 ± 5.2	11 ± 3.5	0	11 ± 6.4	15 ± 1.8	4 ± 2.5	16 ± 5.5
4	**Amb**	15 ± 3.0	1 ± 1.6	13 ± 7.0	20 ± 4.3	5 ± 2.6	7 ± 2.8	20 ± 6.5	4 ± 3.4	9 ± 4.7
8	**10°C**	16 ± 3.4	4 ± 1.1	4 ± 3.6	21 ± 8.9	0	7 ± 4.1	21 ± 9.8	4 ± 1.0	8 ± 1.9
8	**Amb**	9 ± 3.0	2 ±3.2	5 ± 4.2	20 ±7.0	2 ± 0.8	14 ± 7.9	10 ± 1.8	2 ± 2.2	3 ± 3.1
24	**10°C**	23 ± 8.4	4 ± 2.2	9 ± 4.1	16 ± 5.8	2 ± 3.7	12 ± 3.6	10 ± 6.7	6 ± 6.6	12 ± 6.3
24	**Amb**	19 ± 7.6	2 ± 2.3	12 ± 0.6	19 ± 4.0	4 ± 2.0	8 ± 3.1	15 ± 6.1	5 ± 4.0	14 ± 4.3
30	**10°C**	18 ± 10.3	1 ± 2.0	11 ± 6.0	18 ± 3.7	2 ± 2.1	13 ± 9.8	10 ± 1.2	2 ± 3.4	15 ± 7.4
30	**Amb**	5 ± 3.3	0	13 ± 5.7	7 ± 2.1	5 ± 1.3	31 ± 1.1	8 ± 2.3	2 ± 2.2	21 ± 14.4
36	**10°C**	10 ± 4.0	3 ± 3.0	11 ± 6.2	9 ± 2.7	6 ± 1.3	16 ± 15.2	1 ± 0.6	3 ± 5.2	7 ± 11.5
36	**Amb**	5 ± 2.1	6 ± 6.3	7 ± 7.2	5 ± 2.9	5 ± 8.2	16 ± 1.4	6 ± 3.8	0	25 ± 13.6
50	**10°C**	17 ± 6.1	4 ± 1.2	10 ± 4.6	21 ± 7.0	1 ± 1.3	10 ± 4.0	6 ± 4.3	6 ± 6.8	7 ± 9.7
50	**Amb**	7 ± 1.3	2 ± 3.2	11 ± 5.1	7 ± 1.1	3 ± 4.8	22 ± 13.0	6 ± 1.7	20 ± 8.1	25 ± 5.8
64	**10°C**	14 ± 3.7	5 ± 2.2	15 ± 8.4	15 ± 3.7	10 ± 6.6	17 ± 10.2	5 ± 2.0	4 ± 6.8	27 ± 9.4
64	**Amb**	9 ± 3.6	8 ± 4.2	17 ± 9.5	9 ± 2.6	11 ± 5.0	18 ± 2.1	10 ± 1.7	9 ± 2.9	27 ± 7.0

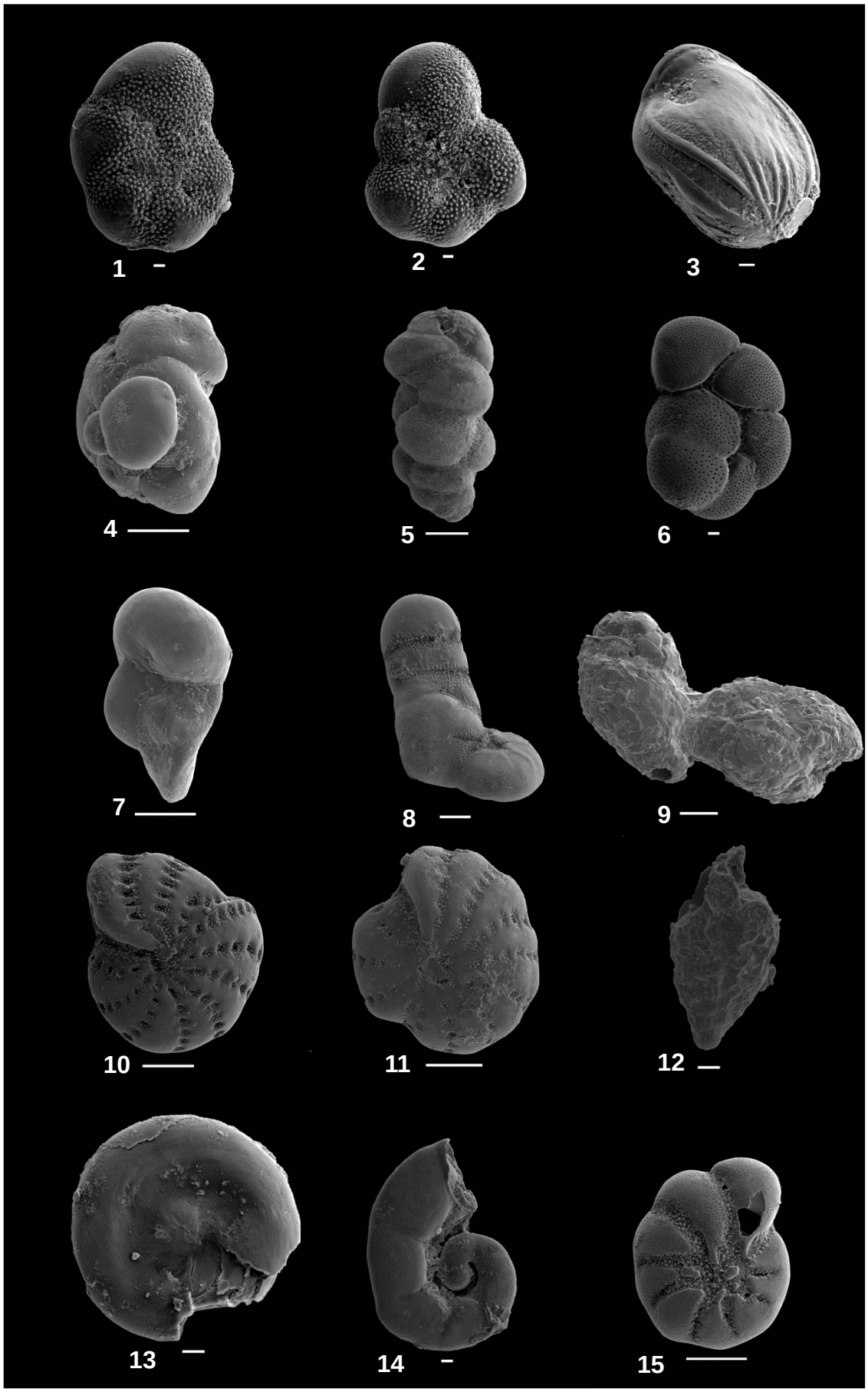
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Figure 5.1 Examples of morphological abnormality (1 - 11) and damage (12 - 15) in foraminiferal specimens as described in Section 5.3.1.

1. *Elphidium albiumbilicatum* with normal chamber arrangement (scale bar = 10 μm).

2. *Elphidium albiumbilicatum* with distorted chamber arrangement (scale bar = 10 μm).

3. *Fissurina* sp. with twisted chamber arrangement (scale bar = 10 μm).

4. *Quinqueloculina* sp. with distorted chamber arrangement (scale bar = 100 μm).

5. Unknown species with twisted chamber arrangement (scale bar = 100 μm).

6. *Ammonia* sp. with additional chamber (scale bar = 10 μm).

7. *Quinqueloculina* sp. with a protruding chamber (scale bar = 100 μm).

8. *Haynesina germanica* with an uncoiled chamber arrangement (scale bar = 100 μm).

9. Two agglutinated foraminiferal specimens with a fused test (scale bar = 100 μm).

10. *Elphidium williamsoni* with one stunted chamber (scale bar = 100 μm).

11. *Elphidium williamsoni* with numerous stunted chambers (scale bar = 100 μm).

12. *Bolivina pseudoplicata* with two broken chambers (scale bar = 10 μm).

13. *Cyclogyra* sp. with a broken section of numerous chambers (scale bar = 10 μm).

14. *Lamarckina haliotedea* with broken final chamber (scale bar = 10 μm).

15. *Elphidium excavatum* with damaged final chamber (scale bar = 100 μm).

Table 5.2 Different modes of abnormality in test morphology observed in living assemblages (1=distorted chamber arrangement, 2= stunted shell or reduction in size of at least one chamber, 3= twisted shell, 4= abnormally protruding or enlarged chamber, 5= additional chamber, 6= double test). • indicates at least one foraminiferal test per sample displayed mode of abnormality.

	380 ppm						**750 ppm**						**1000 ppm**					
Abnormality	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
Week 0 (10°C)	•	•						•		•								
Week 4 (10°C)		•		•										•	•	•		
Week 4 (Amb)		•						•		•				•	•	•		
Week 8 (10°C)		•	•	•										•	•	•		
Week 8 (Amb)		•						•						•				
Week 24 (10°C)	•	•		•				•			•			•	•			
Week 24 (Amb)	•	•	•	•				•		•				•	•		•	
Week 30 (10°C)	•		•						•		•			•		•		
Week 30 (Amb)									•		•					•		
Week 36 (10°C)	•		•					•	•	•				•				
Week 36 (Amb)		•						•										
Week 50 (10°C)		•	•	•			•	•		•					•			
Week 50 (Amb)			•						•					•	•	•	•	
Week 64 (10°C)		•	•				•	•	•	•		•	•					
Week 64 (Amb)	•	•	•				•	•	•					•	•			

Throughout the surveyed time intervals, fluctuations were observed in the percentages of abnormal specimens within the living populations (Fig. 5.2). The percentage of abnormal specimens varied between CO_2 treatments and temperature regimes

The optimal model with percentage of live abnormal specimens as the dependant variable consisted of two two-way interactions (CO_2 x Temperature: L-ratio = 10.849, d.f. = 2, $p < 0.005$) and (CO_2 x Week: L-ratio = 32.412, d.f. = 12, $p < 0.002$).

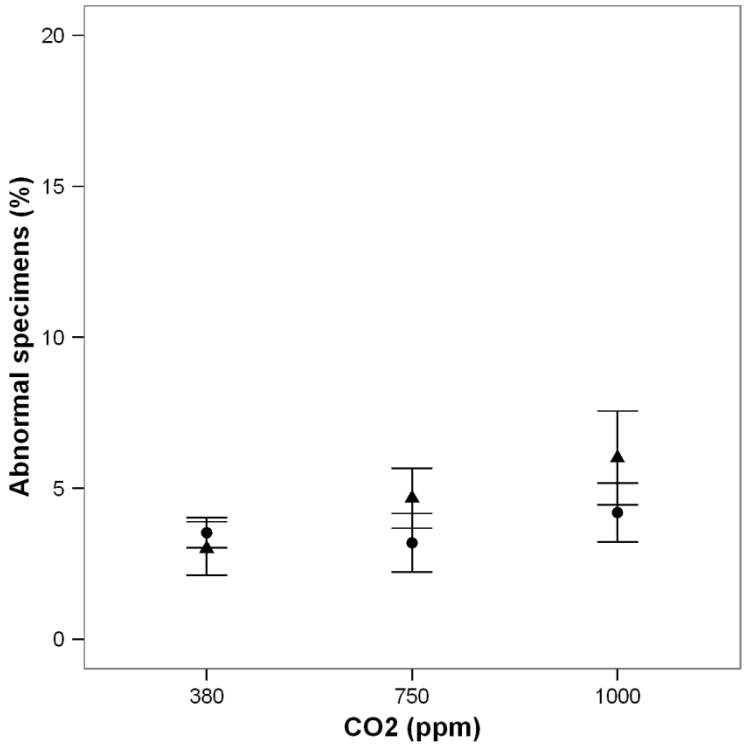

Figure 5.2 Model visualisation of the two-way interaction (CO_2 x Temperature) on the percentage of living abnormal specimens (y-axis) across the two temperature regimes for each CO_2 concentration (380, 750 & 1000 ppm). The visualisation shows the CO_2 concentration along the x-axis and at each concentration the different temperatures are represented by circles (constant temperature) and triangles (ambient temperature).

At 380 ppm CO_2 concentration, the highest percentage of living abnormal specimens were found under the constant temperature regime (Fig. 5.2). At higher CO_2concentrations (750 & 1000 ppm) the highest percentages of living abnormal specimens were found under the ambient temperature regime (Fig. 5.2).

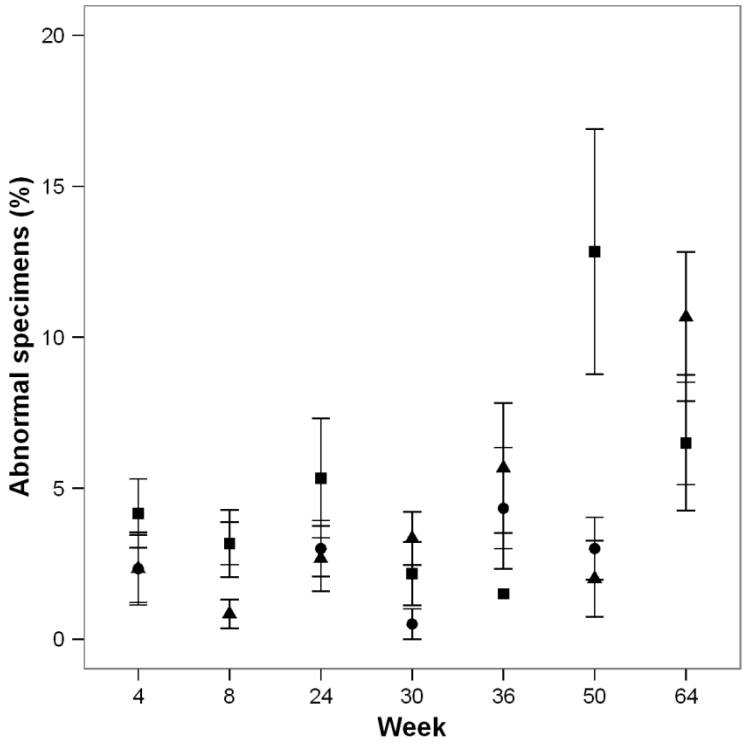

Figure 5.3 Model visualisation of the two-way interaction (CO_2 x Week) on the percentage of living abnormal specimens (y-axis) across the three CO_2 treatments for each time point (week 4, 8, 24, 30, 36, 50, 64). The visualisation shows the time point (week) along the x-axis. The different CO_2 treatments are represented by circles (380 ppm), triangles (750 ppm) and squares (1000 ppm).

The highest percentage of live abnormal specimens were found at the highest CO_2 concentration (1000 ppm) for weeks 4, 8 and 24 (Fig. 5.3). The percentage of living abnormal specimens at 1000 ppm then reduced over weeks 30 and 36, before increasing to its highest percentage at week 50 (Fig. 5.3).

5.3.2 Damaged specimens

The percentages of damaged foraminiferal tests across all CO_2 treatments and under each temperature regime are summarised in Table 5.1.

The optimal model with percentage of live damaged specimens as the dependant variable consisted of a three-way interaction (CO_2 x Temperature Regime x Week: L-ratio = 32.238, d.f. = 12, $p < 0.001$).

Under both the constant and ambient temperature there was an increase in the percentage of damaged specimens between weeks 0 and 64 for each CO_2 treatment (Fig. 5.4). Under the constant temperature regime, there were clear fluctuations in the percentages of damaged specimens under each CO_2 treatment with time (Fig. 5.4a). Although percentages fluctuate over time (Fig. 5.4a), the lowest percentage of damaged specimens were found at the lowest CO_2 treatment (380 ppm) at each time point (with the exception of weeks 36 and 50). After both 4 and 64 weeks of exposure, the highest percentages of damaged specimens were found under the highest CO_2 treatment (1000 ppm) (Fig. 5.4a).

Under the ambient temperature regime there were also fluctuations in percentages of damaged specimens under each CO_2 treatment with time (Fig. 5.4b). The fluctuations continue throughout the experimental exposure until week 30 when the lowest percentages of damaged specimens were found at the lowest CO_2 treatment (380 ppm) (Fig. 5.4b). At the highest CO_2 treatment (1000 ppm), the percentages of damaged specimens increase from week 8 and continue to do so until week 64. The highest percentages of damaged specimens from week 36 until the end of the experimental exposure were found under the highest CO_2 treatment (1000 ppm) (Fig. 5.4b).

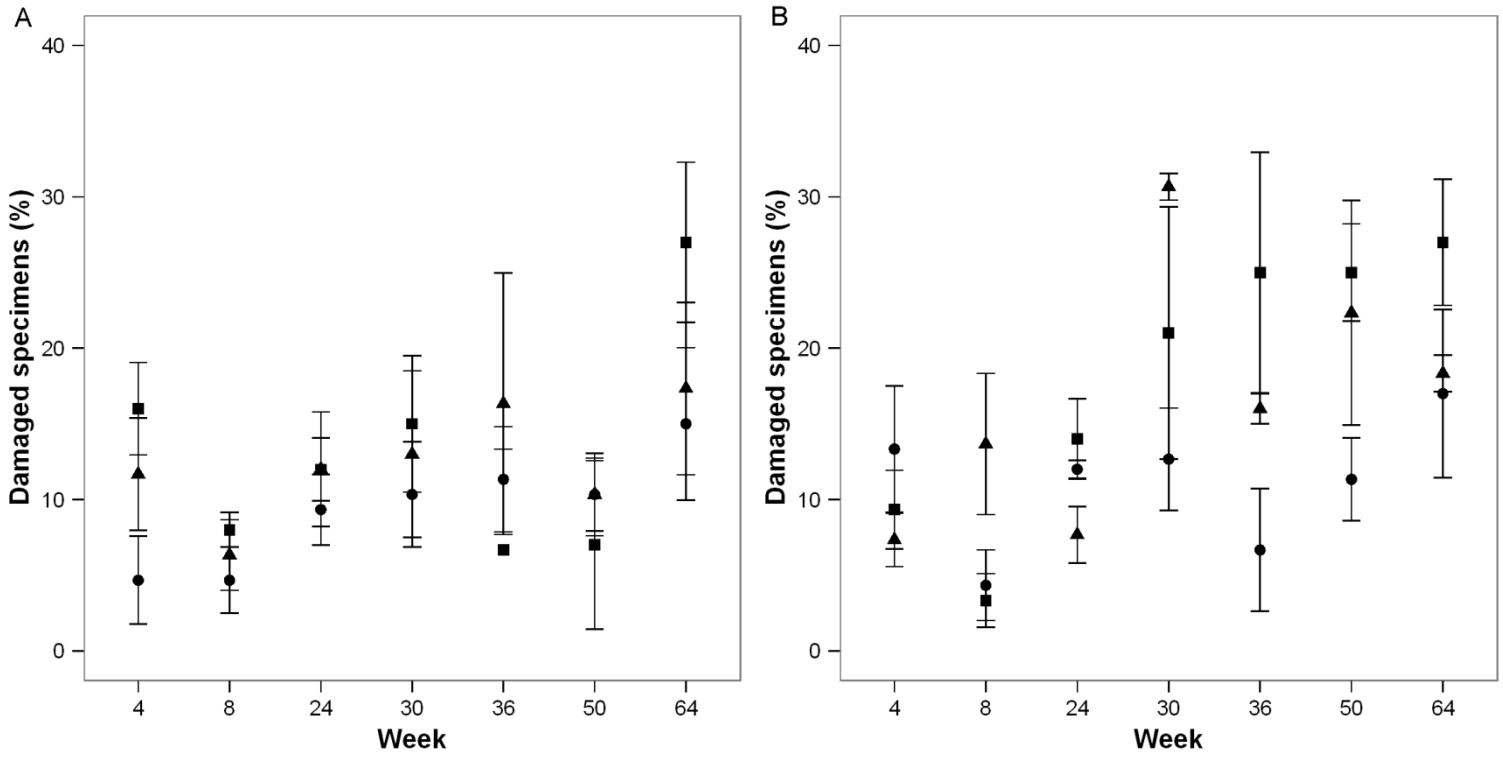

Figure 5.4 Model visualisation of three-way interaction (CO_2 x Temperature Regime x Week) on the percentage of live damaged specimens (y-axis) across each CO_2 treatment at each time point (week 4, 8, 24, 30, 36, 50, 64). **A)** Constant temperature regime, **B)** Ambient temperature regime. The visualisation shows time point along the x-axis. The different CO_2 treatments are represented by circles (380 ppm), triangles (750 ppm) and squares (1000 ppm).

5.3.3 Species-specific responses

Specimens that displayed either an abnormality or some form of damage were categorised based on their wall structure (Tables 5.3 & 5.4). Specimens were grouped into either calcareous hyaline, agglutinated or calcareous porcelaneous forms. Abnormalities and damage were found in specimens in each type of wall structure. The highest percentages of specimens that exhibited either an abnormality or some form of damage often had calcareous hyaline wall structures. The genera *Haynesina* and *Elphidium* were most prone to developing abnormalities. From the total number of abnormal specimens documented across the time series the above genera accounted for 34.4% and 24.6% of total deformation respectively. The highest percentages of abnormality were found in the species *Haynesina germanica*, *Elphidium williamsoni* and *Elphidium albiumbilicatum*. Most commonly damaged specimens also belonged to the genera *Haynesina and Elphidium*. They account for 39.2% and 17% of all damaged specimens, respectively. The most common form of damage in these species was partial damage to a chamber or complete destruction of one or more chambers.

The numbers of abnormal and damaged specimens of *Haynesina* and *Elphidium* were calculated as a proportion of the total live numbers of each genera to identify any potential species-specific sensitivities (Figs. 5.5 & 5.6). In the majority of treatments, higher proportions of abnormal and damaged specimens were observed in *Elphidium*. A qualitative assessment was made on how the proportions of abnormal and damaged specimens of each genera changed in relation to the total living populations (Figs. 5.5 - 5.7) but results were inconclusive.

Table 5.3 Percentages of abnormal Hyaline (H), Agglutinated (A), and Porcelaneous (P) benthic foraminifera for each sample (values represent mean ± SD, n = 3, ***** indicates n = 2). Percentages were calculated on live abnormal populations only (- indicates no abnormal specimens were present in any of the replicate samples). CO_2 concentrations are 380 ppm, 750 ppm or 1000 ppm. Temperature regimes were 10°C (constant regime) or Amb (seasonally varying ambient regime).

