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The Effects of Ocean Acidification on Modern Benthic Foraminifera

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FORAMINIFERA

by

Laura R. Pettit

A thesis submitted to Plymouth University

in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

School of Marine Science and Engineering Doctoral Training Centre

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Abstract

Ocean acidification may cause biodiversity loss, alter ecosystems and impact food security, yet uncertainty over ecological responses to ocean acidification remains considerable. Most work on the impact of ocean acidification on foraminifera has been short-term laboratory experiments on single species. To expand this, benthic foraminiferal assemblages were examined across shallow water CO_2 gradients in the Gulf of California, off the islands of Ischia and Vulcano in Italy and off Papua New Guinea. Living assemblages from the Gulf of California did not appear to show a response across a pH range of 7.55 – 7.88, although the species assemblage was impoverished in all locations and the dead assemblage was less diverse at the lowest pH sites where there was evidence of post mortem dissolution. At Vulcano, the small macroalga, *Padina pavonica*, did not protect calcareous foraminifera from the adverse effects of ocean acidification. Calcareous taxa disappeared from the assemblage and were replaced by agglutinated foraminifera as mean pH reduced from 8.19 to 7.71. Settlement of benthic foraminifera onto artificial collectors off Vulcano was adversely affected in the acidified water, with few species as $p$$CO_2$ increased and evidence of post-mortem dissolution. The foraminiferal tests, collected off Papua New Guinea, had lower $\delta^{11}B$ as mean pH decreased from 7.99 – 7.82 for small (250 – 500 μm) *Amphistegina lessonii*, but not for *A. lessonii* or *Calcarina spengleri* >500 μm. In the larger foraminifera, photosynthetic activity by symbionts may begin to dominate the boron isotopic signature. Overall, the responses of foraminiferal assemblages to ocean acidification are complex, but there was an overall reduction in species diversity in infaunal, epifaunal and epiphytic assemblages as $p$$CO_2$ increased. This raises serious concerns for the survival of shallow water calcareous benthic foraminifera as the oceans continue to acidify, with implications for benthic ecosystems and inorganic carbon cycling.

i

Table of Contents

List of Figures

x

List of Tables

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Author's declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

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

Relevant scientific seminars and conferences were regularly attended at which work was often presented; external institutions were visited for consultation purposes. One paper has been accepted for publication in a refereed journal.

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Publications and conferences

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Conference presentations:

2010

September: Joint EPOCA/BIOACID and UKOARP meeting, Bremerhaven, Germany.

Oral presentation: Assessing the Impacts of Ocean Acidification at Volcanic CO_2 Vents.

December: The Marine Institute 3rd Annual Conference – The Spirit of Discovery, Plymouth University, Plymouth, UK.

Poster presentation: Volcanic CO_2 vents reveal the ecosystem effects of ocean acidification.

2011

January: The First Annual UKOARP Science Meeting, Cambridge, UK.

Oral presentation: Assessing the effects of long term ocean acidification at volcanic CO_2 vents.

December: The Palaeontological Association 55th Annual Conference, Plymouth, UK.

Poster presentation: Foraminifera resist ocean acidification in the Wagner Basin under conditions similar to high CO_2 environments of the Cretaceous-Paleogene.

2012

April: UKOA 2nd Annual Science Meeting, Exeter, UK.

Oral presentation: Assessing the effects of long-term ocean acidification on benthic foraminifera using natural CO_2 gradients.

2013

July: UKOA 3rd Annual Science Meeting, St Andrews, UK.

Poster presentation: Benthic foraminifera along shallow water CO_2 gradients.

Workshops attended:

2010

October: 2nd International Workshop - Research in Shallow Marine and Fresh Water Systems, Milazzo, Italy.

2011

April: UKOARP carbonate chemistry facility training Workshop, Southampton, UK.

May: MedSeA Training Workshop, Vulcano, Italy.

2012

February: Potential environmental effects of CO_2 leakage in the marine and terrestrial environment: Understanding; Monitoring; Mitigation workshop, University of Nottingham, UK.

September: International Summer School for students: Climate Change in the Marine Realm, Bremen, Germany.

2013

March: Multivariate analysis for biologists, ecologists and environmental scientists, using PRIMER v.6, Marine Biological Association, Plymouth, UK.

May: COST Action Training School: Effects of increased CO2/OA on Seagrass Meadows, Vulcano, Italy.

2014

June: International School on Foraminifera, Urbino, Italy.

Abbreviations

Abbreviations, symbols and acronyms used in the text are defined below. Those in equations are defined *in situ*.