Sample		380 ppm			750 ppm			1000 ppm		
Week	**Temp**	**H (%)**	**A (%)**	**P (%)**	**H (%)**	**A (%)**	**P (%)**	**H (%)**	**A (%)**	**P (%)**
0	**10°C**	100.0 (n=1)	0	0	100.0 ± 0.0*	0	0	-	-	-
4	**10°C**	100.0 ± 0.0*	0	0	-	-	-	100.0 ± 0.0	0	0
4	**Amb**	100.0 (n=1)	0	0	100.0 ± 0.0	0	0	100.0 ± 0.0	0	0
8	**10°C**	50.0 ± 50.0	50.0 ± 50.0	0	-	-	-	100.0 ± 0.0	0	0
8	**Amb**	100.0 (n=1)	0	0	100.0 ± 0.0	0	0	100.0 ± 0.0*	0	0
24	**10°C**	100.0 ± 0.0	0	0	100.0 (n=1)	0	0	64.3 ± 50.5*	35.7 ± 50.5*	0
24	**Amb**	87.5 ± 17.7*	12.5 ± 17.7*	0	100.0 ± 0.0	0	0	55.6 ± 50.9	44.4 ± 50.9	0
30	**10°C**	66.7 (n=1)	33.3 (n=1)	0	100.0 ± 0.0*	0	0	100.0 (n=1)	0	0
30	**Amb**	-	-	-	66.7 ± 57.7	33.3 ± 57.7	0	100.0 ± 0.0*	0	0
36	**10°C**	100.0 ± 0.0*	0	0	83.3 ± 28.9	16.7 ± 28.9	0	100.0 (n=1)	0	0
36	**Amb**	100.0 ± 0.0*	0	0	100.0 (n=1)	0	0	-	-	-
50	**10°C**	44.4 ± 50.9	55.6 ± 50.9	0	100.0 ± 0.0*	0	0	12.5 ± 17.7*	87.5 ± 17.7*	0
50	**Amb**	100.0 (n=1)	0	0	0	100.0 (n=1)	0	27.8 ± 25.5	72.2 ± 25.5	0
64	**10°C**	91.7 ± 14.4	8.3 ± 14.4	0	53.3 ± 50.3	13.3 ± 23.1	33.3 ± 57.7	100.0 (n=1)	0	0
64	**Amb**	16.7 ± 28.9	72.2 ± 25.5	11.1 ± 19.2	72.2 ± 25.5	27.8 ± 25.5	0	75.0 ± 25.0	25.0± 25.0	0

Table 5.4 Percentages of damaged Hyaline (H), Agglutinated (A), and Porcelaneous (P) benthic foraminifera for each sample (values represent mean ± SD, n = 3, ***** indicates n = 2). Percentages were calculated on live damaged populations only.CO_2 concentrations are 380 ppm, 750 ppm or 1000 ppm. Temperature regimes were 10°C (constant regime) or Amb (seasonally varying ambient regime).

Sample		380 ppm			750 ppm			1000 ppm		
Week	**Temp**	**H (%)**	**A (%)**	**P (%)**	**H (%)**	**A (%)**	**P (%)**	**H (%)**	**A (%)**	**P (%)**
0	**10°C**	100 ± 0.0	0	0	89.9 ± 19.2	11. 1 ± 19.2	0	100 ± 0.0*	0	0
4	**10°C**	100 ± 0.0*	0	0	91.7 ± 14.4	8.3 ± 14.4	0	94.4 ± 9.6	5.6 ± 9.6	0
4	**Amb**	94.4 ± 9.6	5.6 ± 9.6	0	100 ± 0.0	0	0	83.3 ± 28.9	16.7 ± 28.9	0
8	**10°C**	66.7 ± 57.7	33.3 ± 57.7	0	83.3 ± 14.4	16.7 ± 14.4	0	79.6 ± 26.3	20.4 ± 26.3	0
8	**Amb**	100 ± 0.0*	0	0	100 ± 0.0	0	0	100 ± 0.0*	0	0
24	**10°C**	91.7 ± 14.4	5.6 ± 9.6	2.8 ± 4.8	83.3 ± 16.7	16.7 ± 16.7	0	47.6 ± 50.2	52.4 ± 50.2	0
24	**Amb**	100 ± 0.0	0	0	94.4 ± 9.6	5.6 ± 9.6	0	94.4 ± 9.6	0	5.6 ± 9.6
30	**10°C**	88.9 ± 19.2	11.1 ± 19.2	0	100 ± 0.0	0	0	88.9 ± 19.2	11.1 ± 19.2	0
30	**Amb**	66.7 ± 57.7	0	33.3 ± 57.7	89.6 ± 10.0	10.4 ± 10.0	0	85.9 ± 17.2	11.1 ± 19.2	3.0 ± 5.2
36	**10°C**	100 ± 0.0	0	0	50.0 ± 50.0	33.3 ± 57.7	16.7 ± 28.9	100 ± 0.0*	0	0
36	**Amb**	50.0 ± 70.7*	50 ± 70.7*	0	66.7 ± 28.9	33.3 ± 28.9	0	59.5 ± 36.7	40.5 ± 36.7	0
50	**10°C**	80.0 ± 34.6	20.0 ± 34.6	0	49.4 ± 32.1	4.8 ± 8.2	45.8 ± 40.2	25.0 ± 35.4*	75.0 ± 35.4*	0
50	**Amb**	47.2 ± 41.1	52.8 ± 41.1	0	63.5 ± 33.8	36.5 ± 33.8	0	73.3 ± 23.1	26.7 ± 23.1	0
64	**10°C**	65.1 ± 7.3	9.5 ± 8.2	25.4 ± 9.9	76.9 ± 4.0	0	23.1 ± 40.0	88.9 ± 19.2	0	11.1 ± 19.2
64	**Amb**	85.2 ± 17.0	3.7 ± 6.4	11.1 ± 19.2	84.1 ± 16.7	15.9 ± 16.7	0	73.2 ± 15.3	26.8 ± 15.3	0

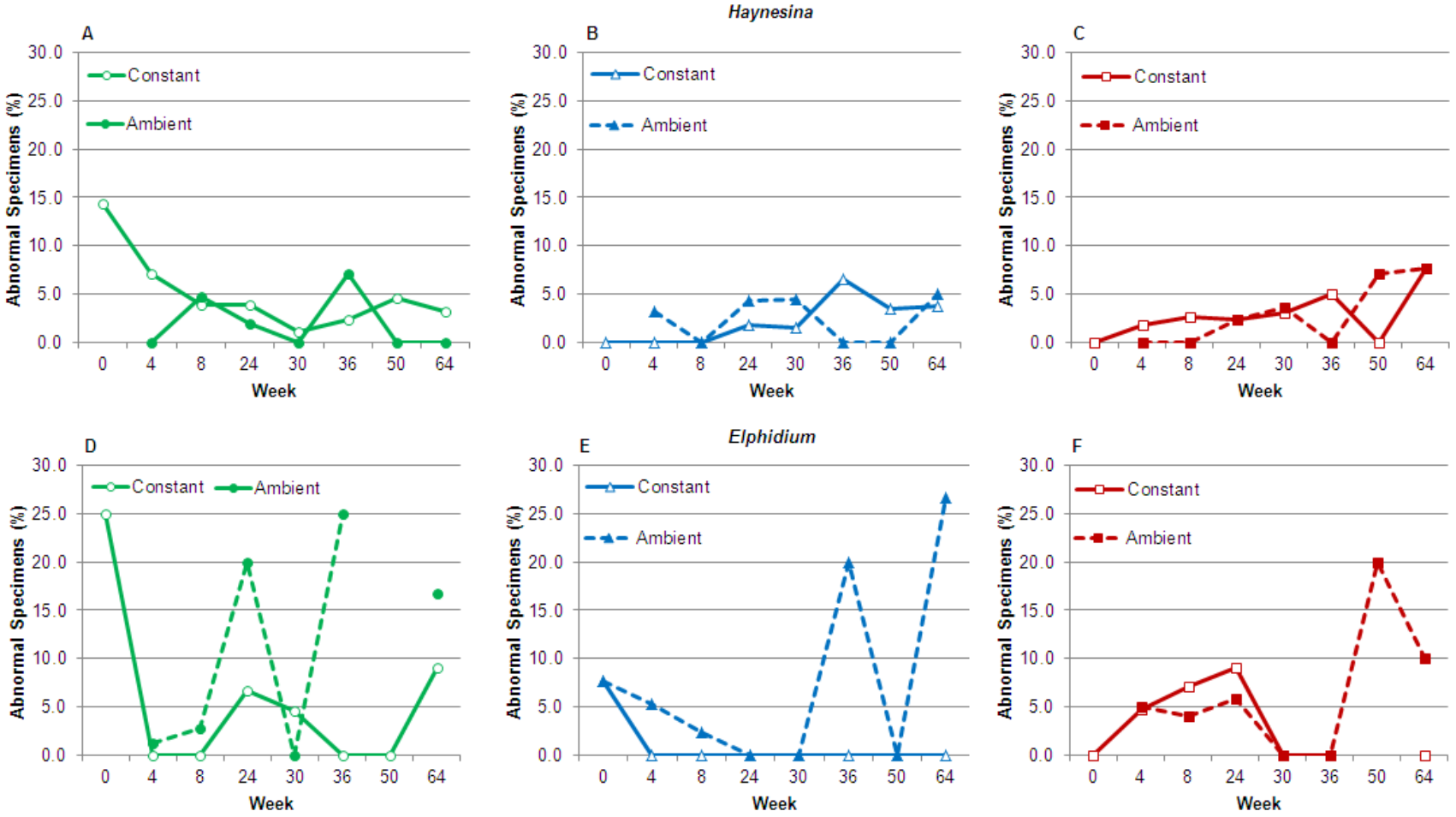

Figure 5.5 Proportions of abnormal specimens of the genus *Haynesina* (A-C) and *Elphidium* (D-F). Proportions of abnormal specimens were calculated from the total live number of each species per treatment. 0% indicates no live abnormal specimens were present in the population. Missing data points indicate zero live (normal or abnormal) individuals present. The CO_2 treatments are represented by green (380 ppm), blue (750 ppm) or red lines (1000 ppm).

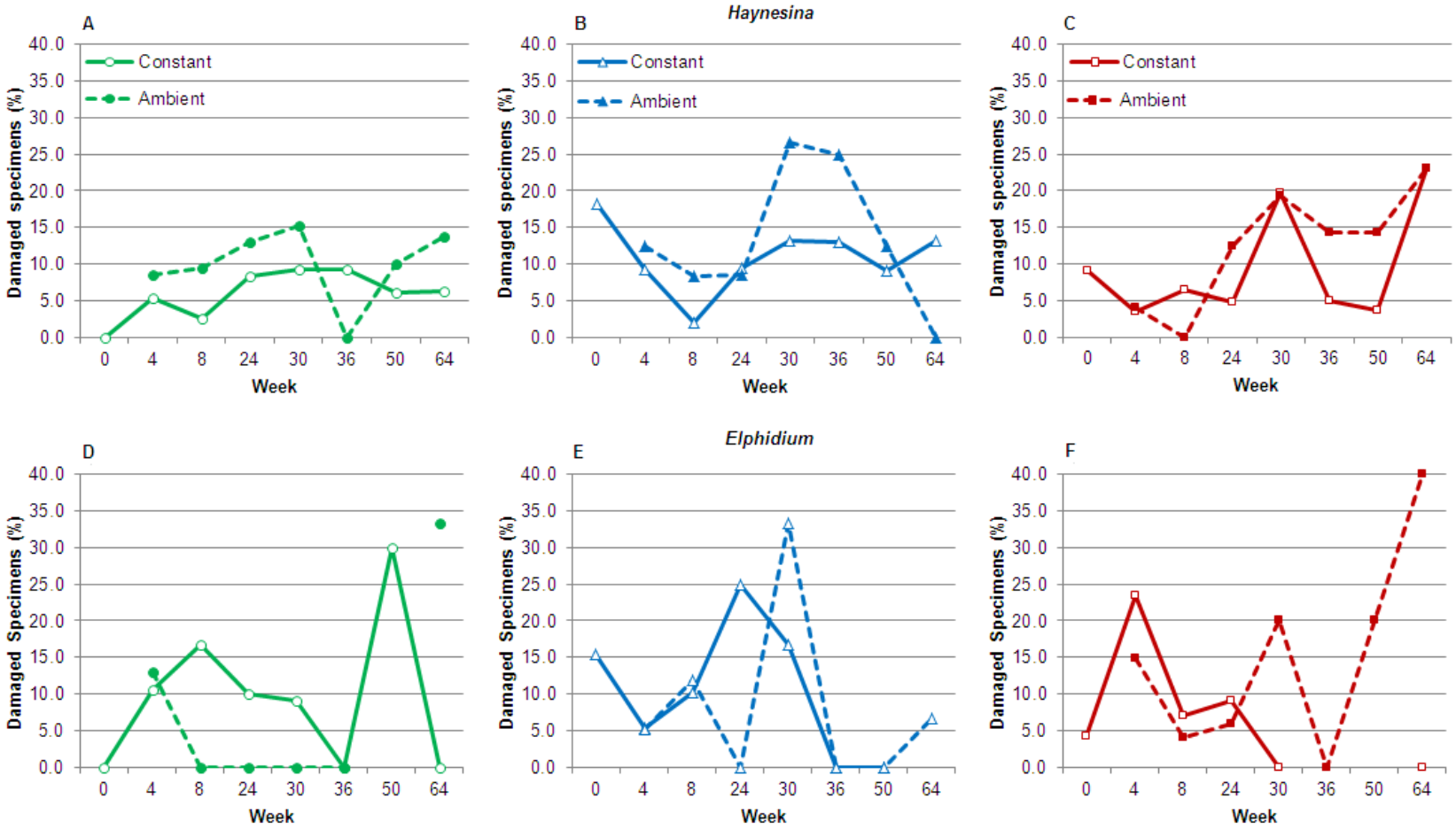

Figure 5.6 Proportions of damaged specimens of the genus *Haynesina* (A-C) and *Elphidium* (D-F). Proportions of damaged specimens were calculated from the total live number of each species per treatment. 0% indicates no live damaged specimens were present in the population. Missing data points indicate zero live (normal or damaged) individuals present. The CO_2 treatments are represented by green (380 ppm), blue (750 ppm) or red lines (1000 ppm).

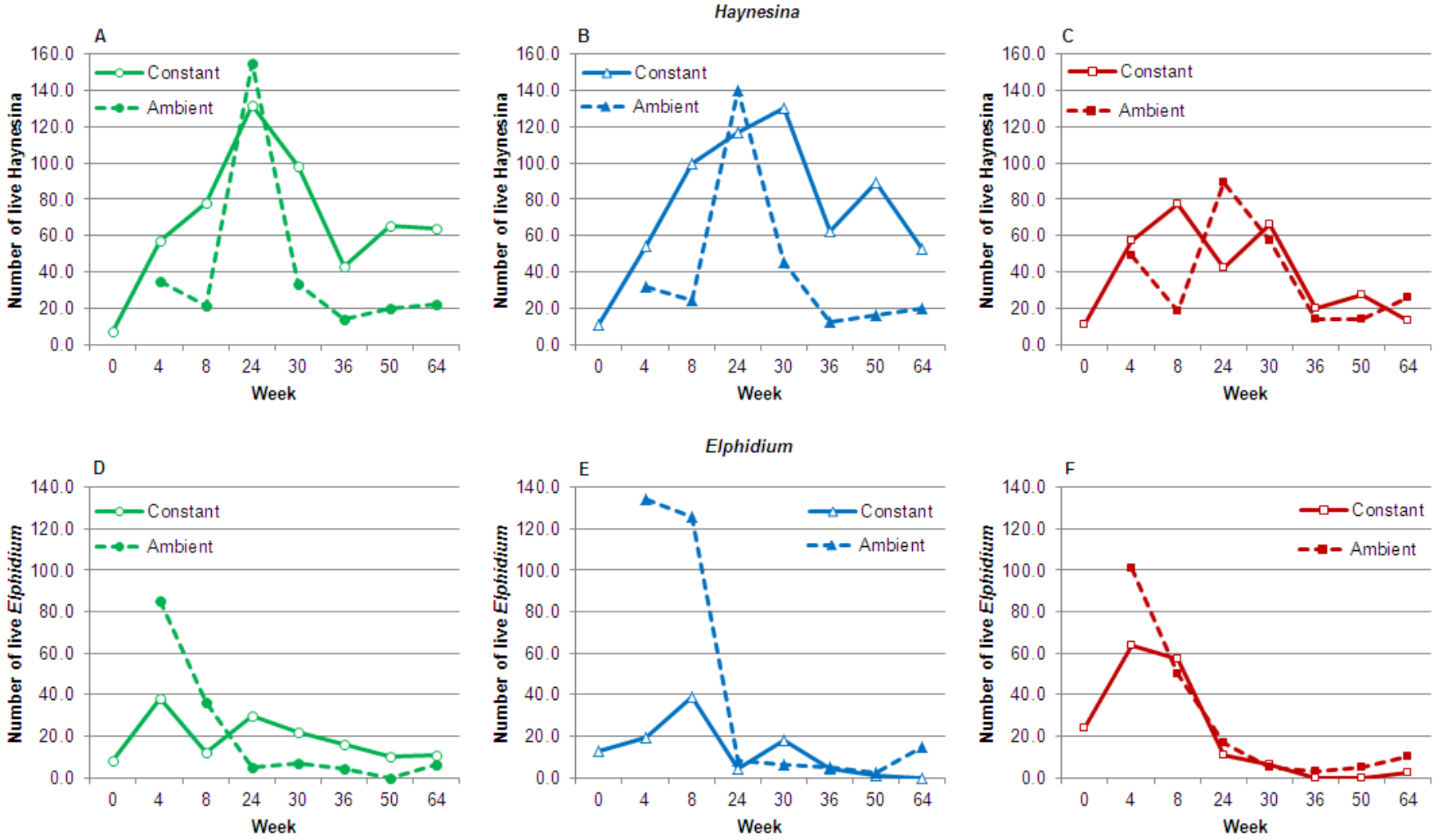

Figure 5.7 Number of live individuals of the genus *Haynesina* (A-C) and *Elphidium* (D-F). The CO_2 treatments are represented by green (380 ppm), blue (750 ppm) or red lines (1000 ppm).

5.4 Discussion

Before using morphological abnormalities of foraminiferal tests as general environmental indicators, a precise understanding of their responses to environmental conditions is necessary (Le Cadre et al., 2003).

5.4.1 Modes of abnormality

The modes of deformation catalogued throughout the time-series are similar to those previously described in the literature (Alve, 1991; Debenay et al., 2000; Geslin et al., 2000, 2002). Abnormalities were most commonly documented in calcareous specimens from the genera *Haynesina* and *Elphidium*. Some types of abnormality were observed more frequently than others. The most common modes of abnormality observed in the above genera were stunted shells or reduction in the size of at least one chamber. Adult tests with abnormal size or shape of chambers have previously been documented in natural environments (Geslin et al., 2000). Geslin et al. (2000) reported that this type of abnormality may result from variability of the natural environment and was typically rare in cultures. Abnormal chamber sizes have also been attributed to seasonal effects. For example, Myers (1943) recorded that the chambers of *Elphidium* added in the winter were usually smaller than those added in the summer, resulting in an uneven peripheral margin.

The least common type of abnormality were double or fused tests. Double tests are typically associated with hypersaline cultures (Stouff et al., 1999a). Only two double specimens were observed across the complete time-series, throughout which stable salinity conditions were maintained.

In some cases abnormalities can be so severe that it is almost impossible to determine which part of the test was affected and sometimes even the foraminiferal

taxon (Debenay et al., 2000). In the experimental samples it was always possible to distinguish between different species. It was sometimes however difficult to determine exactly which part of the test was affected.

5.4.2 Fluctuations in abnormality

It is difficult to differentiate between the impact of natural stress and the potential impacts of ocean acidification on foraminiferal abnormality. It has been inferred that deformation of hyaline tests in coastal environments frequently results from natural conditions (Le Cadre et al., 1999). Significant interactions were documented between"CO_2 x Temperature Regime" and "CO_2 x Week" with respect to the percentages of abnormal specimens. The percentages of abnormal specimens increased with length of exposure, with the highest percentages occurring in the calcareous hyaline forms and under increased CO_2 (1000 ppm). If the calcification process was challenged or compromised under increased CO_2 deformity should be more common. Despite these clear interactions it is important to note that percentages of abnormality fell within the natural range of variability seen at week 0 before the experiments began.

A low percentage of abnormalities cannot be considered as an indicator of 'abnormal' environmental conditions (Geslin et al., 2002). Alve et al. (1991) noted that the normal rate of abnormal tests in a non-stressed population is about 1%. Stouff et al. (1999b) also observed 1% abnormality in laboratory cultures under normal conditions. At maximum ~ 20% abnormality, the percentages from this time-series were found to be much higher than the proportions expected under 'normal' conditions and could therefore indicate a stressed environment.

5.4.3 Damage

The percentages of damaged specimens from the experimental period were significantly different from those observed prior to the experiment at Week 0. Since all samples were processed using the same methodology it is assumed that recorded damage occurred during life and growth under the experimental conditions. A three-way interaction between "CO_2 x Temperature x Week" was observed. The percentages of damaged tests increased with exposure to CO_2, with increased damage occurring at the highest CO_2 treatment (1000 ppm). With increasing dissolution, the thickness of the test decreases (Kucera, 2007). Following early stages of dissolution the tests in planktonic foraminifera become thinner before the more exposed parts become so weakened that the shells disintegrate into fragments (Kucera, 2007).

5.4.4 Species-specific responses

The majority of specimens that suffered from broken or damaged chambers were calcareous hyaline foraminifera. It is feasible that this is due to weakening of the calcareous shells under the experimental conditions. For example, in previous culturing experiments specimens using *Elphidium williamsoni* cultured at 7.6 pH, cells produced less dense and significantly thinner chamber walls that those cultured at pH 8.1 and 8.3 (Allison et al., 2010). Haynert et al. (2011) also found that young and small specimens of calcareous benthic foraminifera had thinner walls and fewer calcite lamellae and could therefore be more easily destroyed or damaged. Decalcification occurs first in the last chambers because the wall, made up of a smaller number of lamellae, was thinner (Le Cadre et al., 2003). This was demonstrated in our present work where the majority of damage and breaks occurred on the ultimate and/or penultimate chambers.

Porcelaneous foraminifera (Miliolida) construct their tests of high-Mg calcite. Incorporation of Mg^{2+} weakens the crystal structure, so that high-Mg calcite shells may be somewhat weaker than those of low-Mg calcite (Hallock, 2000b, and references therein). However, calcareous porcelaneous foraminifera had the lowest percentage of damaged specimens throughout the time-series. This is possibly as a result of these species being present in such low numbers and in the majority of samples the porcelaneous group were entirely absent.

Structural details and abnormality in agglutinated foraminifera are more difficult to discern as structural details can often be obscured. Agglutinated specimens were present throughout the time-series but revealed low percentages of abnormal and damaged specimens. In estuarine and intertidal environments, increasing proportions of agglutinated species may be related to decreasing pH (Debenay et al., 2000). Agglutinated tests consist of accumulated particles cemented together within an organic matrix (Anderson & Lee, 1991). Agglutinated tests may therefore be more resilient as they do not rely on $CaCO_3$ for test construction. This is reflected in the experimental data where agglutinated foraminifera have lower overall percentages of abnormal and damaged specimens.

An initial qualitative investigation into species-specific sensitivities within the calcareous hyaline group proved to be inconclusive. Initial results reveal that specimens from the genus *Elphidium* may be more sensitive than *Haynesina* as they revealed higher proportions of abnormal and damaged specimens across the majority of treatments. However, further investigation as to whether some species are more susceptible to ocean acidification is still required.

6. The effects of long-term exposure to ocean acidification on functional morphology of benthic foraminifera.

6.1 Introduction

The evolutionary and ecological success of shelled foraminifera is supported by the functional significance of the test (Austin et al., 2005; Murray, 2006). The test provides shelter and protection from predators, assistance in cell growth, aids in reproduction, acts as a receptacle for excreted matter, and provides buoyancy control (Marszalek et al., 1969; Murray, 1991b; Hallock et al., 1991).

Morphology is a basic adaptive characteristic since morphological adaptations are typically required to exploit a particular habitat and its resources (Hallock et al., 1991).One of the keys to the evolutionary success of foraminifera are the finely tuned relationships they have developed to exploit the food resources of their community (Lee, 1980). Morphological features are critical in this relationship particularly where food prey species, such as diatoms, have their own protective casing. Hamm et al. (2003) suggested that grazers of diatoms, such as foraminifera, are likely to have evolved specialised tools to break open diatom frustules.

Bernhard and Bowser (1999) documented the significance of foraminiferal test functional morphology in extant species known to sequester chloroplasts. They showed that external test ornamentations facilitate separation of chloroplasts from algal prey and describe a range of test ornamentations from relatively plain tests (e.g. *Nonionella* and *Stainforthia*) to highly ornamented forms (e.g. *Haynesina* and *Elphidium*). Bernhard and Bowser (1999) suggested that each of these species shared ornamentation that may function to break diatom frustules. Chloroplast

retention has been described in five families of foraminifera, almost all of which have sieve-like ornamentation (Correia & Lee, 2000). For example, tubercles and teeth in the apertures (Fig. 6.1) of some species are important in feeding and serve to break up aggregates of food and detritus (Bernhard & Bowser, 1999). Previous studies have shown at least eight species of the foraminiferal genera *Haynesina* and *Elphidium* grazing on diatoms and husbanding their chloroplasts (Pillet et al., 2011, and references therein). All species use surface transport as a principal means of collecting food (Travis & Bowser, 1991). As particles are moved past the tubercles, they are sorted by size, where larger fragments become disaggregated in preparation for later ingestion.

Elphidium have one of the most sculptured and elaborate of all the foraminiferal tests (Marszalek et al., 1969). The genus has no main aperture but has evolved a unique and very complex canal system that is connected to openings (fossae) guarded by denticals (Lee, 2011). The fossettes are studded with short spines or granules similar to those observed in the vicinity of the apertural face (Marszalek et al., 1969). The complicated canal systems and modified apertures and fossae are highly evolved to retain diatom plastids (Lee, 2011). Up to 100,000 chloroplasts have been recorded in one individual of *Elphidium williamsoni*, with the products of their photosynthetic activity providing a considerable energy source (Haynes, 1981). In addition, this ornamentation may serve to prevent entry of foreign material into the passageways of the central system (Marszalek et al., 1969).

Austin et al. (2005) showed a direct relationship between fracturing of diatom frustules and feeding sequestration by *Haynesina germanica* (Ehrenberg).Banner & Culver (1978) proposed that surface transport in *H. germanica* forces aggregates of detritus past sharp tubercles that line specific regions of the test. Alexander &

Banner (1984) further suggested that *H. germanica* could exploit the forces generated by surface transport which are of sufficient magnitude to break open diatom frustules against the tubercles and liberate chloroplasts for incorporation and ingestion. Symbiotic chloroplasts are photosynthetically active and could account for 10 - 20% of respiration in *H. germanica* (Alexander & Banner, 1984). To maintain a steady population of chloroplasts they must ingest more than 20 chloroplasts per individual per hour (Lopez, 1979).

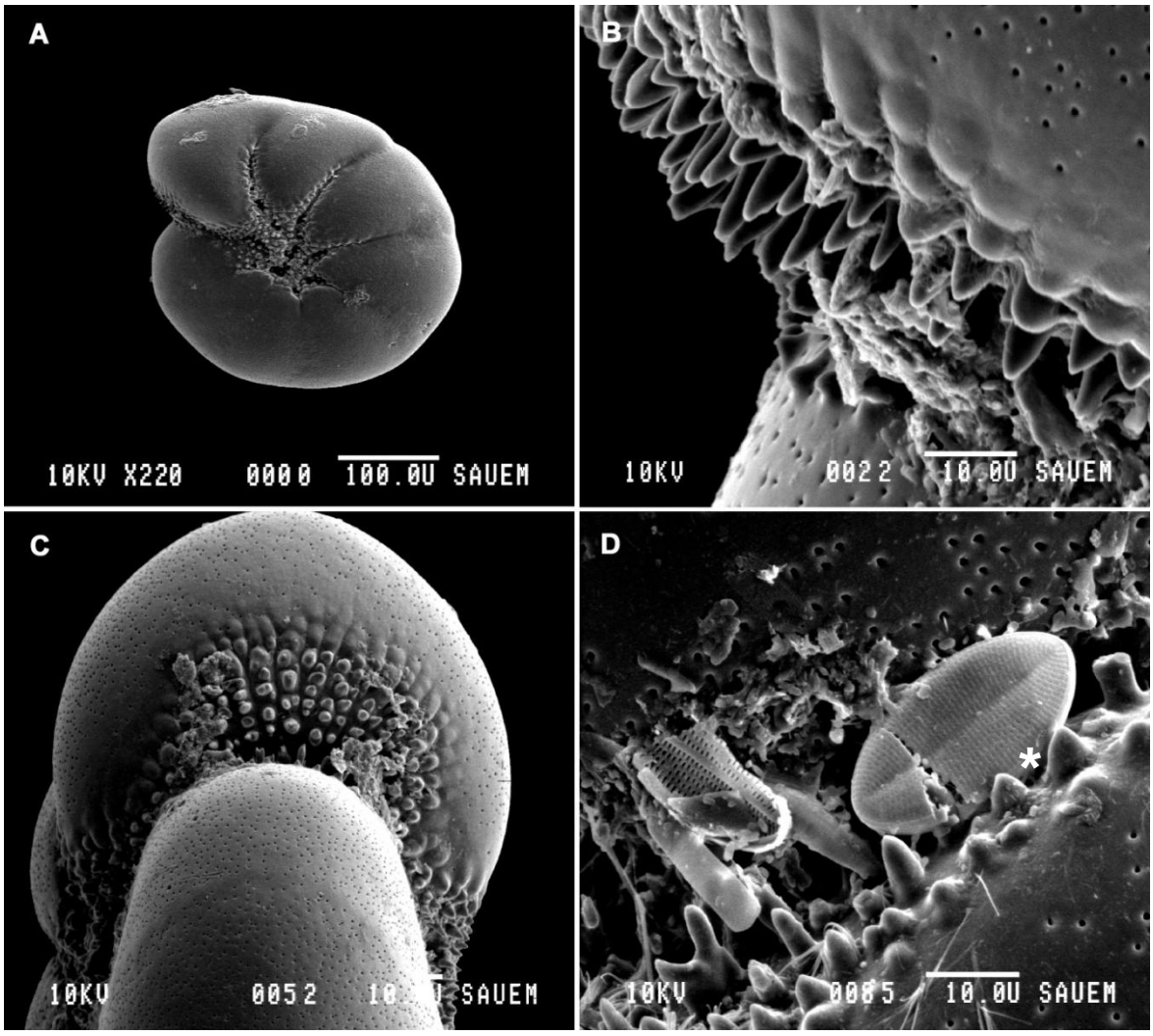

Figure 6.1 Scanning electron micrographs of *Haynesina germanica*. (A) Typical test, side view. (B) Higher magnification view of apertural region, showing tubercles and teeth lining the aperture. (C) Typical test, apertural view. (D) Higher magnification view of apertural region. Note impaled diatom (*).

Tubercles are protrusions that border the chamber margins while teeth are similar protrusions that are found adjacent to the aperture, occupying part of the apertural space (Loeblich & Tappan, 1988).Food particles are transported over the surface of the foraminifera (Fig. 6.2) to areas of high tubercle density (Banner & Culver, (1978). The particulate matter is then partially channelled (by ridges and linearly arranged tubercles) and partly sieved and disaggregated by the tubercles, prior to its selective ingestion (Banner & Culver, 1978).

It is believed that surface transport in feeding may have provided selection pressure for the evolution of certain test ornamentations (Travis & Bowser, 1991).Travis and Bowser (1991) observed live isolated foraminifera drawing a single diatom towards its aperture using pseudopodia. Diatoms were held with their pole towards the aperture and then turned so the valve face was orientated and held adjacent to the foraminiferal aperture (Travis and Bowser, 1991). Some species of *Haynesina* and *Elphidium* seem to preferentially retain chloroplasts from particular diatom clades while other species accept a wide range of diatoms as chloroplast donors (Pillet et al., 2011). Austin et al. (2005) documented a characteristic pattern of fracturing in *Pleurosigma angulatum* diatom frustules by *H. germanica* that was directly attributed to foraminiferal feeding and sequestration mechanism (Austin et al., 2005). In addition to serving a sieving function, the tooth-like projections allow the exclusion of the mineralised tests surrounding the prey (Anderson & Lee, 1991).

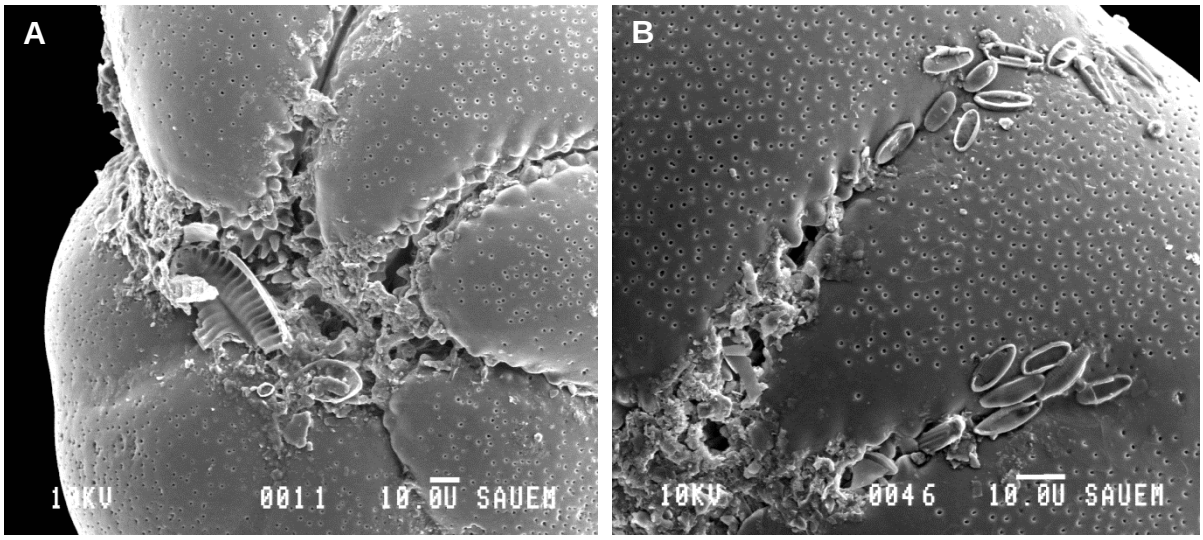

Figure 6.2 Scanning Electron Micrographs of *Haynesina germanica*. (A) Empty frustules of diatom prey around areas of high tubercle density. (B) Diatom transport across test surface.

To date, researchers have examined ocean acidification impacts on benthic foraminifera via test weight, thickness and growth rate (Le Cadre et al., 2003; Dissard et al., 2010; Haynert et al., 2011; Keul et al., 2013). Results from chapter five also reveal increased test damage following exposure to ocean acidification. However, there is currently no information on how foraminiferal test functional morphology might respond to ocean acidification.

Here we present ultrastructural observations using scanning electron microscopy of cultured *H. germanica* to examine the effect of different levels of atmospheric $CO_2$and temperature regime on test morphology, focussing on the apertural region known to have functional ornamentation (Austin et al., 2005).

6.2. Materials and Methods

6.2.1 Sediment collection

Sediment was collected from mesocosms maintained as part of the long-term time-series culturing experiment (Chapter 2.2). For this chapter, samples were taken from mesocosms maintained at either 10°C constant (average local conditions) or seasonally varying ambient temperature. Seawater pH was controlled by bubbling air with a known concentration of CO_2 into each mesocosm (Godbold & Solan, 2013). Throughout a 64 week exposure period, surface scrapes of 1 - 2 mm depth were collected from each of the treatments for the isolation of living benthic foraminifera. Sampling dates, regimes and methodology are provided in detail in Chapter 2.2. All seawater chemistry measurements for the experiment are summarised in Appendix I (Table S1). This chapter is based on surface sediment samples taken following 4, 24, 36 and 64 weeks exposure, to CO_2 treatments of 380 ppm or 1000 ppm (a small number of images were also taken of specimens cultured at CO_2 concentrations of 750 ppm after 36 weeks of exposure but these are not included in the statistical analyses).

6.2.2. Foraminiferal isolation and preparation

Mature specimens of *Haynesina germanica* (300 - 400 µm), were selected using a fine paintbrush under an Olympus® SZ stereo-microscope and dry-stored on micropalaeontological slides prior to imaging with a scanning electron microscope (SEM). At each time point sampled, 5 individuals were selected for each CO_2 treatment (380 ppm and 1000 ppm) under each temperature regime (constant and ambient). Only specimens living at the time of sampling were selected. The foraminiferal specimens were mounted onto SEM stubs using double-sided adhesive

tabs. Samples were prepared and imaged using the methodology outlined in Chapter 2.3. For each specimen, the number of teeth in a randomly selected 20 μm^2 region of the apertural area were counted and measured (SemAfore®) for maximum length and width (Table 6.1).

6.2.3 Statistical analyses

A basic linear regression model (containing all explanatory variables and their interactions) was applied to each of the foraminiferal teeth measures. Data was assessed for normality (Q-Q plots) and homogeneity of variance by plotting the standardised residuals versus fitted values and the standardised residuals versus each individual explanatory variable (Zurr et al., 2007, 2009). To avoid data transformations and in order to account for heterogeneity of variance, a generalised least squares (GLS) mixed modelling approach was adopted (West et al., 2007; Zurr et al., 2007; Zurr et al., 2009). The most appropriate variance-covariance structure for each model was determined using AIC scores and the examination of plots of fitted values versus residuals based on a full model specification using restricted maximum likelihood (REML, West et al., 2007). Once the optimal random component structure was found, the optimal fixed component structure was determined. This was established through a manual backwards stepwise selection approach where explanatory variables that were not significant were dropped using maximum likelihood (ML) estimation. The influence of each independent term was assessed using a likelihood ratio test between the minimum adequate model and reduced models, with all terms involving the relevant independent factor removed (including interactions). All analyses were performed using the statistical programming software ‘R’ (R Development Core Team, 2012), and the additional ‘nlme’ (linear and non-linear mixed effects models package (version 3.1-110,Pinheiro et al., 2012).

6.3 Results

SEM imaging of *Haynesina germanica* revealed distinct differences in surface ornamentation between specimens cultured in each of the CO_2 treatments (Fig.6.3).

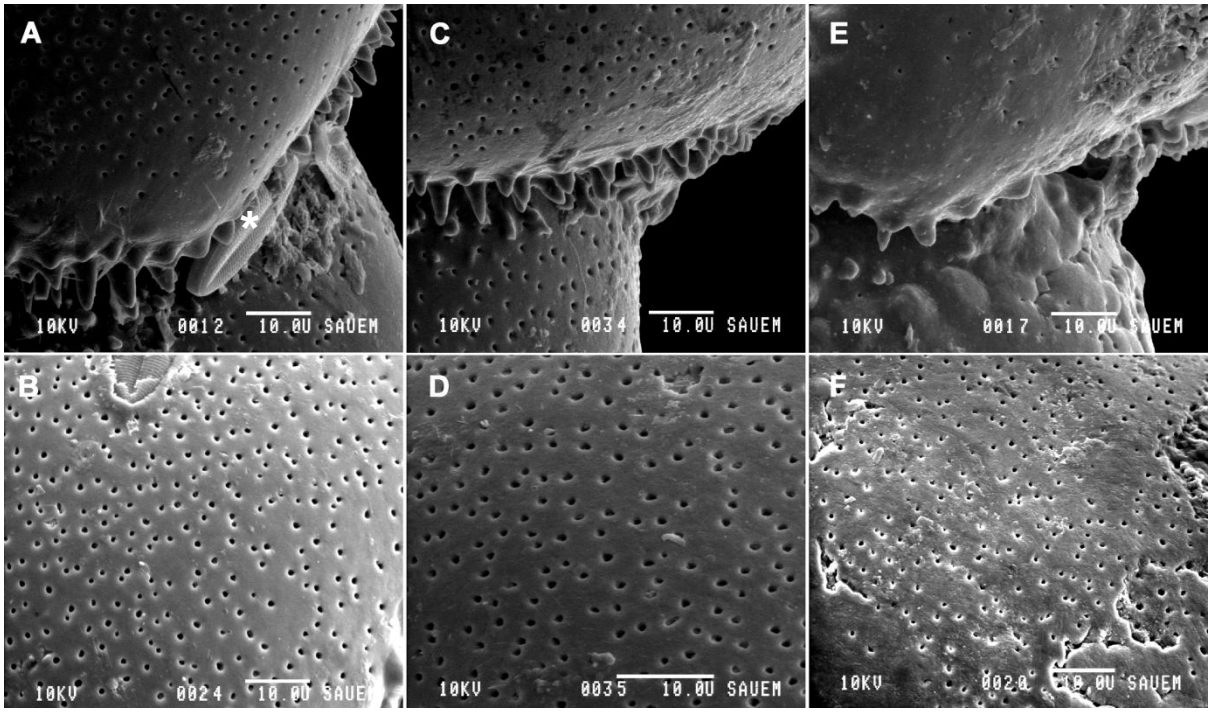

Figure 6.3 Scanning Electron Micrographs of *Haynesina germanica* following exposure to each of the CO_2 treatments. SEM images of specimens were taken at 380 ppm (A & B), 750 ppm (C & D) and 1000 ppm (E & F). (A) Side view of apertural region showing numerous sharp tubercles (380 ppm). Note diatom impaled on ornamentation (*). (B) Test surface of specimen A. (C) Side view of apertural region, showing tubercles and teeth (750 ppm). (D) Test surface of specimen C. (E) Side view of apertural region (1000 ppm). Note distinct absence of numerous conical tubercles present in A. (F) Scanning electron micrograph of test surface of specimen E. Note surface dissolution and cracking damage.

Table 6.1 Summary of main morphological features for each ocean acidification treatment.

Sample				Number of Teeth (mean ± SD)	Average Length of Teeth (mean ± SD)	Average Width of Teeth (mean ± SD)
Week	pCO_2	Temperature	n			
4	380	Constant	5	11.8 ± 3.6	5.1 ± 1.3	3.4 ± 0.8
4	380	Ambient	5	13.6 ± 4.5	6.5 ± 2.7	4.0 ± 1.4
4	1000	Constant	4	9.0 ± 6.6	5.7 ± 0.7	3.4 ± 0.2
4	1000	Ambient	5	9.8 ± 2.7	4.7 ± 0.8	3.4 ± 0.7
24	380	Constant	5	15.0 ± 2.9	6.5 ± 0.8	3.3 ± 0.4
24	380	Ambient	5	18.4 ± 5.3	6.2 ± 1.0	3.2 ± 0.2
24	1000	Constant	5	7.8 ± 2.4	5.9 ± 0.9	3.6 ± 0.5
24	1000	Ambient	5	8.6 ± 2.9	5.8 ± 0.7	3.2 ± 0.1
36	380	Constant	3	13.3 ± 3.2	5.2 ± 0.3	3.3 ± 0.5
36	380	Ambient	4	12.0 ± 1.4	6.2 ± 1.4	3.7 ± 0.6
36	1000	Constant	2	7.0 ± 1.4	3.0 ± 0.2	2.8 ± 0.1
36	1000	Ambient	5	7.8 ± 1.3	5.3 ± 1.2	3.4 ± 0.4
64	380	Constant	4	11.3 ± 2.6	6.3 ± 1.1	3.7 ± 0.7
64	380	Ambient	4	11.5 ± 2.4	5.2 ± 1.2	3.1 ± 0.5
64	1000	Constant	4	8.8 ± 3.6	4.1 ± 0.7	4.1 ± 0.6
64	1000	Ambient	5	6.6 ± 1.8	3.9 ± 1.0	2.6 ± 0.8

Control specimens of *H. germanica*, maintained at 380 ppm, possessed teeth that were both numerous and conical in shape (Fig. 6.3A). Several specimens also had diatoms impaled on teeth close to the apertural region. In addition, the test surface was smooth and unblemished (Fig. 6.3B).

At the highest CO_2 treatment (1000 ppm), there were marked signs of dissolution (Fig. 6.3) and deformation features on multiple individuals in comparison to those maintained at 380 ppm. Dissolution was evident on the test surface and in many cases the teeth were completely absent (Fig. 6.3E). In those specimens from the high CO_2 treatment (1000 ppm) that retained some ornamentation, the shape and appearance of the features was highly irregular. Teeth were less conical and more rounded in shape. In addition, the entire test surface appeared to be partially

dissolved with evidence of cracking (Fig. 6.3F), which was entirely absent from control specimens.