Abbreviation	Definition
$B(OH_3)$	Boric acid
$B(OH_4)^-$	Borate ion
Ca_2^+	Calcium ion
$CaCO_3$	Calcium carbonate
Chl *a*	Chlorophyll *a*
CO_2	Carbon dioxide
CO_3^{2-}	Carbonate ion
CPR	Continuous Plankton Recorder
DBL	Diffusion boundary layer
DIC	Dissolved inorganic carbon
FOCE	Free Ocean CO_2 Enrichment
H^+	Hydrogen ion
HCl	Hydrochloric acid
HCO_3^-	Bicarbonate ion
HEPA	High efficiency particulate air
HNO_3	Nitric acid
H_2CO_3	Carbonic acid
H_2O_2	Hydrogen peroxide
ICPMS	Inductively Coupled Plasma Mass Spectrometer
IPCC	Intergovernmental Panel on Climate Change
MC-ICPMS	Multi Collector - Inductively Coupled Plasma Mass Spectrometer
NH_4OH	Ammonium hydroxide
NIST	National Institute of Standards and Technology
PETM	Palaeocene-Eocene Thermal Maximum
SAHFOS	Sir Alister Hardy Foundation for Ocean Science
SD	Standard deviation
SEM	Scanning Electron Microscope
SNW	Size normalised weight
TA	Total Alkalinity
TPB	Total procedural blank
Ω	Calcium carbonate saturation state
Ω_{Arag}	Aragonite saturation state
Ω_{Calc}	Calcite saturation state

Chapter 1: The impact of ocean acidification on foraminifera

1.1 Ocean acidification

Over the past 200 years the oceans have absorbed approximately 30% of the carbon dioxide produced by anthropogenic emissions (Sabine et al., 2004, Raven et al., 2005, IPCC, 2014). This has resulted in a series of chemical changes to surface waters, including lowering the pH, in a process known as ocean acidification (Caldeira and Wickett, 2003). Surface ocean pH has fallen by approximately 0.1 units since 1800 to a current day global average of approximately 8.2 (Raven et al., 2005). This is equivalent to a 30% increase in the concentration of hydrogen ions (Raven et al., 2005, Guinotte and Fabry, 2008, IPCC, 2014). The rate and magnitude of present day ocean acidification is greater than any inferred from geological records of the past 300 million years with surface ocean pH predicted to fall by up to 0.77 units by 2250 (Caldeira and Wickett, 2003, Hönisch et al., 2012).

When CO_2 enters seawater it dissolves to form carbonic acid (H_2CO_3). A series of equilibrium reactions then occur (Equation 1.1) which results in an increase in hydrogen ions, this lowers the pH.

$$CO_2 + H_2O \leftrightarrow H_2CO_3$$
$$H_2CO_3 \leftrightarrow HCO_3^- + H^+$$
$$HCO_3^- \leftrightarrow CO_3^{2-} + H^+$$
$$CO_3^{2-} + H^+ \leftrightarrow HCO_3^- \qquad\qquad [1.1]$$
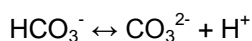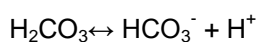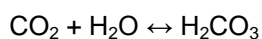
Adding CO_2 to seawater increases the concentration of carbonic acid, bicarbonate ions (HCO_3^-) and hydrogen ions (H^+), whilst decreasing the concentration of carbonate ions (CO_3^{2-}). When compared to pre-industrial levels, the concentration of carbonate ions globally, is predicted to half by the year 2100 (Tyrrell, 2008).

As part of an equilibrium reaction, carbonate ions react with some of the excess hydrogen ions. This reduces the hydrogen ion concentration and is referred to as the

carbonate buffering system, which has been responsible for maintaining surface ocean pH between 8.0 and 8.3 over the last 25 million years (Widdicombe and Spicer, 2008). The ability of the carbonate buffering system to limit changes in pH is dependent upon the supply of carbonate ions.

The present day chemical changes to surface oceans due to ocean acidification are expected to have significant consequences for marine organisms, particularly those that calcify (Guinotte and Fabry, 2008, Doney et al., 2009, Kroeker et al., 2010; 2013a). Calcium carbonate exists in two main structures; calcite and aragonite. Aragonite has orthorhombic symmetry in its structure whereas calcite is trigonal (Raven et al., 2005). The reduction in carbonate ions is lowering the saturation state of calcite and aragonite (Kleypas et al., 1999, IPCC, 2014). The saturation state of seawater with respect to calcium carbonate is defined by Equation 1.2:

$$\Omega = \frac{[Ca^{2+}]_{sw} \times [CO_3^{2-}]_{sw}}{K^*_{sp}} \qquad [1.2]$$