6.3.1 Number of teeth

Both CO_2 treatment and length of exposure had independent effects on the number of teeth present. CO_2 treatment had the most significant effect on the number of teeth (CO_2: L-ratio = 39.562, d.f. = 1, $p < 0.001$). At the highest CO_2 treatment (1000 ppm) the number of teeth was significantly reduced (Fig. 6.4). The length of experimental exposure also had a significant effect on the number of teeth present (Week: L-ratio = 7.913, d.f. = 3, $p < 0.05$). The number of teeth reduced with increased length of exposure to the experimental conditions (Fig. 6.5).

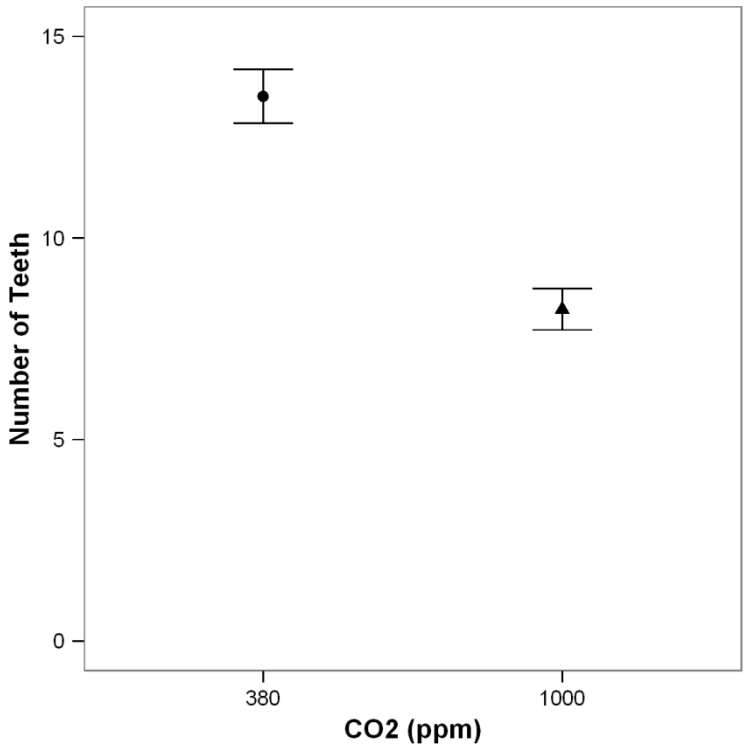

Figure 6.4 Model visualisation of CO_2 treatment effects on the number of teeth (y-axis) present on specimens of *H. germanica* in 20 μm^2 area. The visualisation shows CO_2 concentration along the x-axis. The different CO_2 concentrations are represented by either a circle (380 ppm) or triangle (1000 ppm).

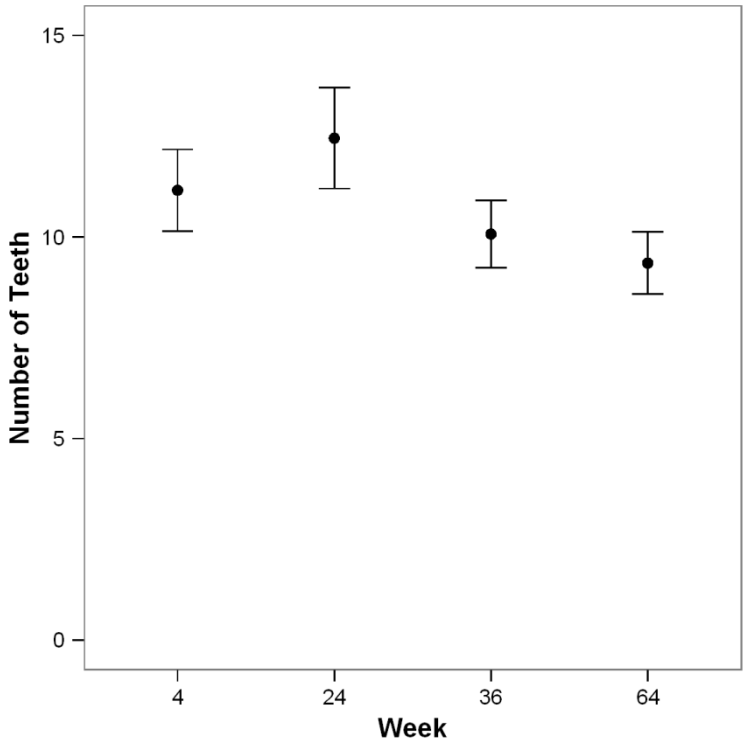

Figure 6.5 Model visualisation of length of exposure effects on the number of teeth (y-axis) present on *H. germanica* specimens in 20 µm^2 area. The visualisation shows the time point (week) along the x-axis (2, 24, 36 & 64).

6.3.2 Shape of teeth

Maximum length and width measurements were taken for each of the foraminiferal teeth counted above. The optimal model with length of teeth as the dependant variable consisted of a three-way interaction (CO_2 x Temperature Regime x Week: L-ratio = 17.873, d.f. = 3, p = 0.0005). Under the constant temperature regime, there was an increased CO_2 effect with time (Fig. 6.6a). After 24 weeks of exposure the length of teeth were reduced under the highest CO_2 treatment (1000 ppm). Under the ambient temperature regime, a CO_2 effect was also observed with time (Fig. 6.6b). Under ambient conditions, teeth length was reduced under the highest CO_2 treatment at every time point. The CO_2 effect under ambient temperatures appears to be smaller than that observed under the constant temperature regime (Fig. 6.6).

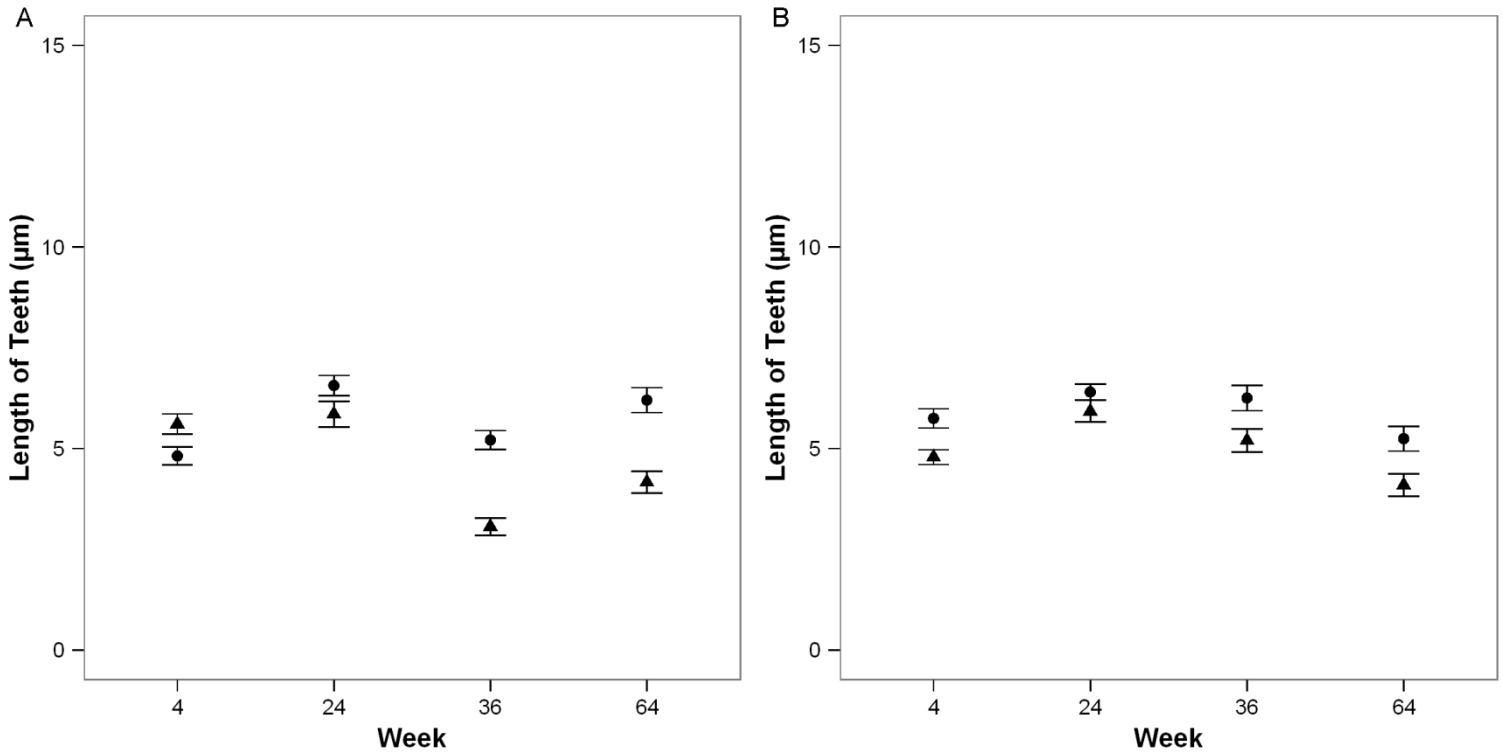

Figure 6.6 Model visualisation of three-way interaction (CO_2 x Temperature Regime x Week) on length of teeth on *H. germanica* specimens (y-axis) across each CO_2 treatment at each time point (week 4, 24, 36,64). **A)** Constant temperature regime, **B)** Ambient temperature regime. The visualisation shows time point along the x-axis. The different CO_2 treatments are represented by circles (380 ppm) and triangles (1000 ppm).

The optimal model with width of teeth as the dependant variable consisted of two two-way interactions (CO_2 x Week: L-ratio = 18.986, d.f. = 3, $p < 0.0005$, Temperature x Week: L-ratio = 13.848, d.f. = 3, $p < 0.005$). An increased CO_2 effect was observed with increased length of exposure to experimental conditions (Fig. 6.7). After 24 weeks of exposure the teeth width was lowest in the highest CO_2 treatment (1000 ppm). The width of the teeth remained lowest in the highest CO_2 treatment for the experimental duration and continued to reduce over time.

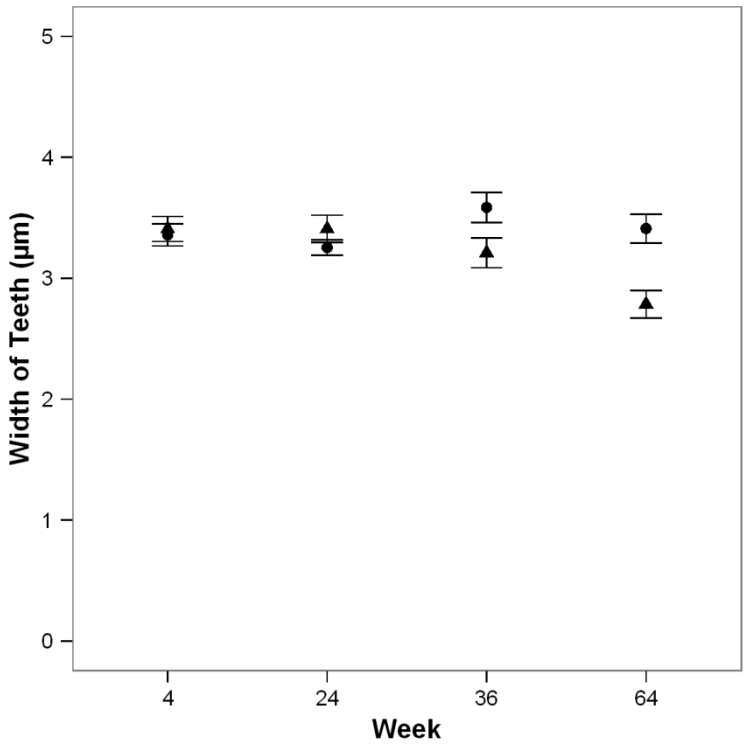

Figure 6.7 Model visualisation of the two-way interaction (CO_2 x Week) on width of teeth on *H. germanica* specimens (y-axis) across the two CO_2 treatments for each time point (week 4, 24, 36, 64). The visualisation shows the time point (week) along the x-axis. The different CO_2 treatments are represented by circles (380 ppm) and triangles (1000 ppm).

The maximum width of teeth also fluctuated with temperature regime over time (Fig. 6.8). An increased temperature effect on the width of teeth was observed at week 36. At this time point the largest teeth width is found under ambient temperature conditions. After 64 weeks of exposure the maximum width of teeth was observed under the constant temperature regime.

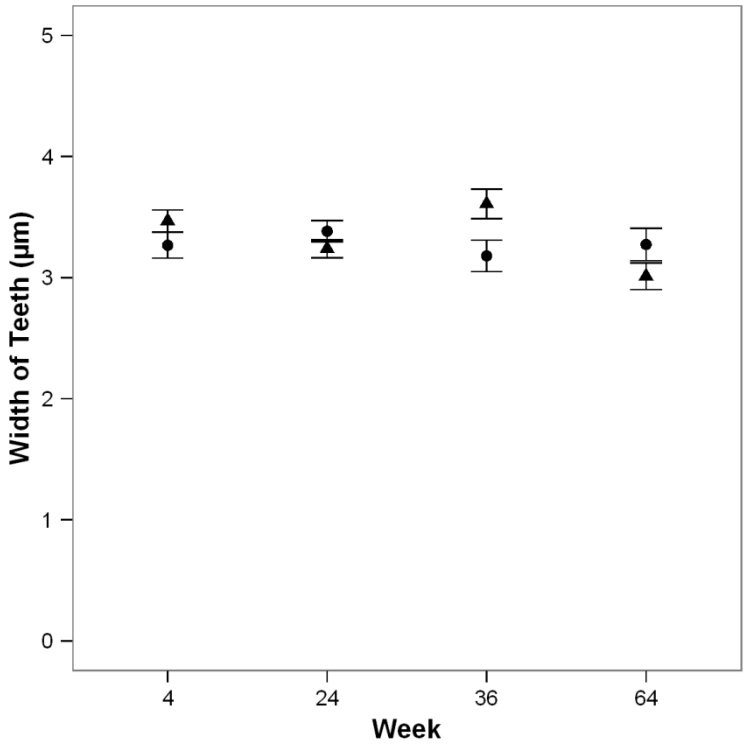

Figure 6.8 Model visualisation of the two-way interaction (Temperature Regime x Length of Exposure) on width of teeth on *H. germanica* specimens (y-axis). The visualisation shows the time point (week) along the x-axis (4, 24, 36 & 64). The different temperature regimes are represented by circles (constant) and triangles (ambient).

6.4. Discussion

The results from this study demonstrate that ocean acidification has a significant effect on the nature of ornamentation in the species *Haynesina germanica*. Test ornamentation of functional importance revealed sensitivity to decreasing seawater pH. Observations of the test surface allowed identification of shell dissolution (Fig. 6.3).

Under ambient CO_2conditions (380 ppm), the test surface of *H. germanica* is smooth with the primary openings along the base of the apertural face being obscured externally by teeth. Foraminifera maintained at ambient CO_2 levels had tubercles and teeth that were numerous and very well developed in both the apertural and umbilical regions. They were typically conical in shape, rimming both the apertural face and chamber margins (Banner & Culver, 1978). The tubercles were often arranged in rows, radiating from the primary aperture. The ornamentation rimming the apertural margin extended up the apertural face to approximately one-third of its height (as described by Banner & Culver, 1978).

Many individuals maintained under ambient CO_2 conditions (380 ppm) had discarded diatoms on the test surface. The patterns of fracturing and distinctive signatures in the discarded diatom frustules described by Austin et al. (2005) were observed in a number of diatom specimens (Fig 6.9). As described by Travis and Bowser (1991) and recorded by Austin et al., 2005, the frustules mostly displayed splits along the raphe from near the mid-point towards one of the poles. In many instances part of the valve wall had been completely removed (Travis & Bowser, 1991; Austin et al,, 2005). Diatom frustules were rarely observed on specimens maintained under the highest CO_2 conditions (1000 ppm). It is unclear from the images alone whether the

foraminiferal specimens in this treatment had stopped feeding or if they were feeding on an alternative food source.

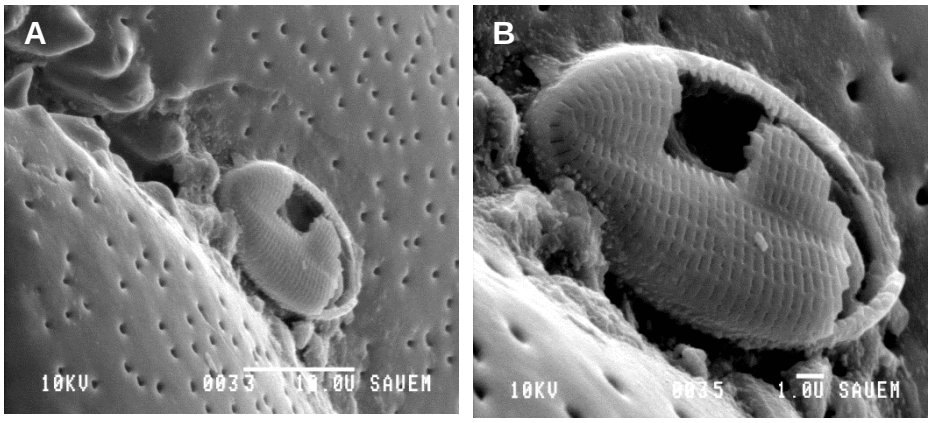

Figure 6.9 Scanning Electron Micrographs of characteristic fracturing pattern in discarded diatom frustules following foraminiferal feeding. (A) Diatom frustule (*Cocconeis* spp.) discarded adjacent to apertural region of *Haynesina germanica* specimen. (B) Detail of damage and cracking to empty diatom frustule following foraminiferal feeding.

At higher CO_2 levels, the functional ornamentation of the apertural area was vastly reduced. In some cases, the extent to which the apertural and umbilical features were dissolved or damaged made the foraminifera almost unrecognizable as *H. germanica* (e.g. Fig 6.3E). Both CO_2 treatment and length of exposure had significant independent effects on the number of teeth that were present in the apertural regions. Individuals suffered from dissolution under the highest CO_2 treatment (1000 ppm) with specimens having fewer teeth when compared to those maintained under ambient CO_2 conditions (380 ppm). The number of teeth present on specimens in the highest CO_2 treatment (1000 ppm) continued to decline with increased exposure time to the experimental conditions. Given the exposure of surface ornamentation, it ought to be particularly prone to dissolution (e.g. Kucera,

2007). This was evident in specimens maintained under the highest CO_2 treatment with foraminifera seemly unable to maintain these important calcified structures.

There were significant interaction effects (Length: CO_2 X Week x Temperature Regime, Width: CO_2 x week and Temperature Regime x Week) on the size and shape of teeth present on each of the specimens. The CO_2 effect on both the length and width of teeth increased with length of exposure to the experimental conditions. The CO_2 effect on the length and width of the teeth was stronger under the constant (10°C) temperature regime and was more pronounced following 24 weeks of exposure. The temperature effect on the width of teeth at week 36 corresponds to an increase in temperatures at weeks 24 (15.2°C) and 36 (13.4°C) under seasonally varying ambient conditions. It is only at these high levels that a temperature effect is observed in the data. This effect breaks down by week 64 when temperatures are reduced.

Although this species of intertidal foraminifera is likely to be adapted to short term fluctuations in pH, it is probably unable to maintain functionally important ornamentation when exposed to the long-term effects of ocean acidification. After just 4 weeks of experimental exposure, the number size and shape of the foraminiferal teeth were reduced at higher CO_2 levels. This reduction becomes more pronounced the longer they are exposed to higher CO_2 levels with specimens exhibiting fewer and smaller calcified structures.

Morphological changes are species-specific (Boltovskoy et al., 1991). Initial observations suggest that species other than *H. germanica* may also be impacted by ocean acidification. The main focus for this chapter was *H. germanica* as they were present in high enough numbers to allow for statistical comparisons between

treatments. Preliminary observations reveal that live *Elphidium williamsoni* also exhibit similar effects in their surface ornamentation in response to ocean acidification. There appears to be test dissolution and a reduction in the number of denticals in specimens incubated at higher CO_2 levels (Fig. 6.10).

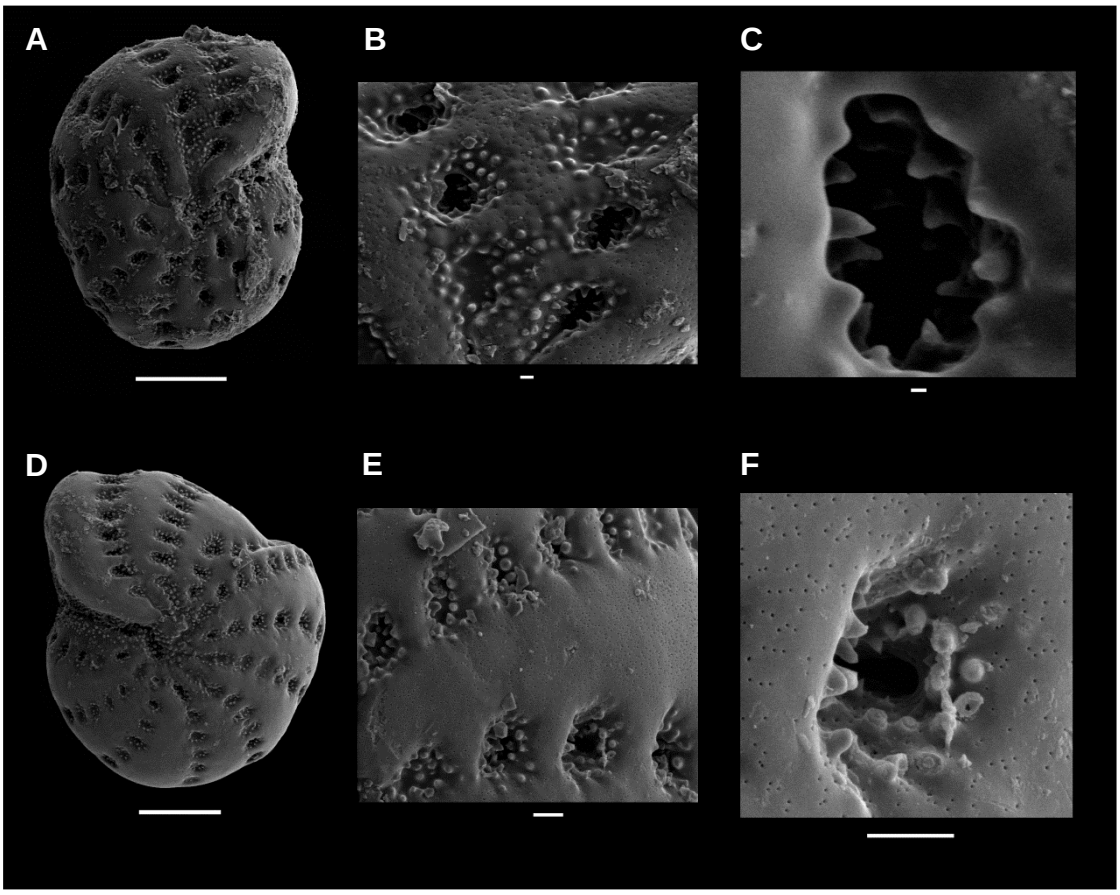

Figure 6.10 Scanning Electron Micrographs images of *Elphidium williamsoni*. Images of A & D show whole specimen, with images B-C and E-F displaying the surface ornamentation, including detail of the fossettes. Specimen A (ornamentation: B & C) was incubated in the 380 ppm CO_2 treatment. Specimen D (ornamentation: E & F) was incubated in the highest CO_2 treatment (1000 ppm). Scale bars: A) 100 µm, B) 10 µm, C) 1 µm, D) 100 µm, E) 10 µm and F) 10 µm.

Test ornamentation, notably around the apertural area, is crucial for feeding and chloroplast acquisition (Bernhard & Bowser, 1991; Austin et al., 2005). Dissolution of

these features at anticipated future levels of atmospheric CO_2 will therefore have a direct negative impact on the long-term fitness and survival of these organisms through a reduction in feeding efficiency. The potential for deformation, dissolution and in some cases absence of these functionally important feeding structures, may result in a shift in competitive advantage towards non-calcifiers in the benthic foraminiferal community under conditions of enhanced ocean acidification. This shift in community structure is likely to be further enhanced as a result of weakened tests under high-CO_2 scenarios, making calcifying foraminifera such as *H. germanica* more vulnerable to predators. Such shifts in community structure will have significant knock-on effects for trophic dynamics, carbon cycling and ecosystem productivity.

6.5 Associated paper

A number of key results from this chapter have been submitted as part of the following article (Appendix III):

Khanna, N., Godbold, J. A., Austin, W. E. N. & Paterson, D. M. (2013).The Impact of Ocean Acidification on the Functional Morphology of Foraminifera. *PLoS ONE***8** (12): e83118. DOI:10.1371/journal.pone.0083118.

Author Contributions:

Conceived and designed the experiment: NK, JAG, DMP.

Performed the experiments: NK, JAG.

Analysed the data: NK.

Contributed reagents/materials/analysis tools: NK, JAG, DP.

Wrote the Paper: NK.

Comments on original manuscript: JAG, DMP, WENA.

7. Study of diatom ingestion by benthic foraminifera following long-term exposure to ocean acidification: ^{13}C-tracer experiment.

7.1 Introduction

There have been a number of studies on the effects of ocean acidification on benthic foraminiferal communities (Dias et al., 2010; Pettit et al., 2013; Uthicke et al., 2013). In areas of acidified seawater, such as natural CO_2 vents, foraminiferal distribution and diversity change markedly (Dias et al., 2010). Chapter 4 discusses in detail how the survival of calcareous species is reduced following long-term exposure to high CO_2 concentrations (reduced pH) following experimental incubations.

Although these studies provide insight into the foraminiferal response at a community level, there is currently no information on how individuals will respond at a functional level. To date, there is just one study on the impacts of ocean acidification on foraminiferal functional morphology (see Chapter 6; Khanna et al., 2013).

Foraminifera utilize a diversity of trophic mechanisms, feeding types and nutritional resources (discussed in Goldstein, 2002). Although many types of potential food are present in the environment, feeding experiments have demonstrated prey selectivity in many foraminiferal species (Boltovskoy & Wright, 1976; Lee et al., 1988). When testing the feeding reactions of selected foraminifera to more than 50 species of microflora, all species tested were shown to be selective (Lee et al., 1966). Three species of littoral benthic foraminifera also demonstrated selective feeding with only 4 - 5 of the 28 species of algae tested being consumed in significant quantities (Lee & Muller, 1973). Moodley et al. (2000) demonstrated selective feeding among

foraminiferal species in labelling experiments with the green algae *Chlorella*. They stated the next step would be to use locally-dominant diatoms. In shallow environments, benthic diatoms form an important source of carbon for foraminifera (Lee et al., 1966; Moodley et al., 2000).

Some benthic foraminiferal species, such as *Haynesina germanica*, typically have surface ornamentation (Fig. 7.1) that is used in feeding (Banner & Culver, 1978; Alexander & Banner, 1984; Austin et al., 2005). Forces generated by manipulation of food items among this ornamentation allow the foraminiferal specimen to break open diatom frustules and liberate chloroplasts and cell content (see Chapter 6 for details).

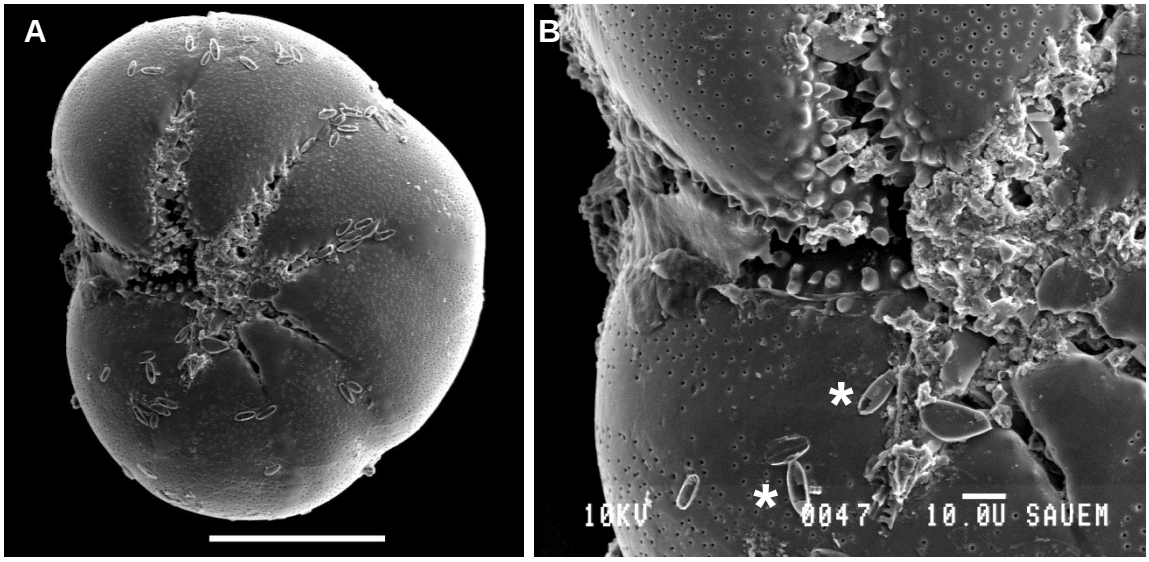

Figure 7.1 Scanning electron micrographs (SEM) of *Haynesina germanica* incubated at the lowest CO_2 treatment (380 ppm) for 64 weeks. (A) SEM of a typical test showing the umbilical view and active foraminiferal selection of diatom prey on the test surface (scale bar = 100 µm). (B) Higher magnification of test surface showing characteristic cracking damage (*) described by Austin et al. (2005) made by foraminifera during feeding (scale bar = 10 µm).

The mechanism by which foraminifera manipulate their prey and interact with their surroundings has been shown to involve the extension and contraction of pseudopodia (Goldstein, 2002). The pseudopodial network plays an indispensable role in foraminiferal nutrition (Lee, 1980), serving as a microscopic basket or filter for trapping, gathering and accumulating food particles (Travis & Bowser, 1991). Reticulopods participate in all known foraminiferal trophic mechanisms while their associated cytoplasmic transport contributes to the metabolic exchange process (Travis & Bowser, 1991). Digestion in benthic foraminifera begins in the reticulopodial network, in masses of gathered food, or near the aperture (Lee et al., 1991).

Under high CO_2 scenarios, functionally important ornamentation is vastly reduced in number and size, and in some cases becomes deformed (Khanna et al., 2013). The question therefore arises as to whether foraminifera that rely on calcified structures for feeding and chloroplast assimilation are still able to uptake food following exposure to high CO_2 concentrations. One way to assess this is through the use of stable isotopes.

Stable carbon isotopes have been used in both field and laboratory labelling experiments to trace different food sources (e.g. Herman et al., 2000) through the trophic web and to provide information on flux processes or feeding habits (Aberle et al., 2005). Enrichment with stable isotopes is a more convenient method as compared to radioactive isotopes, since they can be used *in situ* without negative environmental effects and legal restrictions (Pascal et al., 2008).

Ratios of carbon isotopes are closely linked to the origin of the organic matter (Carabel et al., 2006). The isotopic signature of a heterotroph is a function of the diet, the stable isotope composition of the different food sources and the

fractionation of food during processing (Herman et al., 2000). ^{13}C-enriched algal carbon has been used as a tracer over short-term time scales to quantitatively examine ingestion rates. For example, ^{13}C-labelled feeding experiments have demonstrated rapid ingestion and assimilation of phytodetritus by foraminifera (Blair et al., 1996; Levin et al., 1997; Moodley et al., 2000). Moodley et al. (2000) demonstrated rapid transfer of label from algal material to foraminiferans within hours. Benthic foraminifera have shown high assimilation rates of ^{13}C-labelled algae (Nomaki et al., 2005a, b) when compared to metazoan meiobenthos,

To date, no studies have been found that assess foraminiferal feeding abilities following long-term exposure to environmental stress. In this study, the ability of benthic foraminifera to ingest food following long-term exposure to ocean acidification was investigated using ^{13}C-labelled algae.

7.2 Materials and Methods

7.2.1 Long-term cultures

Prior to the grazing experiment, sediment communities from the Ythan Estuary, N. E. Scotland (57°20'N, 01°57'W), were maintained under conditions of long-term (18 months) ocean acidification incubation. The experimental system is described in detail in Godbold & Solan (2013). In brief, sieved sediment (500 µm) was added to experimental mesocosms (12 X 12 X 33 cm) to a depth of 12 cm, with 20 cm of overlying seawater. Mesocosms were exposed to seasonally varying light and temperature regimes (average local conditions). Seawater pH was controlled by bubbling air with a known concentration of CO_2 into each mesocosm and was maintained at 380, 750 or 1000 ppm (Godbold & Solan, 2013). Seawater pH, temperature and salinity were monitored weekly (Appendix I: Table S1). Following a long-term 18 month exposure, surface scrapes of 1 - 2 mm depth were collected from each of the treatments for the isolation of living benthic foraminifera.

7.2.2 Isolation of living benthic foraminifera

For the grazing experiment, only live mature individuals (300 – 400 µm) of *Haynesina germanica* were selected from the experimental culture material. Foraminifera were considered alive if they displayed cytoplasmic colouration alongside pseudopodial activity and movement (Murray, 2006). Foraminifera were picked out using a fine paintbrush under an Olympus® SZ stereo-microscope. Each foraminiferal specimen was washed in sterile seawater in 3 - 5 serial baths, before being starved for one week prior to the grazing experiment (Mojtahid et al., 2011). During the starvation period, specimens were incubated in the dark (Lee et al., 1966).

7.2.3 Grazing Experiment

7.2.3.1 Algal collection and ^{13}C labelling

Diatoms were collected from natural field assemblages in July 2012 (Eden Estuary, N. E. Scotland, 56°22'N, 2°50'W) as they have been shown to be a suitable food source for this species of foraminifera (Austin et al., 2005). Diatoms were collected during low tide by scraping the surface of the sediment and extracting them following the methods used by Austin et al. (2005). Two layers of lens tissue (Whatman® lens tissue 105) were placed on the sediment surface to allow benthic diatoms to migrate into them (Austin et al., 2005). After 1 hour the top layer of lens tissue was removed and washed in a beaker of filtered seawater to release the diatoms (Austin et al., 2005). Cells for the non-labelled culture were maintained in seawater containing f/2 medium. For the labelled culture, cells were maintained in seawater containing f/2 medium supplied with labelled $NaH^{13}CO_3$ (CO_3^{2-} sole added C source, 99% ^{13}C; 4mM) (Moodley et al., 2000; Pascal et al., 2008; Leroy et al., 2012). Both non-labelled and labelled cultures were grown in 1 litre glass batches for six days (Leroy et al., 2012). Both non-labelled and labelled cultures were cultivated under a permanent light regime (Aberle& Witte, 2003).

Labelled and non-labelled diatoms were harvested from the cultured seawater by centrifugation and washed several times in sterilized seawater to remove excess ^{13}C-bicarbonate.After an aliquot was counted, food was diluted to the desired concentration. Algal concentrations were estimated by the mean number of cells obtained by direct counts using a counting cell (Neubauer®) under a light microscope. The algae were stored at -20°C until the grazing experiment (Nomaki et al., 2006).

7.2.3.2 Experimental setup

The grazing experiment was performed in a CT room with a subdued light regime maintained at 13°C (corresponding to the temperature prevailing in the field at the time of the experiment). Foraminiferal feeding experiments have been found to be most accurate when of short duration (2-3 days) and when done in the dark or subdued light (Lee et al., 1966).

Incubations of algae and foraminifera were performed in 6-well tissue culture plates. Each replicate was held in a separate insert. Twenty living foraminiferal specimens were added to each replicate culture well. The condition of the foraminifera was checked daily and at the end of the experiments to ensure specimens were alive. At time point zero (T_0), 100 µL of either non-labelled or labelled concentrated algae was added to each culture well (~10^6 cells ml^{-1}).

Cultures were maintained at either 380 ppm, 750 ppm or 1000 ppm for the duration of the feeding experiment. Seawater was refreshed daily to maintain the respective conditions. Under each CO_2 concentration, three grazing treatments were run (Fig. 7.2). Culture inserts with deposited non-labelled (D^-) diatoms but without added foraminifera were used as controls (treatment 1). These diatom samples were analysed under a light microscope as a procedural control to assess any damage that had occurred when no foraminifera were present. For treatment 2, culture wells contained non-labelled diatoms (D^-) and foraminifera (F^+) while in treatment 3 culture wells contained labelled diatoms (D^+) and foraminifera (F^+).

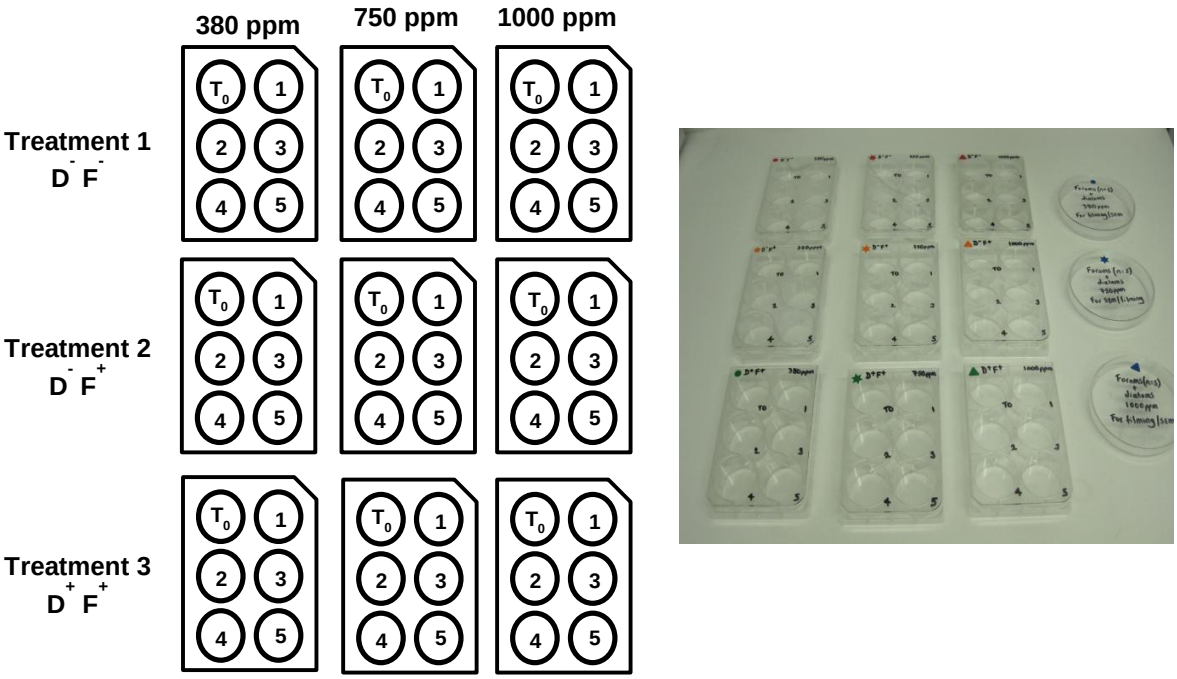

Figure 7.2 Schematic diagram and photograph displaying experimental design with all three treatments: Treatment 1 – non-labelled diatoms with no foraminifera (D^-F^-), Treatment 2 – non-labelled diatoms with foraminifera (D^-F^+) and Treatment 3 – labelled diatoms and foraminifera (D^+F^+).

Six replicates were run at each CO_2 treatment. Immediately after algal addition, one of the replicates was sampled as a time zero (T_0) control. Replicate time zero controls of non-labelled (n = 5) and labelled (n = 5) diatoms from each stock batch were also taken. The grazing experiment ran for three days.

At the end of the experiment (T_f), foraminifera were picked out individually from the inserts and stored in Petri dishes at -20°C (Moodley et al., 2000). Diatoms from each treatment were fixed in 2.5% glutaraldehyde in seawater for later analysis.

7.2.4 Analytical techniques

Prior to analysis, foraminiferal specimens were cleaned vigorously under the microscope with a brush to remove any adhering particles (Moodley et al., 2000). Cleaned specimens were then transferred to pre-combusted silver boats and decalcified with 2.5% HCl (Moodley et al., 2000). To prevent loss by leaching, samples were not washed with water after acidification (Carabel et al., 2006). Excess acid was removed by drying overnight at 40°C (Moodley et al., 2002). For each replicate sample, 20 individuals were combined for stable-isotope determinations in order to obtain a representative sample (Herman et al., 2000).

Assimilation rates of ^{13}C-labelled algae were calculated by measuring the $\delta^{13}C$ values in foraminiferal protoplasm (Nomaki et al., 2005a). The carbon isotopic composition of the samples was measured by elemental analyser-isotope ratio mass spectrometry (EA-IRMS).The carbon isotope ratios were expressed in the delta notation ($\delta^{13}C$) relative to the Vienna Pee Dee Belemnite (PDB) standard for carbon. Laboratory working standards were tailored to suit very low C samples (Woulds et al., 2007) and were run for every 5 samples.

The terms ‘lighter’ or ‘depleted’ are used for lower δ, values, and ‘heavier’ or ‘enriched’ for higher δ values, while Δ represents the difference between the δ-values of two samples (Rolff, 2000). Incorporation of the^{13}C label was calculated as excess (above background) ^{13}C, expressed in terms of specific uptake ($\Delta\delta^{13}C$) (Herman et al., 2000; Moodley et al., 2000; Leroy et al., 2012). Specific uptake was calculated as $\Delta\delta^{13}C = \delta^{13}C_{Tf} - \delta^{13}C_{T0}$, where positive values indicate that foraminifera incorporated some of the introduced label (Leroy et al., 2012).

7.3 Results

The number of replicates available at the final time point were reduced in a number of treatments due to foraminiferal mortality over the course of the grazing experiment. A total of five, three and three replicates ran the full length of experiment under the CO_2 treatments 380ppm, 750 ppm and 1000 ppm, respectively.

Before labelling, the diatom cultures had an average $\delta^{13}C$ of -14.9 ± 0.8‰ (n = 5). After six days of labelling, a significant ^{13}C-enrichment ($\delta^{13}C$ = 20663.4 ± 2061.6‰; n = 5) was recorded (paired t test, t = -22.434, d.f. = 4, $p < 0.0001$). Little natural variability was observed in the diatom stock of the non-labelled treatment, while high variability was observed for the labelled stock (Table 7.1).

Prior to the grazing experiment, foraminifera from each of the CO_2 treatments showed differing initial values of $\delta^{13}C$ (Fig. 7.3). At 380 ppm, foraminifera had a $\delta^{13}C$ of -19.7‰ (n = 1) in the non-labelled and -17.9‰ (n = 1) in the labelled treatment. At 750 ppm, foraminifera had a $\delta^{13}C$ of -24.3‰ (n = 1) and -18.8‰ (n = 1) in the non-labelled and labelled treatments, respectively. At 1000 ppm, foraminifera had a $\delta^{13}C$ of -28.2‰ (n=1) in the non-labelled and -27.6‰ (n=1) in the labelled treatment.

Foraminifera supplied with non-labelled food showed an increase to positive^{13}C values after three days grazing as compared to their initial values (Fig. 7.3). Final $\delta^{13}C$ values in the non-labelled treatments were -16.1 ± 1.4‰ (n = 5), -21.9 ± 1.2‰ (n = 3) and -26.9± 0.2‰ (n = 3) for CO_2 treatments 380, 750 and 1000 ppm, respectively. The $\delta^{13}C$ values increased by 3.6‰ at 380 ppm, 2.4‰ at 750 ppm and 1.3‰ at 1000 ppm.

When supplied with ^{13}C-enriched diatoms, foraminifera showed an increase in $\delta^{13}C$ in all CO_2 treatments after three days (Fig. 7.4). For all treatments, the uptake of

label after its incorporation into diatoms can be quantitatively demonstrated (Fig. 7.4). At 380 ppm, $\delta^{13}C$ values increased to 160.7‰ ± 75.1‰ (n = 5). At 750 ppm, $\delta^{13}C$ values increased to 299.1‰ ± 79.8‰ (n = 3). At the highest CO_2 treatment (1000 ppm), $\delta^{13}C$ values increased to 235.3‰ ± 41.5‰ (n = 3).

A Friedman test revealed no significant difference in specific uptake of diatoms between CO_2 treatment (Friedman chi-squared=1, d.f. = 2, p-value = 0.6065) or labelling treatment (Friedman chi-squared=3, d.f. = 1, p-value = 0.08326).

Table 7.1 Comparison of $\delta^{13}C$ (range values) of the (a) diatoms and (b) foraminifera (n: pooled 20 individuals). Non-labelled diatom (D^-) and labelled diatom (D^+) treatment values displayed for the start (T_0) and end of the grazing experiment (T_f). (n: number of samples, -: not sampled).