Where: Ω is the saturation state with respect to calcium carbonate

      $[Ca^{2+}]_{sw}$ is the calcium ion concentration in seawater

      $[CO_3^{2-}]_{sw}$ is the carbonate ion concentration in seawater

      K^*_{sp} is the stoichiometric solubility product and is calculated from $[Ca^{2+}]_{sat} \times [CO_3^{2-}]_{sat}$ which refers to the equilibrium ion concentrations in seawater that is saturated with $CaCO_3$

Calcium ions within seawater are abundant (contributing approximately 1.2% of salts in seawater) and their concentration remains stable on timescales of thousands of years (Tyrrell, 2008). This means that it is primarily the variation in carbonate ion concentration which leads to differences in the saturation state (Tyrrell, 2008, Doney et al., 2009). Saturation states (Ω) vary locally depending on salinity, temperature and pressure (Feely et al., 2004). Ocean waters with a Ω <1.0 are termed undersaturated and lead to carbonate dissolution, whereas those >1.0 are classed as supersaturated

and the precipitation of inorganic carbon is favoured (Guinotte et al., 2006, Fabry et al., 2008, Tyrrell, 2008, Jackson et al., 2014). Calcite and aragonite have different K^*_{sp} values and they have different propensities for dissolution. The saturation state of calcite is always higher than the saturation state of aragonite (Tyrrell, 2008). The solubility of high-magnesium calcite (high-Mg calcite), which some organisms secrete, can exceed that of aragonite (Morse et al., 2006), making it even less stable with future ocean acidification, if directly exposed to low pH conditions. As the saturation state reduces, biomineralisation is expected to become more energetically expensive (Miller et al., 2009). The response of marine calcifiers to decreasing calcium carbonate saturation state will be species-specific and will depend on environmental parameters such as light, temperature and available nutrients, the form of carbonate mineralogy and the mechanism of calcification (Feely et al., 2004). The substrate used by calcifying organisms for calcification is the subject of some debate. For some organisms it is not clear whether they use CO_3^{2-} or HCO_3^- (Paasche, 2001, Ries et al., 2009). If the substrate is HCO_3^- then calcification rates could increase with ocean acidification as the concentration of HCO_3^- increases. We know that some organisms are able to calcify in low pH environments, but dissolution rates increase dramatically at $\Omega < 1.0$ (Rodolfo-Metalpa et al., 2011).

Changes to seawater carbonate chemistry as a result of increasing carbon dioxide, are well documented (Doney et al., 2009). The biological effects, however, are much less understood and subject to current debate (e.g. Duarte et al., 2013, Harvey et al., 2013, Kroeker et al., 2013a). Broecker and Takahashi (1966) first proposed that increasing atmospheric carbon dioxide levels may affect the ability of marine organisms to calcify, but experiments on the biological response of organisms to ocean acidification did not begin until the late 1990s. Even small reductions in pH can have a significant impact on organisms and ocean acidification is known to impact reproduction (e.g. Albright et al., 2010, Ross et al., 2011, Doropoulos et al., 2012), growth (e.g. Albright et al., 2008, Dupont et al., 2008; 2010a, Range et al., 2011, Milazzo et al., 2014), photosynthesis

(e.g. Semesi et al., 2009a; 2009b, Johnson et al., 2012, Cornwall et al., 2014), metabolism (e.g. Albright and Langdon, 2011, Melatunan et al., 2011, Suggett et al., 2012, Calosi et al., 2013), behaviour (e.g. de la Haye et al., 2011, Donohue et al., 2012, Nilsson et al., 2012) and species interactions (e.g. Hall-Spencer et al., 2008, Martin et al., 2008, Kroeker et al., 2011; 2013b). Ocean acidification can affect an organism both through the effects of reduced pH and through the effects of increased carbon dioxide (Pörtner et al., 2004, Wood et al., 2008). Some photosynthetic organisms that are carbon limited can benefit from increased CO_2 (Connell and Russell, 2010, Manzello et al., 2012, Koch et al., 2013). Photosynthetic organisms that have been found to benefit from increased CO_2 include seagrasses (e.g. Hall-Spencer et al., 2008, Martin et al., 2008), macroalgae (e.g. Porzio et al., 2011, Johnson et al., 2012) and organisms with symbiotic algae (e.g. Hikami et al., 2011, Suggett et al., 2012, Vogel and Uthicke, 2012).

Increasingly, it is being recognised that impacts will be species specific and vary locally (Feely et al., 2004, Ries et al., 2009, Range et al., 2011). It is expected that the main impacts will be indirect knock-on effects of ocean acidification, such as those resulting from changes in biotic interactions (Gaylord et al., 2015). It is also thought that ocean acidification will act in combination with other environmental stressors, such as increased temperatures caused by climate change (e.g. Anthony et al., 2011, Rodolfo-Metalpa et al., 2011, Harvey et al., 2013, Kroeker et al., 2013a, Sinutok et al., 2014).