	T_0		Tf	
	$\delta^{13}C$	**n**	**$\delta^{13}C$**	**n**
(a) Diatoms				
Non-labelled (D^-)	-16.2 to -13.9	5	-	
Labelled (D^+)	18491.2 to 23247.0	5	-	
(b) Foraminifera				
380 ppm (D^-)	-19.7	1	-19.2 to -14.5	5
380 ppm (D^+)	-17.9	1	87.2 to 250.2	5
750 ppm (D^-)	-24.3	1	-23.2 to -20.9	3
750 ppm (D^+)	-18.8	1	246.2 to 390.9	3
1000 ppm (D^-)	-28.2	1	-27.1 to -26.7	3
1000 ppm (D^+)	-27.6	1	211.3 to 283.3	3

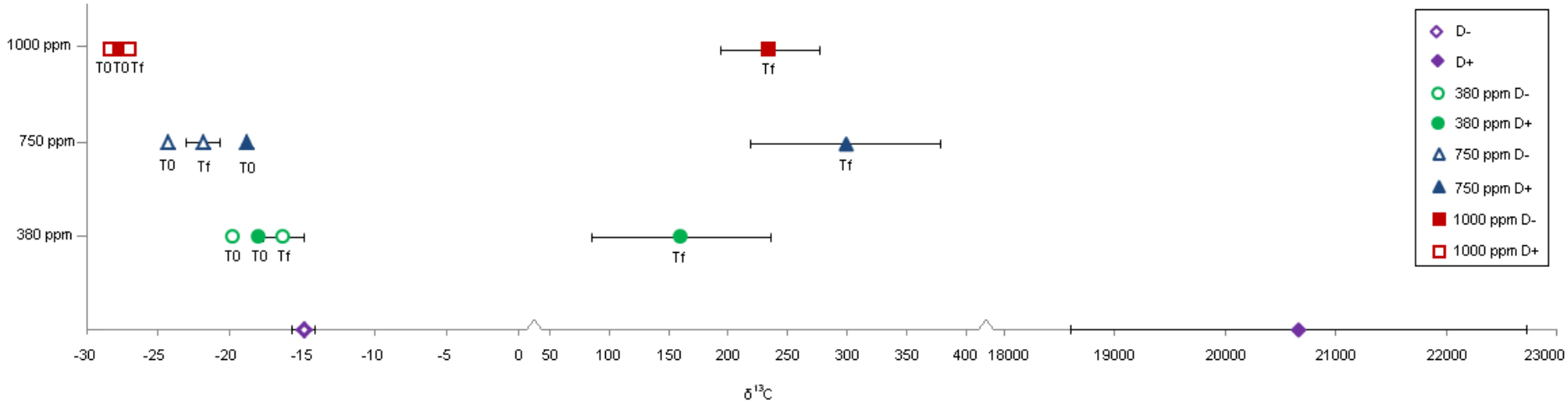

Figure 7.3 Carbon isotope ratios ($\delta^{13}C$) for both non-labelled and labelled treatments at the start (T_0) and end (T_f) of the grazing experiment at each CO_2 concentration (380, 750 & 1000 ppm). T_0 measurements are based on single point measurements. T_f values represent mean ± SD (n = 5 for 380 ppm and n = 3 for 750 & 1000 ppm). Error bars at T_f for 1000 ppm are small and therefore not visible.

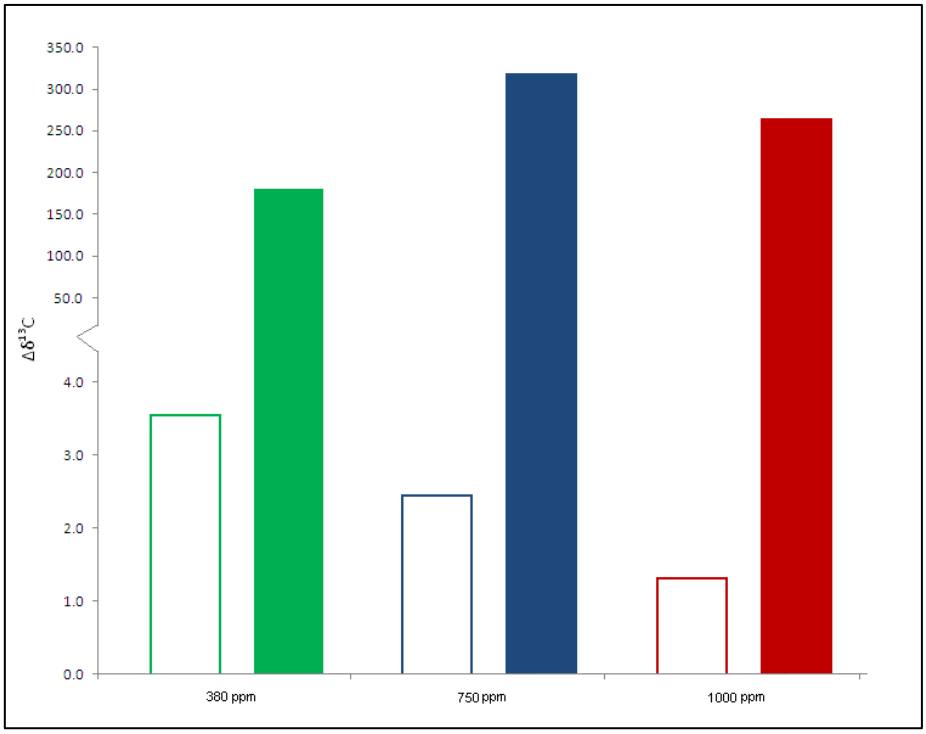

Figure 7.4 Specific uptake (calculated as $\Delta\delta^{13}C = \delta^{13}C_{Tf} - \delta^{13}C_{T0}$) by *Haynesina germanica* supplied with non-labelled and labelled food. The CO_2 treatments 380ppm, 750 ppm and 1000 ppm are coloured with green, blue and red, respectively. Open bars represent non-labelled diatoms and closed bars represent ^{13}C-labelled diatoms.

7.4 Discussion

By labelling algal food with stable isotopes we were able to detect uptake in specimens of *Haynesina germanica* across all CO_2 treatments.

7.4.1 Labelling

The experiment first showed that the diatoms incorporated the ^{13}C-label after six days. All labelled diatom stock samples significantly increased their composition of ^{13}C as compared to the non-labelled diatom stock samples.

The average $\delta^{13}C$ value for non-labelled field collected diatoms from the intertidal mudflat was -14.49‰.This is close to $\delta^{13}C$ reported for benthic diatoms in other mudflats and intertidal areas. For example, average $\delta^{13}C$ values of -14.4‰ (Créach et al., 1997) and values ranging from -17.3‰ to -16.9‰ (Riera&Hubas, 2003) have been reported for benthic microalgae in intertidal mudflat areas. Although field collections are typically mixtures of various diatom species which may differ strongly in isotopic composition (Fry, 1996, and references therein), little variability was shown in the field collected specimens for this study. More variable values have been reported in the literature, with microalgae typically showing $\delta^{13}C$ values of -23.3‰ to -19.4‰ (Hecky & Hesslein, 1995; James et al., 2000; Aberle et al., 2005). The $\delta^{13}C$ values can vary, for example, because of temporal or spatial changes in $\delta^{13}C$ values of dissolved inorganic carbon (Peterson, 1999).

The average $\delta^{13}C$ value for the labelled diatom stock cultures was 20663.4‰ ± 2061.6‰. Although the labelled stock culture was homogenised prior to sampling there was still huge variability between samples. As there was little natural $\delta^{13}C$ variability in non-labelled stock culture the high variability in the labelled stock culture cannot be attributed to the original community but instead to the uptake of ^{13}C

associated with the labelling process itself. Different species of diatom will have different uptake rates and some species of diatom may have not taken up the label within the time available. For example, ^{13}C isotope enrichment was initially very different between two algal species used in a labelled feeding experiment (Aberle et al., 2005).

Growth may have also had an effect on label uptake. Different species of marine phytoplankton have different optimal growth rates (Wong & Sackett, 1978). Increasing growth rate increases carbon demand and hence carbon flux into the cell (Rau et al., 1996). High growth rate appears to cause enrichment of ^{13}C in marine phytoplankton, with growth rates being the best predictor of isotopic fractionation and algal $\delta^{13}C$ (Fry & Wainwright, 1991). Enrichment is also expected to be more pronounced in larger size-classes of phytoplankton since their less favourable surface to volume ratio should cause steeper CO_2 gradients (Rolff, 2000).If populations were at different growth phases they would have therefore had different uptake rates.

7.4.2 Background signatures

Following long-term experimental incubation (prior to the grazing experiment), foraminifera had differing $\delta^{13}C$ values for each of the three CO_2 treatments. Foraminifera exposed to 380 ppm were most enriched in ^{13}C, while those at 1000 ppm were most depleted in ^{13}C.

The differences can be attributed to either the food source changing composition orthe foraminifera changing their food resource under the different CO_2concentrations. Each of these possible explanations is discussed below. The difference between T_0 and T_f values in the non-labelled treatment indicates that

foraminifera were grazing on less enriched food in the long-term mesocosm incubations prior to being introduced to the grazing experiment.

7.4.2.1 Long-term effects of ocean acidification on diatom ^{13}C

Different elemental compositions at T_0arelikely to have arisen from differences in the dietary composition between the CO_2 treatments as they become enriched in $\delta^{13}C$ relative to the food items used. The natural stable-isotope composition of a species integrates over relatively long periods of time and therefore represents a weighted signal (Herman et al., 2000). Kukert & Riebesell (1998) show that changes in environmental conditions are imprinted in the carbon isotopic composition of the particulate organic matter produced. For example, CO_2 availability can affect carbon uptake and fixation by cells and may therefore influence the elemental composition of the organic matter produced (Burkhardt & Riebesell, 1997).

Under controlled conditions with sufficient light and excess nutrients, variation in CO_2 concentrations appeared to be the cause of changes in the chemical composition of cells in a common marine diatom species (Burkhardt & Riebesell, 1997).External CO_2 concentrations have been demonstrated to have the strongest effect on $^{13}C/^{12}C$ distributions and isotope fractionation, with the largest discriminations found under high CO_2 conditions (Fry, 1996).

Diatoms can actively take up both CO_2 and bicarbonate (HCO_3^-) for photosynthesis (Burkhardt et al., 2001; Rost et al., 2003). At high pH, a greater proportion of dissolved inorganic carbon occurs as bicarbonate, and the use of bicarbonate by phytoplankton would further enrich their carbon isotopic composition (Rolff, 2000, and references therein). Experiments have demonstrated that a decline in CO_2 supply is accompanied by the gradual induction of HCO_3^- uptake, whereas species

of diatom favour the uptake of CO_2 if present at high concentrations (Burkhardt et al., 2001). Ocean acidification may also cause diatoms to increase growth, down-regulate their CO_2 concentrating mechanism (CCM), and experience enhanced photo-inhibition and dark respiration (Wu et al., 2010).Cells grown under elevated CO_2 in the low-pH cultures can save some energy due to down-regulation of CCM, but at the same time, they need additional energy to cope with the external pH decrease (Wu et al., 2010).The preference for CO_2 uptake at higher CO_2 concentrations is maintained for energetic reasons (Burkhardt et al., 2001).

Isotope fractionation may also have been influenced by taxon-specific differences, nutritional status of the cells, growth rate, cell size and shape and culture conditions (Wong & Sackett, 1978; Kukert & Riebesell, 1998;Gervais & Riebesell, 2001, and references therein).A fast growing organism will show a smaller apparent fractionation relative to a slow grower if they both have the same mechanisms for CO_2 fixation (Wong & Sackett, 1978). There is, however, considerable scatter in the isotope fractionation for any given ratio of diatom growth rate and CO_2 concentration (Kukert & Riebesell, 1998). Photosynthetic carbon isotope fractionation is thought to be a function of the ratio of carbon demand to carbon supply, so that a low demand (i.e. low growth rate) and a high supply (e.g. high CO_2 concentration) favours high fractionation (Wolf-Gladrow et al., 1999).

7.4.2.2 Long-term effects of ocean acidification on foraminiferal feeding

In addition to possible CO_2 effects on diatom fractionation, long-term exposure to ocean acidification could have also impacted the foraminifera. A number of explanations could account for the differences observed between CO_2 treatments at T_0. Firstly, it is possible that the foraminiferal specimens selected the same species of diatoms across all treatments but fixed them differently depending on the

CO_2concentration they were exposed to. Secondly, the foraminiferal specimens could have eaten the same species of diatoms but fixed differing amounts. Finally, foraminifera could have been feeding at differing rates under each of the CO_2 treatments.

It is possible that the CO_2 levels at 1000 ppm changed the grazing behaviour of the foraminifera resulting in them eating less during long-term experimental incubation. At 1000 ppm, the CO_2 levels could have reduced or stopped foraminiferal feeding or influenced their metabolism. If foraminifera were all feeding in the same manner (i.e. same uptake rate, amounts, assimilation rates)at all CO_2 concentrations during the long-term incubations, then they must have been feeding on food sources that had different compositions (shown in differences in $\delta^{13}C$ at T_0).

7.4.3 Uptake of ^{13}C

Most importantly these results indicate that foraminifera fed on diatoms across all CO_2 concentrations during the three day grazing experiment. When foraminifera were supplied with both non-labelled and labelled diatoms, $\delta^{13}C$ values became more positive indicating uptake of the introduced food.

High variability in labelled diatom stocks masks any uptake in any of the treatments. This therefore makes it difficult to quantify exact uptake rates. However, the data demonstrates the foraminifera ate diatoms in all treatments (backed up by D^- data), but cannot discern if the foraminifera ate more or less depending on the CO_2 concentration.

As mentioned previously, selective feeding in foraminifera is well-known (Lee & Muller, 1973) and a number of factors have been shown to affect feeding: (1) the age

of the food organism, (2) the age of the foraminifer or its stage in its life cycle, (3) the concentration of food and (4) relative crowding (Lee et al., 1966; Muller, 1975).

Abundance relationships of algae are important in feeding experiments. Theoretically, food uptake by a grazer increases with abundance of food. Above a certain threshold value of prey concentration, however, uptake rate remains constant, e.g. Holling's prey-dependent type II functional response (Holling, 1959). During feeding experiments with littoral foraminifera, feeding below a concentration of 1 x 10^3 microphytes/10 ml was erratic but above this threshold was directly proportional to the concentration (Lee et al., 1966). Saturation was reached at ~1 x 10^6 or 1 x 10^7 organisms/10 ml (Lee et al., 1966). At the concentrations used in this grazing experiment and given that food availability did not vary during the grazing experiment, there should have been no effect of food concentration on the foraminiferal feeding between each of the CO_2 treatments.

Care was also taken not to crowd the foraminifera as this can also affect the feeding rates of some species (Muller, 1975). For example, at high densities (50 animals/10 ml) foraminifera ate at a 44% lower rate than single individuals (Muller, 1975). During the grazing experiment, 20 individuals/10 ml were maintained so crowding should not have influenced the feeding response observed for each of the CO_2 treatments.

Uptake can also be affected by the life stage of the species (Lee 1980) or the size and structure of the benthic community (Moodley et al., 2000). For example, differences in feeding rates between newly released agamonts (~150-200 µm) and older, larger stages (350-400 µm) in *Allogromia laticollaris* was found to be as great as 200% (Lee et al., 1969). Care was therefore taken in this study to select specimens of approximately the same size (typically 300 – 400 µm) and growth phase (Lee & Muller, 1973) to maintain a consistent feeding potential.

Selection of mature individuals was combined with a short-term grazing experiment. Tracer experiments with littoral benthic foraminifera have been found to be most accurate when of short duration (2-3 days) (Lee et al., 1966). Short-term incubations can be used to both detect natural feeding preferences and avoid adaptive feeding that might occur over time (Aberle et al., 2005; Leroy et al., 2012). In this instance, three days was enough to show uptake of the experimental labelled diatoms. In feeding experiments with *Peneroplis planatus*, assimilation rates for some algal species were very high (~100%) for the first 24 hours (Faber & Lee, 1991). Similarly, specimens of *Amphistegina lobifera* and *Amphisorus hemprichii* that had been starved prior to feeding experiments showed initial voracious feeding, which slowed down after 8 - 24 hours (Lee, 2011).

Feeding in many benthic foraminifera is episodic (Anderson & Lee, 1991). Importantly, everything that is captured is not ingested, digested or assimilated (Lee, 2011). Particle ingestion and nutrient assimilation by benthic foraminifera has been measured in the laboratory (Lee et al., 1966; Lee & Muller, 1973; Lee, 1980), however, quantitative information on the ingestion rates of organic carbon by benthic foraminifera remains scarce (Nomaki et al., 2006).

A number of studies have reported on benthic foraminiferal assimilation efficiency. In previous labelling experiments, benthic foraminiferal communities were able to process up to 6 mg Cmg^{-2} within 35 hours (Moodley et al., 2002). Moodley et al. (2000) examined foraminiferal ingestion rates after 3, 6, 12 and 53 hour incubations. The uptake of algal carbon was $\Delta\delta^{13}C$ 240‰ ± 22‰ within three hours for *Ammonia* spp. demonstrating rapid uptake (~1 - 7 % of added carbon being ingested within 3-53 hours). Uptake appeared linear over the first few hours but then seemed to level off, possibly due to satiation (Moodley et al., 2000). Over a two day experimental

period, changes in $\delta^{13}C$ values in foraminiferal cytoplasm clearly showed ingestion of labelled algae (Nomaki et al., 2005a). Similarly, Rivkin & DeLaca (1990) reported rapid assimilation of ingested algal carbon in benthic foraminifera within hours.

In *Ammonia* spp. it was reported that a significant portion of algal carbon was rapidly ingested and a large part was probably also assimilated (Moodley et al., 2000). In addition to the tracer recovered, however, the algal carbon could also be immediately burnt for energy, with diatom carbon released as $^{13}CO_2$ (Levin et al., 1997). For example, Linke et al. (1995) reported activation of benthic foraminiferal metabolism such as respiratory activity and ATP content after the foraminifera were fed. Moodley et al. (2000) recognised that a large part of the incoming carbon was rapidly respired but in the case of *Ammonia*, the uptake probably reflected ingestion of added carbon (Moodley et al., 2000).

Moodley et al. (2000) also recorded an absence of distinct uptake and large variation among individuals of *Haynesina germanica*. They suggested a possible preference for another food source (e.g. locally produced diatoms or more refractory material) as an alternative to the green algae, *Chlorella*, food source.

This present study has demonstrated obvious and preferential uptake of labelled diatoms using locally-dominant diatom species from an intertidal area. Although many of the above studies have reported uptake and assimilation over short durations it seems unlikely that the foraminifera underwent complete turnover in this grazing experiment as the foraminifera were not more enriched than the diatom compositions.

Given the variability surrounding the ^{13}C labelling in this grazing experiment it was not possible to determine the significance of CO_2 concentration effects on

foraminiferal uptake. However, insights into benthic foraminifera still being able to feed after long-term exposure to high CO_2 scenarios have provided a basis for further experiments on feeding preferences and efficiency. The initial starting points for future experimentation are discussed below.

7.4.4 Future work

The study demonstrated the direct transfer of carbon from diatoms to the foraminifera, with an increase in $\delta^{13}C$ for all treatments. However, the extent of CO_2 effects on feeding still needs to be assessed in greater detail. Biomass estimates would allow us to accurately estimate uptake. In this example, specific uptake was calculated but without biomass measures total uptake could not be determined.

It is probable that working with mono-cultures of different diatom species would aid in limiting the variability during labelling. This would also allow us to assess any changes in dietary preference between different species of diatom. With known nutritional differences between different diatom species (Lee et al., 1969; Muller & Lee, 1969), additional experimentation is required to assess prey selection under different CO_2 scenarios. It is of great importance to understand how benthic foraminifera will discriminate in their feeding in the future as nutritional differences in food are reflected in both growth and reproductive responses (Lee, 1980) and will have a potential cascade effect along the trophic web.

8.1 Discussion

The initial experiments (Chapter 3) were conducted to determine the short-term responses of two dominant intertidal species of foraminifera (*Haynesina germanica* and *Elphidium williamsoni*) to ocean acidification. Multiple species and multiple stressors (elevated CO_2 and seasonal temperature regime) were then incorporated in a series of mesocosm experiments to investigate community level responses in benthic intertidal foraminifera (Chapters 4 - 7). The results associated with each of the approaches and related hypotheses are discussed below.

Hypothesis One: Survival and growth of calcareous foraminiferal species (*Haynesina germanica* and *Elphidium williamsoni*) will reduce with decreasing pH.

Survival of both *H. germanica* and *E. williamsoni* was significantly reduced under low pH conditions. Live specimens of both species were however found in the lowest pH treatment but in significantly lower numbers than controls. It is important to note that some of these calcareous specimens did survive and grow under reduced pH conditions.

Hypothesis Two: The proportion of agglutinated foraminiferal species will increase while calcareous species will decline under long-term exposure of natural mixed assemblages to elevated CO_2.

Live populations were still dominated by calcareous foraminifera following long-term exposure to ocean acidification. The original hypothesis suggested agglutinated taxa, which do not rely on calcification, would replace calcifying species at elevated CO_2 (Uthicke et al., 2013). This however was not the case, with low live numbers of

agglutinated specimens recorded under increased CO_2. The mesocosm experiments were closed systems and if the propagules for the agglutinated species were not present at the time of initial sediment collection they would not be available to grow to maturity (Alve & Goldstein, 2003, 2010) or take advantage of reduced competition from calcareous species. Juvenile specimens of agglutinated species were not recorded during the time-series suggesting that reproduction in these species did not occur within the experimental period.

Further work is required to examine the impacts of ocean acidification on agglutinated foraminifera. This group use a number of different wall materials with various kinds of cement and therefore show a great variety of form and structure. It is still unclear as to how the biochemistry of the organic lining or the cements of agglutinated foraminifera will be impacted by ocean acidification. As pH change can interfere with pseudopodial movement, there could be knock on effects for rhizopodal streaming and shell construction in these non-calcareous foraminifera.

Hypothesis Three: Overall survival of foraminifera will be reduced by elevated CO_2 and temperature.

Survival, measured as number of live individuals, was reduced under elevated CO_2 and temperature. The general trend of benthic foraminiferal species diversity, as measured by richness (species count), was one of decreasing diversity with increasing CO_2.

Hypothesis Four: Multiple stressors will have a greater detrimental effect on community structure and population survival than single stressors.

The number of living individuals was lowest under the ambient temperature regime when compared to the constant temperature regime. Foraminiferal diversity (as measured by richness) was highest under the ambient temperature regime when compared to the constant regime. A number of species may have required a temperature cue for growth and reproduction and this would have been absent under the constant (10°C) temperature regime. The largest CO_2 effect was observed under ambient temperature conditions. The observed temperature effect broke down under elevated CO_2 conditions (1000 ppm). Similar effects have been observed in other organisms (e.g. *Alitta virens*), where effects were mostly driven by absolute temperature, rather than enhanced levels of CO_2, and were exacerbated by supplementary warming (Godbold & Solan, 2013).