With the increasing number of investigations published concerning the impact of ocean acidification, a number of meta-analysis investigations have now been conducted in an attempt to assess the overall impact of ocean acidification on biological organisms (e.g. Dupont et al., 2010b, Hendriks et al., 2010, Kroeker et al., 2010; 2013a, Harvey et al., 2013). In the most thorough of the early meta-analyses of the effects of ocean acidification (based on 73 studies, amounting to 251 unique experiments), Kroeker et al. (2010) found that the effects of ocean acidification were generally large and

negative, but that there was significant variation in the sensitivity of organisms. This has important implications for ecosystem responses as some species may experience a competitive advantage as a result of ocean acidification. Surprisingly, they found that organisms with more soluble forms of calcium carbonate could be more resilient than those with less soluble forms.

In contrast to the findings of Kroeker et al. (2010), Hendriks et al. (2010) also conducted a meta-analysis and concluded that most biological processes were not significantly affected by near future ocean acidification. They suggested that marine biota are likely to show a greater resilience to ocean acidification than previously expected. There is, however, much criticism of this study within the scientific community (Dupont et al., 2010c), one disparagement being that they did not use rigorous meta-analysis techniques. When Dupont et al. (2010b) used a similar meta-analysis approach to Hendriks et al. (2010) they found that this approach would lead to the conclusion that echinoderms were robust to ocean acidification, yet results from laboratory based research suggested the opposite. Dupont et al. (2010b) argue that although meta-analysis can be useful to get an overview of investigations, effects of ocean acidification will be species specific, vary according to the developmental stage, and that ecological effects are likely to be hidden in single-species experiments and negative impacts may be hidden in short-term experiments. The authors were, however, agreed that a paucity of data on the impacts of ocean acidification was hindering a robust assessment of the effects (Hendriks and Duarte, 2010). In response to Dupont's et al. (2010c) criticism, Hendriks and Duarte (2010) argue that it is important not to overstate the impact of ocean acidification on biological organisms and conclusions should be supported by the results published.

More recent meta-analysis investigations indicate that organisms show increased sensitivity to ocean acidification when exposed to elevated seawater temperature at the same time (Harvey et al., 2013, Kroeker et al., 2013a). Kroeker et al. (2013a)

synthesised the results from 228 studies on the biological responses to ocean acidification. Although the magnitude of responses varied between taxonomic groups, when marine organisms were pooled together, results indicated reduced survival, calcification, growth, development and abundance. Variability in response increased when species were exposed to acidification in multi-species assemblages. The results indicated enhanced sensitivity to ocean acidification when taxa were exposed to elevated temperature at the same time as ocean acidification. Harvey et al. (2013) found that combining the stressors of acidification and warming led to a stronger biological (either positive or negative) effect.

Calcium carbonate saturation horizons, the limit between supersaturation (in surface waters) and undersaturation (at depth) (Orr et al., 2005), are important as they determine the preservation of calcium carbonate on the sea floor and the amount buried within sediments (Sabine et al., 2002). They also determine the distribution of certain organisms such as deep-sea corals (Guinotte et al., 2006, Tittensor et al., 2010, Jackson et al., 2014). The level of saturation with respect to aragonite and calcite decreases as water depth increases (Sabine et al., 2002) and the aragonite saturation horizon is always shallower than the calcite saturation horizon because aragonite is more soluble. The depth of both saturation horizons varies spatially (Bindoff et al., 2007).

Increasing carbon dioxide levels are causing the aragonite saturation horizon to shoal rapidly in all ocean basins (Feely and Chen, 1982, Sabine et al., 2002, Sarma et al., 2002, Olafsson et al., 2009). This means that the limit between undersaturated and supersaturated waters is becoming shallower, exposing vast areas of seabed sediment to water that is corrosive to calcium carbonate. Sabine et al. (2002) estimate that, since pre-industrial times, the aragonite saturation horizon has shoaled by as much as 33% in the northern Indian Ocean. Olafsson et al. (2009) calculated that the aragonite saturation horizon in the Iceland Sea was shoaling at 4 m yr^{-1}, exposing another 800

km^2 of seafloor to undersaturated conditions each year. The surface waters of the Southern Ocean are expected to become undersaturated with respect to aragonite by the year 2050 (Caldeira and Wickett, 2005, Orr et al., 2005). The saturation horizon has varied significantly in the geologic past (e.g. Van Andel, 1975, Coxall et al., 2005, Zachos et al., 2005, Hönisch et al., 2012), but rates of change have been much slower than those experienced today.

Modelling work has examined the potential impact of shoaling carbonate saturation horizons on biological communities (Guinotte et al., 2006, Tittensor et al., 2010, Jackson et al., 2014). Guinotte et al. (2006) reviewed the distribution of deep-sea scleractinian corals and found that >95% of 410 coral locations occurred in areas that would have had waters that were saturated with respect to aragonite during pre-industrial times. Projections indicated that ~ 70% of these locations will be in waters that are corrosive to aragonite by 2099. Although trawling is considered currently to be the main threat to North East Atlantic cold-water coral reefs, Jackson et al. (2014) predicted that 86% of these reefs will be exposed to corrosive waters by 2060. Tittensor et al. (2010) put forward the argument that seamounts in particular, require protection from bottom trawling since they may serve as refugia for cold-water stony corals as saturation horizons shoal.

1.2 Foraminifera

Foraminifera are single celled protists with granuloreticulopodia; fine strands of cytoplasm, with a granular texture, that split and merge to form a net (e.g. Murray, 1979, Anderson and Lee, 1991, Murray, 1991a, Goldstein, 1999). They usually produce a shell, also known as a test, which can have one or more chambers made of organic matter, sediment grains or calcium carbonate (e.g. Murray, 1991b, Goldstein, 1999, de Nooijer et al., 2014). Foraminifera have a life history characterised by alternation of sexual and asexual generations. Reasonably complete life cycles are known for fewer than 30 species of foraminifera, but there is known to be significant

variation in the life cycle (Lee et al., 1991, Goldstein, 1999, Murray, 2006). The adult gamont typically produces biflagellated gametes that are liberated directly into the surrounding seawater. Fertilization then takes place by the fusion of two gametes, which are generally from different parents. Although hundreds of thousands of gametes can be released by a single gamont, it is very difficult for a gamete to fuse with a gamete from another parent (Hohenegger, 2011). Most gamonts will release gametes at different times and low settlement densities may mean that distance are too far to detect a partner. The short survival time of gametes (up to a few days), once released from the parent, means that the fusion of two gametes from different parents is unlikely (Hohenegger, 2011). If fusion does occur, the resulting zygote may spend a brief phase as a shell-less amoeba during which it feeds and grows before it calcifies, eventually forming the adult agamont.

In foraminifera, meiosis, which halves the diploid number of chromosomes, occupies an intermediate position in the life cycle and occurs as an integral part of multiple fission, by which the adult agamont produces multiple young. The resulting young have half the number of chromosomes (haploid) and typically grow to become adult gamonts (Goldstein, 1999) (Figure 1.1). The higher survival rate of gamonts, compared to gametes means that adult gamonts are much more abundant than agamonts, varying from one agamont per 100 gamonts to one agamont per 1000 gamonts (Hohenegger, 2011). In some species, the life cycle also includes a schizont, which is produced from the agamont and reproduces asexually (Lee et al., 1991, Goldstein, 1999).
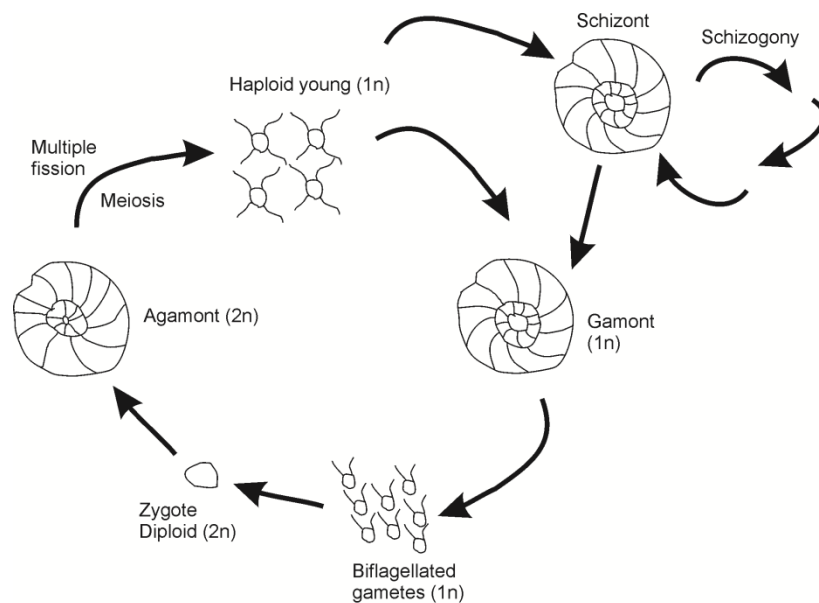
Figure 1.1: Generalised life cycle of foraminifera, which includes alternation between a haploid gamont and a diploid agamont. Meiosis occurs in the agamont as part of multiple fission and gametes are produced by mitosis. Two parent gametes then fuse to form a diploid zygote. The life cycle can also include a schizont which reproduces asexually and is produced from the agamont. The schizont can interject numerous successive asexual cycles, but the type of nuclear divisions in the schizont have not been documented for any species. 1n refers to haploid cells that have one set of chromosomes. 2n refers to diploid cells that have two sets of chromosomes. Not to scale. Adapted from Goldstein (1999).