Hypothesis Five: Damaged and deformed foraminiferal specimens will increase in frequency with elevated CO_2.

Significant interactions were recorded between "CO_2 x Temperature" and "CO_2 x Length of Exposure" on percentages of abnormal foraminiferal specimens. The increase in percentage of abnormal specimens at higher CO_2 levels (750 ppm & 1000 ppm) could indicate a stressed environment. Ocean acidification also had significant effects (significant 3-way interaction: CO_2 x Temperature x Length of Exposure) on the percentage of damaged foraminiferal tests. The percentages of damaged specimens increased with exposure to increased CO_2 and temperature over time. Highest percentages of damaged specimens were recorded for calcareous forms, with percentages falling outside the range of natural variability. An

increase in percentage of damaged specimens could indicate weakening of the tests or compromised calcification mechanisms under ocean acidification scenarios.

Although this chapter provides initial insights, the interpretation must be cautious due to the low numbers of live individuals. With individuals (and many species) not surviving the duration of the experiment (Chapter 4) it is difficult to fully assess the impacts of ocean acidification on foraminiferal deformation and damage.

Hypothesis Six: Calcareous morphological features and ornamentation of functional importance will be reduced under elevated CO_2 conditions.

After just four weeks of exposure, the number, size and shape of calcified structures used in feeding by *Haynesina germanica* were significantly reduced under high CO_2 levels (1000 ppm). This reduction in ornamentation became more pronounced with increased length of exposure. Further study is required to establish if these calcified structures simply dissolve under elevated CO_2 or if their formation is also affected.

Hypothesis Seven: Feeding function will be compromised in calcareous foraminiferal species under elevated CO_2.

Following 18 months exposure, foraminifera from all three CO_2 conditions (380, 750 and 1000 ppm) were able to feed on diatoms. High variability in the ^{13}C-labelled diatom stocks made it difficult to quantify exact uptake in each of the treatments and the extent of CO_2 effects on feeding still needs to be fully understood.

8.2 Future Work

Experimental incubations, counting procedures and morphological measurements conducted as part of this thesis were a function of both financial and human resources. The following approaches would have been addressed if time and the above restrictions were not present.

8.2.1 Effects of ocean acidification on foraminiferal population sizes

Shell size is easy to determine and existing records, e.g. planktic foraminifera, show that it is highly sensitive to environmental forcing (Kucera, 2007). Under environmental change, where stress increases, the mean size of individuals will decrease (Murray, 2000). Calcite dissolution increases fragmentation and therefore alters the size spectrum of the assemblage (Kucera, 2007). For example, young and small specimens of foraminifera have thinner walls and fewer calcite lamellae and could therefore be more easily destroyed as a result of ocean acidification (Haynert et al., 2011).

Although changes in foraminiferal diversity were documented as part of this study, time restrictions did not permit assessment of foraminiferal size distributions. In the future, foraminiferal specimens recorded as part of this work will be measured to assess how population sizes differ under elevated CO_2 and temperature.

8.2.2 Effects of ocean acidification of foraminiferal test porosity

Initial observations on *H. germanica* revealed test dissolution and reduction of functional morphology under elevated CO_2. Time and financial restrictions meant that other important areas of the test, e.g. pores, were not investigated at this time. Pore size and shape are considered important in taxonomic considerations of

foraminifera (Bé et al., 1979). Both porosity and pore arrangement are relatively constant within a species (Hofker, 1951). The pores in species such as *Elphidium* may also serve a digestive function as they are fossettes lined with tooth-like borders (Faber & Preisig, 1994).

Ocean acidification effects on foraminiferal shell porosity could have implications for both foraminiferal feeding and gas exchange. Acidified seawater probably diffuses through the pores of the external walls towards the inner organic lining. To identify porosity responses to ocean acidification the pores of live foraminiferal specimens could be assessed with pore diameters measured for chambers constructed under each of the experimental conditions.

8.2.3 Effects of ocean acidification on foraminiferal reproduction

The effects of ocean acidification on reproduction and different life stages can sum to significant impacts on population success (Barry et al., 2011). It is important to include the whole life cycle of an organism to understand ocean acidification effects on various life-history stages, including carry over effects between consecutive life stages. Early life stages are often the most vulnerable part of an organism's life cycle so the impacts of ocean acidification could be hardest felt by juveniles (Widdicombe et al., 2009). For example, the juvenile life stages in fish became impaired under elevated pCO_2 (Chambers et al., 2013).

Foraminifera have short reproductive cycles (month to year) (Coccioni, 2000). During our 18 month incubation juvenile foraminiferal specimens were documented. In the future, it would be useful to assess these juveniles to establish if the reproductive strategy (asexual or sexual) and growth was affected by elevated pCO_2.

8.3 Conclusions

Following long-term exposure to elevated CO_2 and a seasonal temperature regime, living populations of foraminifera were still dominated by calcareous forms. During short-term exposure to reduced pH foraminiferal specimens (both alive and dead) were still intact with no obvious signs of fragmentation. Similar observations have been recorded in individual corals (*Montipora capitata*). This species was still able to deposit significant amounts of $CaCO_3$ and appeared healthy despite a low seawater carbonate saturation state, but was however at lower calcification rates compared to those grown under ambient conditions (Andersson et al., 2009).

Test dissolution was evident upon closer examination of functional ornamentation. It is unclear at this time whether these important morphological features are solely affected by dissolution under elevated CO_2 conditions or if the calcification process is also compromised. Further work is required in this area to make a distinction between ocean acidification effects on calcification and those on dissolution. Similar results have been observed in other organisms, e.g. the common limpet (*Patella vulgata*). In this species, dissolution of radula was recorded which could compromise the organisms feeding (Marchant et al., 2010). The reduction in test strength caused by thinning due to dissolution could lead to increased mechanical damage and breakages in the high energy intertidal environment. This in turn would lead to a further reduction in survival. Changes in the relative abundance and activities of species can also affect biological interactions (e.g. food web function) and habitat quality.

pH reduction has also been shown to induce the withdrawal of pseudopodia (Travis & Bowser, 1991). A reduction in pseudopodial activity and damage to morphological

ornamentation essential for feeding could result in a reduction in feeding efficiency (Le Cadre et al., 2003). We know very little about the trophic interactions of most foraminifera and the potential roles of competition and predation in structuring foraminiferal communities (Goldstein & Alve, 2011). Species that grew under experimental conditions e.g. *Haynesina* and *Elphidium*, utilize a range of nutritional resources with some degree of overlap and potential for competition (Goldstein & Alve, 2011). A reduced ability to assimilate food under elevated CO_2 conditions would have major implications for trophic interactions and inter-species competition. Further feeding experiments are required to assess any changes in dietary preference of these species under elevated CO_2.

Temperature is often the most important factor distinguishing experimental assemblages (Goldstein & Alve, 2011). A number of organisms are able to tolerate elevated CO_2 until increased temperature regimes are also included. As the abundance of dissolution-prone species gradually becomes reduced and biological and trophic interactions are affected, future oceans will have a different ecological balance. Many scientists predict that future oceans will be lacking in calcifiers e.g. no more oysters, bivalves etc. However, it is clear from current literature that there will be both winners and losers. Experimental time-series provide a valuable insight into how fauna changes through time in response to changing environmental (CO_2 and temperature) conditions. Data can be extrapolated for future ocean scenarios in addition to providing better interpretation and insight into the geological record. Future research is required to better understand which species will benefit from ocean acidification and the resulting shifts in community dynamics. For further research, there is a need to move towards more complex experimental designs that mimic realistic environmental conditions (Dupont & Pörtner, 2013).

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Appendix I. Seawater measurements from experimental mesocosms

Table S1. The measured values of temperature, salinity and pH (NBS scale) and total alkalinity (A_T) were used to calculate the values of dissolved inorganic carbon (DIC), pCO_2, saturation states of calcite ($\Omega_{Calcite}$) and aragonite ($\Omega_{Aragonite}$), bicarbonate (HCO_3^-) and carbonate concentration (CO_3^{2-}) using CO_2Calc with appropriate solubility constants (Mehrbach, 1973, refit by Dickson &Millero, 1987) and KSO_4 (Dickon, 1990).

Month	Date	Target CO_2 (ppm)	n	Temperature (°C)	Salinity	pH_{NBS}	A_T (µmol kg^{-1})	DIC (µmol kg^{-1})	pCO_2 (µAtm)	$\Omega_{Calcite}$	$\Omega_{Aragonite}$	HCO_3^-	CO_3^{2-}
1	11/02/11	380	3	10.27 ± 0.09	33.40 ± 0.06	8.13 ± 0.056	2665 ± 19	2479 ± 45	506 ± 71	3.54 ± 0.38	2.25 ± 0.24	2310 ± 58	147 ± 16
1	11/02/11	750	3	10.10 ± 0.00	33.60 ± 0.06	8.04 ± 0.021	2661 ± 12	2518 ± 19	633 ± 38	2.89 ± 0.12	1.83 ± 0.07	2370 ± 22	120 ± 5
1	11/02/11	1000	3	10.10 ± 0.06	33.53 ± 0.13	7.78 ± 0.015	2913 ± 221	2867 ± 224	1331 ± 142	1.82 ± 0.10	1.15 ± 0.06	2732 ± 214	76 ± 4
1	11/02/11	380	3	5.90 ± 0.06	33.50 ± 0.10	8.08 ± 0.016	2535 ± 27	2401 ± 22	513 ± 17	2.65 ± 0.11	1.67 ± 0.07	2265 ± 20	110 ± 5
1	11/02/11	750	3	5.80 ± 0.12	33.33 ± 0.12	7.89 ± 0.014	2596 ± 19	2533 ± 17	858 ± 27	1.79 ± 0.06	1.13 ± 0.04	2415 ± 15	74 ± 3
1	11/02/11	1000	3	5.80 ± 0.10	33.40 ± 0.06	7.74 ± 0.004	2692 ± 47	2677 ± 46	1271 ± 15	1.35 ± 0.04	0.85 ± 0.02	2556 ± 44	56 ± 2
3	06/04/11	380	4	9.58 ± 0.08	33.28 ± 0.09	8.10 ± 0.032	2836 ± 38	2665 ± 51	582 ± 53	3.42 ± 0.18	2.17 ± 0.12	2497 ± 56	142 ± 8
3	06/04/11	750	4	9.48 ± 0.05	33.26 ± 0.05	7.92 ± 0.043	2987 ± 55	2886 ± 39	953 ± 82	2.53 ± 0.29	1.60 ± 0.18	2738 ± 32	105 ± 12
3	06/04/11	1000	4	9.55 ± 0.05	33.13 ± 0.05	7.76 ± 0.011	2930 ± 59	2893 ± 61	1377 ± 56	1.74 ± 0.04	1.10 ± 0.02	2759 ± 58	72 ± 2
3	06/04/11	380	4	7.98 ± 0.02	33.23 ± 0.02	8.04 ± 0.060	2700 ± 20	2565 ± 41	642 ± 99	2.81 ± 0.34	1.77 ± 0.21	2418 ± 50	117 ± 14

Table S1 continued:

3	06/04/11	750	4	7.88 ± 0.03	33.20 ± 0.04	7.88 ± 0.048	2735 ± 20	2665 ± 35	975 ± 107	1.98 ± 0.21	1.25 ± 0.14	2537 ± 38	82 ± 9
3	06/04/11	1000	4	7.90 ± 0.06	33.23 ± 0.07	7.70 ± 0.010	2864 ± 25	2856 ± 24	1541 ± 34	1.40 ± 0.04	0.88 ± 0.02	2725 ± 23	58 ± 2
5	03/06/11	380	3	9.93 ± 0.26	33.37 ± 0.24	7.99 ± 0.083	3089 ± 95	2949 ± 61	845 ± 159	3.15 ± 0.59	2.00 ± 0.38	2781 ± 46	131 ± 25
5	03/06/11	750	3	10.23 ± 0.19	32.50 ± 0.50	7.89 ± 0.041	2997 ± 32	2911 ± 26	1057 ± 93	2.36 ± 0.21	1.49 ± 0.13	2767 ± 24	98 ± 8
5	03/06/11	1000	3	10.17 ± 0.12	32.77 ± 0.12	7.74 ± 0.014	3083 ± 31	3053 ± 31	1550 ± 55	1.76 ± 0.06	1.11 ± 0.04	2912 ± 30	73 ± 2
5	03/06/11	380	3	13.67 ± 0.12	33.17 ± 0.18	8.00 ± 0.026	2980 ± 121	2822 ± 112	801 ± 51	3.36 ± 0.26	2.14 ± 0.17	2651 ± 103	139 ± 11
5	03/06/11	750	3	13.63 ± 0.07	33.07 ± 0.03	7.94 ± 0.082	3816 ± 485	3651 ± 421	1194 ± 110	4.04 ± 1.26	2.58 ± 0.80	3437 ± 373	168 ± 52
5	03/06/11	1000	3	13.67 ± 0.07	33.00 ± 0.10	7.73 ± 0.009	3261 ± 148	3215 ± 148	1730 ± 90	2.06 ± 0.10	1.31 ± 0.06	3062 ± 140	85 ± 4
6	05/07/11	380	4	9.83 ± 0.08	31.85 ± 0.16	8.04 ± 0.033	3057 ± 135	2913 ± 132	749 ± 74	3.19 ± 0.24	2.02 ± 0.15	2748 ± 126	132 ± 10
6	05/07/11	750	4	9.55 ± 0.09	31.53 ± 0.14	7.90 ± 0.004	3163 ± 93	3076 ± 89	1068 ± 26	2.45 ± 0.10	1.55 ± 0.06	2927 ± 84	101 ± 4
6	05/07/11	1000	4	9.58 ± 0.05	31.40 ± 0.06	7.76 ± 0.009	3217 ± 72	3185 ± 72	1527 ± 44	1.85 ± 0.06	1.17 ± 0.04	3040 ± 69	76 ± 2
6	05/07/11	380	4	15.90 ± 0.00	32.13 ± 0.09	7.97 ± 0.043	3309 ± 272	3140 ± 242	975 ± 37	3.81 ± 0.64	2.44 ± 0.41	2948 ± 217	157 ± 26
6	05/07/11	750	4	15.95 ± 0.03	31.75 ± 0.10	7.89 ± 0.035	3767 ± 361	3633 ± 339	1393 ± 77	3.57 ± 0.55	2.28 ± 0.35	3435 ± 316	146 ± 23
6	05/07/11	1000	4	15.98 ± 0.02	31.68 ± 0.05	7.83 ± 0.022	3988 ± 360	3881 ± 343	1708 ± 79	3.32 ± 0.46	2.12 ± 0.30	3682 ± 321	136 ± 19
9	21/10/11	380	3	9.70 ± 0.06	30.07 ± 0.03	8.01 ± 0.000	2743 ± 98	2631 ± 95	725 ± 25	2.58 ± 0.10	1.62 ± 0.06	2493 ± 90	105 ± 4
9	21/10/11	750	3	9.77 ± 0.09	30.33 ± 0.03	7.85 ± 0.014	2993 ± 60	2933 ± 58	1161 ± 36	2.05 ± 0.08	1.29 ± 0.05	2796 ± 55	84 ± 3
9	21/10/11	1000	3	9.70 ± 0.00	30.47 ± 0.03	7.72 ± 0.005	2699 ± 19	2685 ± 18	1430 ± 11	1.39 ± 0.02	0.88 ± 0.01	2563 ± 17	57 ± 1
9	21/10/11	380	3	10.60 ± 0.06	30.60 ± 0.06	7.92 ± 0.039	2376 ± 103	2294 ± 97	778 ± 71	2.00 ± 0.00	1.24 ± 0.13	2180 ± 91	80 ± 8

Table S1 continued:

9	21/10/11	380	3	10.60 ± 0.06	30.60 ± 0.06	7.92 ± 0.039	2376 ± 103	2294 ± 97	778 ± 71	2.00 ± 0.00	1.24 ± 0.13	2180 ± 91	80 ± 8
9	21/10/11	750	3	10.60 ± 0.00	30.63 ± 0.13	7.82 ± 0.008	2799 ± 25	2748 ± 25	1187 ± 24	1.84 ± 0.04	1.16 ± 0.02	2621 ± 23	75 ± 2
9	21/10/11	1000	3	10.63 ± 0.03	30.57 ± 0.07	7.73 ± 0.015	3037 ± 274	3016 ± 275	1601 ± 162	1.65 ± 0.15	1.04 ± 0.10	2879 ± 262	67 ± 6
11	01/12/11	380	3	9.43 ± 0.03	33.00 ± 0.00	7.90 ± 0.007	2620 ± 52	2537 ± 50	874 ± 18	2.07 ± 0.06	1.31 ± 0.04	2412 ± 47	86 ± 3
11	01/12/11	750	3	9.40 ± 0.00	33.07 ± 0.03	7.78 ± 0.004	2852 ± 56	2808 ± 56	1269 ± 33	1.76 ± 0.03	1.11 ± 0.02	2678 ± 54	73 ± 1
11	01/12/11	1000	3	9.40 ± 0.00	32.97 ± 0.03	7.67 ± 0.009	2798 ± 121	2794 ± 119	1644 ± 39	1.34 ± 0.08	0.85 ± 0.05	2664 ± 114	56 ± 4
11	01/12/11	380	3	7.73 ± 0.03	32.80 ± 0.00	7.95 ± 0.032	2469 ± 54	2381 ± 46	727 ± 47	2.03 ± 0.17	1.28 ± 0.10	2262 ± 42	84 ± 7
11	01/12/11	750	3	7.80 ± 0.00	33.00 ± 0.06	7.70 ± 0.014	2505 ± 137	2494 ± 140	1330 ± 106	1.23 ± 0.06	0.77 ± 0.04	2380 ± 134	51 ± 2
11	01/12/11	1000	3	7.90 ± 0.06	32.97 ± 0.09	7.63 ± 0.005	2651 ± 106	2667 ± 105	1700 ± 48	1.10 ± 0.06	0.69 ± 0.04	2540 ± 101	45 ± 2
12	09/01/12	380	4	9.73 ± 0.03	33.23 ± 0.02	8.03 ± 0.022	2890 ± 59	2747 ± 52	700 ± 34	3.05 ± 0.19	1.93 ± 0.12	2589 ± 47	127 ± 8
12	09/01/12	750	3	9.70 ± 0.00	33.30 ± 0.06	7.87 ± 0.004	2974 ± 74	2894 ± 73	1067 ± 31	2.25 ± 0.05	1.42 ± 0.03	2753 ± 70	93 ± 2
12	09/01/12	1000	4	9.63 ± 0.09	33.28 ± 0.09	7.81 ± 0.012	2989 ± 64	2932 ± 61	1249 ± 30	1.98 ± 0.09	1.25 ± 0.05	2794 ± 58	82 ± 4
12	09/01/12	380	4	5.95 ± 0.03	33.15 ± 0.03	8.03 ± 0.021	2738 ± 27	2619 ± 22	634 ± 31	2.57 ± 0.13	1.62 ± 0.08	2480 ± 19	107 ± 5
12	09/01/12	750	4	6.05 ± 0.03	33.23 ± 0.06	7.86 ± 0.011	2992 ± 21	2933 ± 18	1064 ± 25	1.96 ± 0.06	1.23 ± 0.04	2799 ± 17	81 ± 3
12	09/01/12	1000	4	6.00 ± 0.04	33.13 ± 0.03	7.80 ± 0.011	3078 ± 78	3043 ± 76	1273 ± 38	1.76 ± 0.07	1.11 ± 0.05	2906 ± 73	73 ± 3

Table S1 continued:

15	27/04/12	380	3	10.60 ± 0.00	32.40 ± 0.00	8.31 ± 0.155	2555 ± 55	2274 ± 107	341 ± 120	5.07 ± 1.53	3.21 ± 0.97	2049 ± 159	210 ± 63
15	27/04/12	750	3	10.67 ± 0.03	32.50 ± 0.06	7.86 ± 0.012	3018 ± 67	2943 ± 69	1149 ± 55	2.24 ± 0.03	1.42 ± 0.02	2800 ± 66	92 ± 1
15	27/04/12	1000	3	10.60 ± 0.00	32.27 ± 0.23	7.72 ± 0.021	2927 ± 61	2903 ± 62	1548 ± 83	1.62 ± 0.08	1.02 ± 0.05	2769 ± 59	67 ± 3
15	27/04/12	380	3	11.47 ± 0.03	32.43 ± 0.03	8.02 ± 0.042	2836 ± 251	2690 ± 223	713 ± 30	3.11 ± 0.57	1.98 ± 0.36	2531 ± 201	129 ± 24
15	27/04/12	750	3	11.43 ± 0.03	32.47 ± 0.09	7.84 ± 0.037	3256 ± 216	3178 ± 197	1296 ± 33	2.43 ± 0.36	1.54 ± 0.23	3023 ± 183	100 ± 15
15	27/04/12	1000	3	11.50 ± 0.00	32.37 ± 0.07	7.79 ± 0.089	2947 ± 161	2891 ± 156	1375 ± 260	2.03 ± 0.45	1.29 ± 0.28	2749 ± 150	84 ± 18
16	25/05/12	380	3	10.70 ± 0.00	32.50 ± 0.00	8.21 ± 0.020	2589 ± 90	2374 ± 78	397 ± 14	3.98 ± 0.29	2.52 ± 0.18	2192 ± 67	165 ± 12
16	25/05/12	750	3	10.77 ± 0.03	32.50 ± 0.00	7.80 ± 0.033	3027 ± 88	2970 ± 75	1311 ± 66	2.03 ± 0.20	1.29 ± 0.20	2829 ± 69	84 ± 8
16	25/05/12	1000	3	10.70 ± 0.00	32.60 ± 0.00	7.76 ± 0.020	2973 ± 41	2932 ± 36	1419 ± 55	1.81 ± 0.00	1.15 ± 0.06	2795 ± 34	75 ± 4
16	25/05/12	380	3	11.03 ± 0.09	32.43 ± 0.07	8.08 ± 0.099	2488 ± 63	2327 ± 19	556 ± 125	3.13 ± 0.70	1.99 ± 0.44	2174 ± 8	130 ± 29
16	25/05/12	750	3	10.90 ± 0.00	32.73 ± 0.07	7.78 ± 0.047	3040 ± 193	2988 ± 175	1385 ± 70	1.99 ± 0.31	1.26 ± 0.19	2846 ± 165	82 ± 13
16	25/05/12	1000	3	10.93 ± 0.03	32.73 ± 0.13	7.76 ± 0.028	2692 ± 157	2653 ± 154	1305 ± 96	1.64 ± 0.15	1.04 ± 0.10	2529 ± 147	68 ± 6
17	21/06/12	380	3	10.57 ± 0.03	33.27 ± 0.03	8.12 ± 0.055	2633 ± 38	2454 ± 47	520 ± 76	3.43 ± 0.36	21.7 ± 0.23	2289 ± 54	142 ± 15
17	21/06/12	750	3	10.60 ± 0.00	33.13 ± 0.03	7.97 ± 0.148	2907 ± 64	2773 ± 20	914 ± 256	3.05 ± 1.08	1.94 ± 0.68	2607 ± 46	127 ± 45
17	21/06/12	1000	3	10.60 ± 0.00	33.20 ± 0.00	7.74 ± 0.009	2830 ± 11	2797 ± 13	1429 ± 34	1.64 ± 0.03	1.04 ± 0.02	2667 ± 13	68 ± 1
17	21/06/12	380	3	13.27 ± 0.03	33.10 ± 0.00	7.54 ± 0.352	2627 ± 301	2676 ± 428	3768 ± 1884	1.66 ± 1.12	1.06 ± 0.72	2458 ± 393	69 ± 47
17	21/06/12	750	3	13.20 ± 0.00	33.00 ± 0.00	7.76 ± 0.058	2822 ± 230	2765 ± 209	1361 ± 92	1.95 ± 0.37	1.24 ± 0.24	2630 ± 196	81 ± 15
17	21/06/12	1000	3	13.20 ± 0.00	33.00 ± 0.00	7.72 ± 0.049	2638 ± 145	2599 ± 129	1417 ± 91	1.64 ± 0.24	1.05 ± 0.15	2475 ± 123	68 ± 10