Foraminifera are an extremely important group of marine organisms, as well as being very abundant. They occur from coastal habitats to the deep-sea (Murray, 1991a, Goldstein, 1999) and they have a geologic history reaching back to the beginning of the Palaeozoic and possibly earlier (Murray, 1979). Their extensive fossil record makes them suitable for use in palaeoceanographic investigations (e.g. Walker et al., 1981, Caldeira and Rampino, 1991, Lea, 1995, Elderfield and Ganssen, 2000, Palmer and Pearson, 2003, Zachos et al., 2005, Penman et al., 2014) and in the petroleum industry to determine sedimentary environments in reservoir studies (e.g. Culver, 1988, Athersuch et al., 1994).

Foraminifera have a variety of life-histories and obtain food in a variety of ways which include; deposit feeding, parasitism, symbiosis and suspension feeding (Murray, 2006). They have been able to specialise by differentiating subcellular components, or organelles, to perform various functions such as feeding, digestion, metabolism,

secretion, excretion, growth and reproduction (Anderson and Lee, 1991, Goldstein, 1999). The majority of modern foraminifera are benthic, estimations of the number of extant benthic species range from ~ 4000 (Murray, 2007) to ~ 10 000 (Vickerman, 1992), with only 40 – 50 planktonic species (Sen Gupta, 1999a).

The taxonomy of foraminifera has traditionally been based on test morphology and composition (e.g. Loeblich and Tappan 1987). More recently, phylogenetic studies have revealed some of the limitations of morphology-based classification (Pawlowski et al., 2013). Pawlowski et al. (2013) proposed a new classification system based on phylogenetics. In the new system, Orders and Families are separated according to differences in chamber shape, distance between successive apertures and prevailing mode of coiling. Foraminifera are considered to be a Phylum made up of two Classes; the Globothalamea (multi-chambered species with globular chambers), the Tubothalamea (multi-chambered species with tubular chambers), plus a "monothalamid" grouping (single chambered species with organic and agglutinated walls). The new classification system suggests that the major step in the evolution of foraminifera was the transition from single to multi-chambered tests rather than a transition from organic-walled to agglutinated and then calcareous tests (Pawlowski et al., 2013).

Calcareous foraminiferal tests can be porcelaneous (miliolid) or hyaline. Miliolids generally have high-magnesium calcite tests (Bentov and Erez, 2006), which can be more soluble than aragonite tests (Morse et al., 2006), potentially making them even more vulnerable to the effects of ocean acidification. Agglutinated foraminifera are expected to be more resilient to ocean acidification, and may even benefit from the loss of calcified competitors, as they do not produce calcium carbonate tests. The materials used to construct their test, however, may still be cemented together by calcite (Sen Gupta, 1999b).