Table S1 continued:

18	04/07/12	380	4	10.18 ± 0.12	33.30 ± 0.08	8.07 ± 0.026	2738 ± 80	2578 ± 76	599 ± 41	3.20 ± 0.21	2.03 ± 0.13	2419 ± 71	133 ± 9
18	04/07/12	750	3	10.07 ± 0.03	33.53 ± 0.03	7.89 ± 0.003	3086 ± 97	2992 ± 96	1054 ± 39	2.48 ± 0.07	1.57 ± 0.05	2843 ± 91	103 ± 3
18	04/07/12	1000	3	10.10 ± 0.06	33.50 ± 0.00	7.77 ± 0.014	2922 ± 11	2877 ± 15	1346 ± 48	1.81 ± 0.04	1.15 ± 0.03	2743 ± 15	75 ± 2
18	04/07/12	380	4	14.70 ± 0.00	33.75 ± 0.06	7.99 ± 0.031	2598 ± 182	2448 ± 167	713 ± 50	3.02 ± 0.36	1.93 ± 0.23	2296 ± 153	126 ± 15
18	04/07/12	750	4	14.68 ± 0.02	33.80 ± 0.04	7.80 ± 0.043	2946 ± 167	2864 ± 158	1314 ± 109	2.32 ± 0.27	1.49 ± 0.18	2718 ± 149	97 ± 11
18	04/07/12	1000	4	14.70 ± 0.00	33.75 ± 0.05	7.73 ± 0.042	2848 ± 167	2795 ± 152	1508 ± 71	1.93 ± 0.25	1.24 ± 0.16	2657 ± 144	80 ± 10

Appendix II. Faunal List of Foraminifera

Ammonia falsobeccarii (Rouvillois, 1974)

Amphicoryna scalaris (Batsch, 1791)

Bolivina difformis (Williamson, 1858)

Bolivina pseudoplicata (Heron-Allen and Earland, 1930)

Bolivina pseudopunctata (Höglund, 1947)

Bolivina skagerrakensis (Qvale and Nigam, 1985)

Bulimina elongata (d'Orbigny, 1826)

Bulimina gibba (Fornasini, 1902)

Bulimina marginata (d'Orbigny, 1826)

Cassidulina obtusa (Williamson, 1858)

Cassidulina reniforme (Nørvangi, 1945)

Cassidulinoides bradyi (Norman, 1881)

Cibicides lobatulus (Walker and Jacob, 1798)

Cyclogyra involvens (Reuss, 1850)

Eggerella scabra (Williamson, 1858)

Elphidium albiumbilicatum (Weiss, 1954)

Elphidium excavatum (Terquem, 1875)

Elphidium oceanensis (d'Orbigny, 1826)

Elphidium williamsoni (Haynes, 1973)

Epistominella exigua (Brady, 1884)

Fissurina elliptica (Cushman, 1923)

Fissurina lucida (Williamson, 1848)

Fissurina serrata (Schlumberger, 1894)

Haynesina germanica (Ehrenberg, 1840)

Heterohelix striata (Ehrenberg, 1838)

Lagena gracillima (Seguenza, 1862)

Lagena sp. cf. *L. laevis* (Montagu, 1803)

Lagena mollis (Cushman, 1944)

Lagena setigera (Millet, 1901)

Lagena sulcata var. *torquiformis* (Haynes, 1973)

Lamarckina haliotidea (Heron-Allen and Earland, 1911)

Miliammina fusca (Brady, 1870)

Nonionella turgida (Williamson, 1858)

Oolina hexagona (Williamson, 1848)

Quinqueloculina bicornis (Walker and Jacob, 1798)

Quinqueloculina sp. cf. *Q*. *lata* (Terquem, 1876)

Quinqueloculina seminulum (Linnaeus, 1758)

Rosalina anomala (Terquem, 1875)

Textularia earlandi (Parker, 1952)

Stainforthia fusiformis (Williamson, 1848)

Trochammina sp. aff. *T. ochracea* (Williamson, 1858)

Appendix III: Associated Paper

The following article is included as Appendix III:

Khanna, N., Godbold, J. A., Austin, W. E. N. & Paterson, D. M. 2013. The impact of ocean acidification on the functional morphology of foraminifera. *PLoS ONE* **8** (12): e83118. DOI:10.1371/journal.pone.0083118.

An error occurred during production in which a sentence was deleted in the second paragraph of the Results section.

The first sentence of the paragraph currently reads: ‘2A).’

It should read: ‘Control specimens of H. germanica maintained at 380 ppm possessed teeth that were both numerous and conical in shape (Fig, 2A)’.

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PLOS ONE

The Impact of Ocean Acidification on the Functional Morphology of Foraminifera

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Abstract

Culturing experiments were performed on sediment samples from the Ythan Estuary, N. E. Scotland, to assess the impacts of ocean acidification on test surface ornamentation in the benthic foraminifer *Haynesina germanica*. Specimens were cultured for 36 weeks at either 380, 750 or 1000 ppm atmospheric CO_2. Analysis of the test surface using SEM imaging reveals sensitivity of functionally important ornamentation associated with feeding to changing seawater CO_2 levels. Specimens incubated at high CO_2 levels displayed evidence of shell dissolution, a significant reduction and deformation of ornamentation. It is clear that these calcifying organisms are likely to be vulnerable to ocean acidification. A reduction in functionally important ornamentation could lead to a reduction in feeding efficiency with consequent impacts on this organism's survival and fitness.

Citation: Khanna N, Godbold JA, Austin WEN, Paterson DM (2013) The Impact of Ocean Acidification on the Functional Morphology of Foraminifera. PLoS ONE 8(12): e83118. doi:10.1371/journal.pone.0083118

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Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Elevated atmospheric concentrations of carbon dioxide (CO_2), primarily driven by anthropogenic activity, are driving down the pH of the oceans [1]. It is estimated that the oceans have absorbed half of the total anthropogenic CO_2 produced in the last 200 years, reducing oceanic pH by 0.1 units [1] and simultaneously affecting the carbonate ion concentration [2]. A further decrease of 0.3–0.5 pH units is predicted by 2100 [3]. One of the most significant implications is the likely reduction in calcifying capacity of marine organisms [4,5]. The experimental responses of organisms that produce carbonate structures have however been variable depending on the type and length of exposure [6,7].

Foraminifera are amoeboid protozoa that constitute one the most widespread and diverse groups of shelled microorganisms in the modern oceans. They occur in planktic and benthic habitats from the intertidal coastal habitats to the deep sea [8]. The majority of benthic foraminifera build their tests (shells) with calcium carbonate ($CaCO_3$) [9] playing an important role in the carbon cycle of intertidal estuarine sediments [10].

The evolutionary and ecological success of shelled foraminifera depends on the functional significance of the test [11,12]. The test provides shelter and protection from predators, assistance in cell growth, aids in reproduction, acts as a receptacle for excreted matter, and provides buoyancy control [13–15]. There are also morphological features and ornamentation on the test that are of functional importance. For example, tubercles and teeth in the apertures (Fig. 1) of some species are important in feeding and serve to break up aggregates of food and detritus [16]. Tubercles are protrusions that border the chamber margins while teeth are similar protrusions that are found adjacent to the aperture, occupying part of the apertural space [17]. Hamm et al. [18] speculated that diatom grazers are likely to have evolved specialised tools to break open frustules. Austin et al. [12] documented extracellular cracking and removal of diatom contents, aided by the presence of 'tooth-like' dentition in *Haynesina germanica* (Ehrenberg).

To date, researchers have examined ocean acidification impacts on benthic foraminifera via test weight, thickness and growth rate [19–21]. However, there is currently no information on how foraminiferal test functional morphology might respond to ocean acidification.

Here we present ultrastructural observations using scanning electron microscopy of cultured *H. germanica* to examine the effect of different levels of atmospheric CO_2 on test morphology, focussing on the apertural region known to have functional ornamentation [12].

Materials and Methods

Sediment was collected from the Ythan Estuary, N.E. Scotland (57°20′N, 01°57′W), in December 2010. No specific permissions were required for sediment sampling at this location and the sampling did not involve endangered or protected species. Sieved sediment (500 μm), was added to acrylic mesocosms (12×12×33 cm) to a depth of 12 cm and 20 cm of overlying seawater. Mesocosms were exposed to a seasonally varying light cycle and maintained at 10°C (average local conditions). Seawater

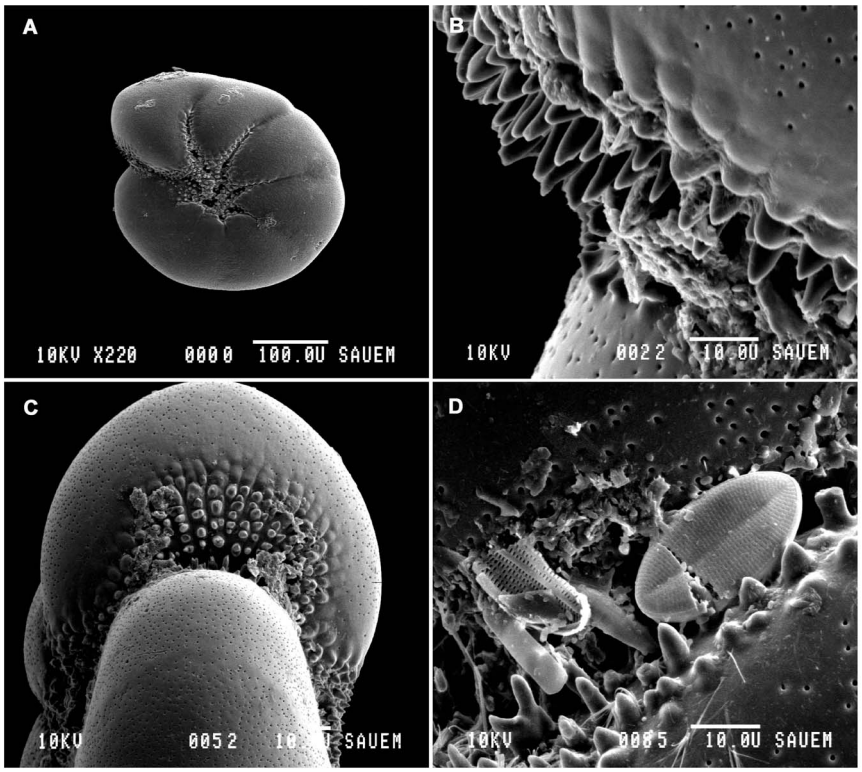

Figure 1. Scanning electron micrographs of *Haynesina germanica*. (A) Scanning electron micrograph of typical test, side view. (B) Higher magnification view of apertural region, showing tubercles and teeth lining the aperture. (C) Scanning electron micrograph of typical test, apertural view. (D) Higher magnification view of apertural region. Note impaled diatom (*).
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pH was controlled by bubbling air with a known concentration of CO_2 (380, 750, 1000 ppm) into each mesocosm [22]. Seawater pH, temperature and salinity were monitored weekly and atmospheric CO_2 was maintained at 380, 750 or 1000 ppm (Table S1). After 36 weeks of exposure, surface scrapes of 1–2 mm depth were collected from each of the treatments for the isolation of living benthic foraminifera.

The surface scrapes were fixed in ethanol and stained with rose Bengal (to distinguish live individuals). After 24 h, samples were washed over a 63 μm sieve and the coarse fraction dried (<40°C). 15 live adult specimens of *Haynesina germanica* (300–400 μm), five from each CO_2 treatment, were selected using a fine paintbrush under an Olympus® SZ stereo-microscope and dry-stored on micropalaeontological slides prior to imaging with a scanning electron microscope (SEM). The foraminiferal specimens were mounted onto SEM stubs using double-sided adhesive tabs. Samples were prepared with an Emscope® SC 500 sputter coater and imaged with a scanning electronic microscope (Joel® JSM-35CF SEM). For each specimen, the number of teeth in a 20 μm^2 region of the apertural area were counted and measured (SemAfore®) for maximum length and width (Table 1). Once assumptions were confirmed, a one-way ANOVA was used to determine the effects of CO_2. Analyses were performed using the statistical programming software 'R' [23].

Results

SEM imaging of *Haynesina germanica* revealed differences between specimens cultured in each of the CO_2 treatments. There was a statistically significant difference in the average number ($df = 2$, $F = 9.8369$, $p<0.05$), length ($df = 2$, $F = 9.6467$, $p<0.0001$) and width ($df = 2$, $F = 4.0073$, $p<0.05$) of teeth.

. 2A). Several specimens also had diatoms impaled on teeth close to the apertural region. In addition, the test surface was smooth and unblemished (Fig. 2B).

At 750 ppm, specimens displayed similar shell appearance to those observed at 380 ppm (Fig. 2C). The teeth were of a similar size and shape but in many cases there was a reduction in overall number (10.80 ± 3.96) in comparison to the control (15.20 ± 2.49). The test surfaces also appeared smooth although some signs of cracking were evident (Fig. 2D).

At the highest CO_2 treatment (1000 ppm), there were marked signs of dissolution and deformation features on multiple individuals in comparison to those cultured at 380 and 750 ppm. Dissolution was evident on the test surface (Fig. 2E, F) and in many cases the teeth were completely absent (Fig. 2E). In those specimens from the high CO_2 treatment that retained some ornamentation, the shape and appearance was highly irregular. Teeth were less conical and more rounded in shape. In addition, the entire test surface appeared to be partially dissolved with evidence of cracking (Fig. 2F), which was entirely absent from control specimens.

Discussion

Measurement data demonstrate that test ornamentation of functional importance for *Haynesina germanica* is sensitive to decreasing seawater pH. Under ambient conditions, the test surface of *H. germanica* is smooth with the primary openings along the base of the apertural face being obscured externally by teeth that are usually conical in shape. Foraminifera maintained at

Table 1. Summary of main morphological features at each CO_2 treatment.

CO_2 Treatment (ppm)	average number of *"teeth" (mean± SD)*	average length of *"teeth" (mean± SD)*	average width of *"teeth" (mean± SD)*	observational notes on *"teeth"*	test surface observations	number of diatom frustules in apertural region
380	15.20±2.49 (n = 76)	6.53±2.13	3.27±1.00	Teeth predominantly conical in shape	Test surfaces smooth with little evidence of dissolution	7
750	10.80±3.96 (n = 54)	5.26±1.79	3.27±1.16	Teeth conical in shape. A number of broken teeth also observed.	Some small areas of dissolution and pitting evident.	2
1000	6.60±2.51 (n = 33)	5.01±1.94	3.99±1.97	Teeth tended to be fewer and generally more rounded in shape. Of those counted a number were broken or deformed.	Dissolved patches more extensive and numerous in addition to visible cracking	0

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ambient CO_2 levels had tubercles and teeth that were numerous and very well developed in both the apertural and umbilical regions. At higher CO_2 levels the functional ornamentation of the apertural area was vastly reduced and the extent to which these apertural and umbilical features were dissolved or damaged makes the foraminifera almost unrecognizable as *H. germanica* (Fig. 2).

One of the keys to evolutionary success of foraminifera are the finely tuned relationships they have developed to exploit the food resources of their community [24]. Bernard and Bowser [16] document the significance of foraminiferal test functional morphology in species known to sequester chloroplasts, and showed that the external test ornamentations facilitate separation of chloroplasts from algal prey. As particles are moved past the tubercles, they are sorted by size, where larger fragments become disaggregated in preparation for later ingestion. Austin et al. [12]

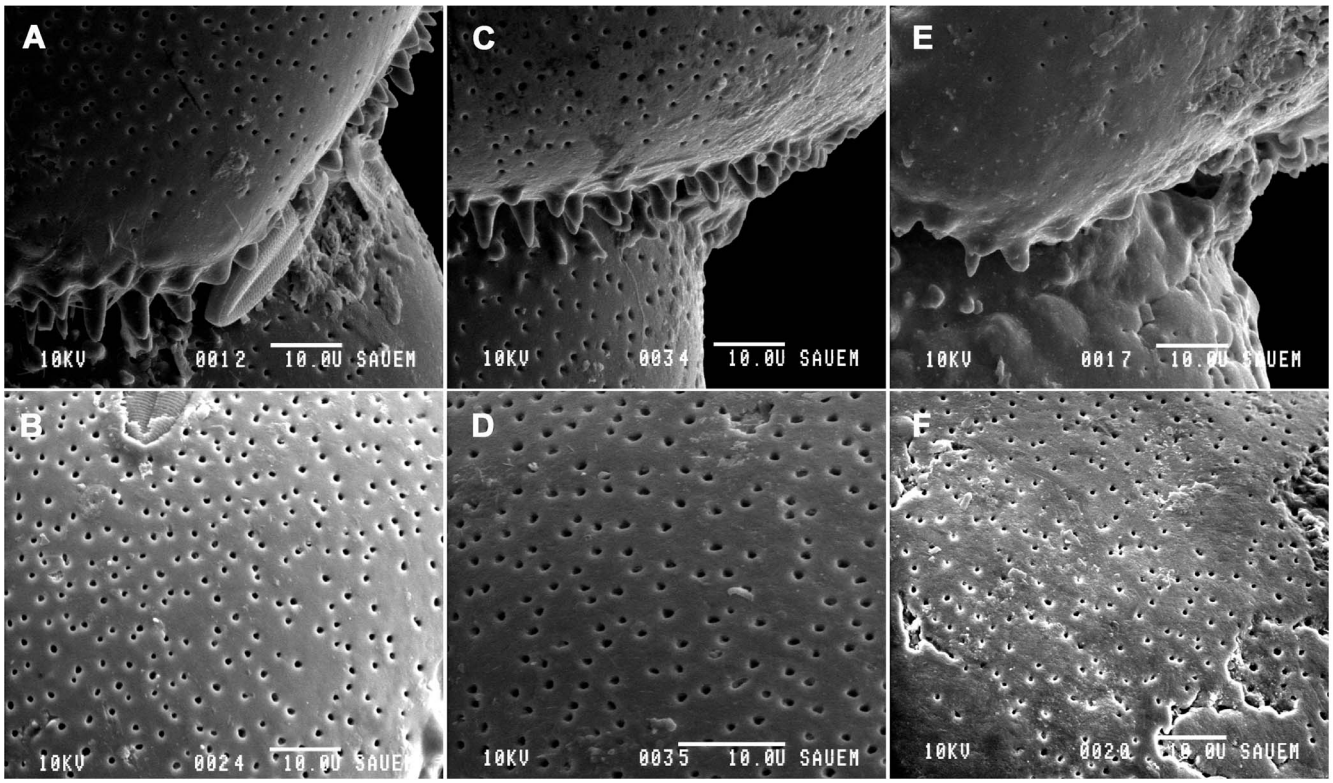

Figure 2. Scanning electron micrographs of *Haynesina germanica* following exposure to each of the CO_2 treatments. SEM images of specimens taken at 380 ppm (A & B), 750 ppm (C & D) and 1000 ppm (E & F). (A) Side view of apertural region showing numerous sharp tubercles. Note diatom impaled on ornamentation (*). (B) Scanning electron micrograph of test surface of specimen 2A. (C) Side view of apertural region, showing tubercles and teeth. (D) Scanning electron micrograph of test surface of specimen 2C. (E) Side view of apertural region. Note the distinct absence of the numerous conical tubercles present in 2A. (F) Scanning electron micrograph of test surface of specimen 2E. Note surface dissolution and cracking damage.
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showed a direct relationship between fracturing in diatoms and feeding sequestration by *H. germanica*.

Test ornamentation, notably around the apertural area, is therefore crucial for feeding and chloroplast acquisition in *H. germanica*. Dissolution of these features at anticipated future levels of atmospheric CO_2 will therefore have a direct negative impact on the long-term fitness and survival on these organisms through a reduction in feeding efficiency.

The potential for deformation, dissolution and in some cases absence of these functionally important feeding structures, may result in a shift in competitive advantage towards non-calcifiers in the benthic foraminiferal community under conditions of enhanced ocean acidification. This shift in community structure is likely to be further enhanced as a result of weakened tests under high-CO_2 scenarios, making calcifying foraminifera such as *H. germanica* more vulnerable to predators. Such shifts in community structure will have significant knock-on effects for trophic dynamics, carbon cycling and ecosystem productivity.

Supporting Information

Table S1 Seawater measurements from experimental mesocosms. The measured values of temperature, salinity and pH (NBS scale) and total alkalinity (A_T) were used to calculate the values of dissolved inorganic carbon (DIC), pCO_2, saturation states of calcite ($\Omega_{calcite}$) and aragonite ($\Omega_{aragonite}$), bicarbonate (HCO_3) and carbonate concentration (CO_3^{2-}) using CO_2Calc.
(PDF)

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Author Contributions

Conceived and designed the experiments: NK JAG DP. Performed the experiments: NK JAG. Analyzed the data: NK JAG DP WENA. Contributed reagents/materials/analysis tools: NK JAG. Wrote the paper: NK JAG WENA DP.

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