1.3 Foraminifera and ocean acidification
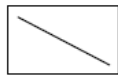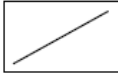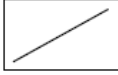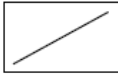
Foraminifera are an interesting group to examine in relation to ocean acidification as they occur worldwide, are environmentally sensitive, have short life-histories, are important ecosystem engineers (they are capable of modulating the availability of resources to other species by causing physical state changes in resources (Jones et al., 1994)), have an excellent fossil record and many have calcium carbonate tests. Foraminifera also play an important role in the Earth's CO_2/CO_3^{2-} budget (Lee and Anderson, 1991, Langer et al., 1997), so their response to ocean acidification may have important consequences for inorganic carbon cycling (Dissard et al., 2010a). A reduction in the calcification of foraminifera may act as a negative feedback on atmospheric CO_2 levels, as less calcification may result in less CO_2 released to the atmosphere (Riebesell et al., 2000, Zondervan et al., 2001, Ridgwell et al., 2007). The process of calcification takes up two moles of alkalinity in the form of HCO_3^- and one mole of dissolved inorganic carbon (DIC), causing a shift in the equilibrium of the carbonate system towards higher CO_2 concentration. Biogenic calcification, therefore, represents a potential source of CO_2 to the environment (Riebesell et al., 2000, Zondervan et al., 2001) and if constant annual global carbonate production is assumed, as a result of the decrease in the buffering capacity of seawater due to increasing anthropogenic CO_2, biogenic calcification would act as an additional source for CO_2 (Zondervan et al., 2001). If global calcification decreases, however, the expected positive feedback on increasing CO_2 concentrations is completely reversed and the capacity of the surface ocean to absorb CO_2 would increase (Zondervan et al., 2001, Ridgwell et al., 2007). By decreasing calcification, anthropogenic CO_2 emissions will cause a reduction in the rate of CO_2 release from $CaCO_3$ precipitation and accelerate the removal of CO_2 from the atmosphere (Ridgwell et al., 2007).
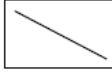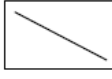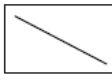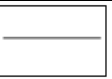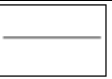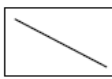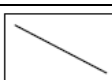
The study of foraminifera is complicated by the range of ecological niches that they occupy, varying life histories and the range of materials from which they can construct their test wall (Murray, 2006). Calcifying foraminifera have been a particular focus of
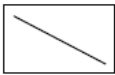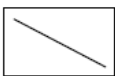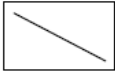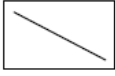
investigations into ocean acidification effects with the majority of investigations conducted in the last four years (e.g. Allison et al., 2010, Dias et al., 2010, Naik et al., 2010, Fujita et al., 2011, Haynert et al., 2012, Beare et al., 2013, Keul et al., 2013a, Khanna et al., 2013, Uthicke et al., 2013). Investigations into the effects of ocean acidification on foraminifera have reported variable responses (Table 1.1), which could be due to the different species examined, different test types, different life modes, different durations of the experiments or different experimental methods. Out of the studies listed in Table 1.1, thirty showed a negative response to increased pCO_2, ten showed no response and only five showed a positive response to increased pCO_2. The positive responses tended to be for foraminifera with algal symbionts, raising the possibility that higher CO_2 levels benefit the algae, which in turn benefit their hosts, or those that do not have a calcium carbonate test. Generally, calcareous species are expected to be more severely affected by ocean acidification than agglutinated or allogromiid forms (Uthicke et al., 2013).
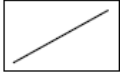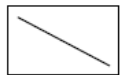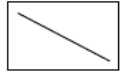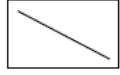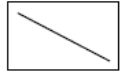
The short duration of many laboratory experiments makes it harder to predict responses over the longer-term. For example, it is possible that short-term experiments present an artificial shock to foraminifera that is not representative of ocean acidification and that these organisms may be able to adapt to gradual increases in CO_2 (Munday et al., 2013, Sunday et al., 2014). Examination of foraminifera from natural environmental settings can be used to address this problem because the organisms may be subjected to elevated CO_2 for multiple generations and those present will have had time to acclimate to chronic effects of increased CO_2.
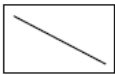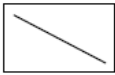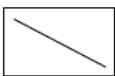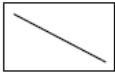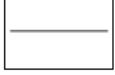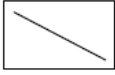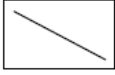
Table 1.1: Overview of response patterns of foraminifera to changes in carbonate chemistry. Responses to pCO_2 are represented by trend plots. TA = total alkalinity, DIC = dissolved inorganic carbon, SNW = size-normalised weight. Updated from Keul et al. (2013a).
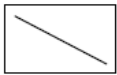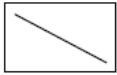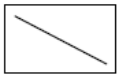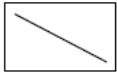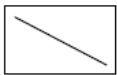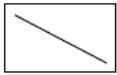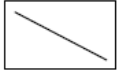
Study	Species	Type of investigation	Methods	Response to pCO_2	Response parameter
Allison et al. (2010)	*Elphidium williamsoni*	Culture experiment	TA manipulation 8 weeks 3 pH levels (7.7 – 8.3)	[decreasing trend]	Chambers formed at low pH significantly thinner than at high pH
Barker and Elderfield (2002)	*Globigerina bulloides*	Palaeo	Comparison of down core SNW and carbonate system	[decreasing trend]	Highest shell weights during glacial periods Shell weights appear to be related to carbonate ion concentration
Beare et al. (2013)	Foraminiferal assemblage	Plankton tows 1958 – 2010	Comparison of abundance of foraminifera and carbonate system	[increasing trend]	Abundance increased since 1950 Tolerant of changes in pH that have occurred since 1950
Beaugrand et al. (2013)	Foraminiferal assemblage	Plankton tows 1960 – 2009	Comparison of frequency of occurrence of foraminifera and carbonate system	[flat trend]	Response to temperature change overrode signal from decreased pH
Beer et al. (2010)	*Globigerina bulloides*	Plankton tows	Comparison of plankton tow SNW and carbonate system	[decreasing trend]	Strong inter/intra species variations with different response of SNW to $[CO_3^{2-}]$
	Globigerinoides ruber			[increasing trend]	Difference in relative abundance
Bernhard et al. (2009a)	Foraminiferal assemblage	*In situ* experiment	DIC manipulation *in situ* at 3.1 and 3.3 km water depth 5 weeks 3 pCO_2 treatments	Thecate and agglutinated foraminifera not impacted Survivorship of calcareous foraminifera significantly lower	
Bernhard et al. (2009b)	*Allogromia laticollaris*	Culture experiment	DIC manipulation 10 – 14 days 6 pCO_2 levels (375 – 200000 ppm)	[increasing trend]	Populations survived highest pCO_2 levels
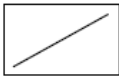
Study	Species	Type of investigation	Methods	Response to pCO_2	Response parameter
Bijma et al. (1999, 2002)	*Orbulina universa*	Culture experiment	TA, DIC and pH-stable manipulation	*(graph: decreasing line)*	Increase in shell weight with increase in carbonate ion concentration
	Globigerinoides sacculifer		TA manipulation		
Cigliano et al. (2010)	Foraminiferal assemblage	Shallow water CO_2 seeps	1 month settlement study at different natural pH levels (7.1 – 8.2)	*(graph: decreasing line)*	Fewer individuals and number of taxa in low pH conditions
de Moel et al. (2009)	*Globigerinoides ruber*	Palaeo	Comparison of shell weights	*(graph: decreasing line)*	Light, thin-walled shells younger than heavier thicker-walled shells
de Villiers (2003)	*Globigerinoides sacculifer* *Globigerinoides ruber*	Palaeo	Down core shell weights	*(graph: flat line)*	Shell weights only weakly correlated with climate indicators Controls on shell weight complex
de Villiers (2004)	*Neo-globoquadrina pachyderma* *Globigerina bulloides* *Globorotalia truncatulinoides*	Palaeo	Shell weight measurement on core top samples	*(graph: flat line)*	Calcification rates not related to calcite saturation state Weights influenced by complex interplay of environmental parameters
Dias et al. (2010)	Foraminiferal assemblage	Shallow water CO_2 seeps	Assemblage study at different natural pH levels (6.6 – 8.1)	*(graph: decreasing line)*	Reduction in diversity and abundance Shift from 24 to 4 species (all agglutinated) with decreasing pH
Dissard et al. (2010)	*Ammonia tepida*	Culture experiment	DIC manipulation 2 pCO_2 levels (230 + 1990 µatm)	*(graph: decreasing line)*	Heavier shell weights in low pCO_2 treatments

Study	Species	Type of investigation	Methods	Response to $p\mathrm{CO_2}$	Response parameter
Fabricius et al. (2011)	Foraminiferal assemblage	Shallow water CO_2 seeps	Assemblage study at different natural pH levels (7.8 – 8.1)	[graph: decreasing line]	High $p\mathrm{CO_2}$ sites free of calcareous foraminifera At $p\mathrm{CO_2}$ 494 ppm many foraminifera tests corroded or pitted
Fujita et al. (2011)	*Baculogypsina sphaerulata* *Calcarina gaudichaudii*	Culture experiment	DIC manipulation 12 weeks 5 $p\mathrm{CO_2}$ levels (260 – 970 µatm)	[graph: hump-shaped curve]	Weight increases at intermediate $p\mathrm{CO_2}$ levels, then decreases
	Amphisorus hemprichii			[graph: decreasing line]	Weights decreased under higher $p\mathrm{CO_2}$ levels
Glas et al. (2012)	*Marginopora vertebralis* *Heterostegina depressa* *Amphistegina radiata* *Peneroplis* sp. *Quinqueloculina* sp. *Miliola* sp.	Culture experiment	Short-term incubation 3 $p\mathrm{CO_2}$ levels (ca. 430 – 2150 µatm) micro - electrode measurement	N/A	Photo-synthetic increase of surface pH insufficient to compensate for seawater pH decreases
Gonzalez-Mora et al. (2008)	*Globigerina bulloides* *Globigerinoides ruber*	Palaeo	Comparison of down core weights and Vostok $p\mathrm{CO_2}$ and Mg/Ca – temperature	[graph: decreasing line]	Decrease in down-core shell weight associated with high $p\mathrm{CO_2}$ values
	Neo-globoquadrina pachyderma			[graph: flat line]	Weights more influenced by temperature
Haynert et al. (2011)	*Ammonia aomoriensis*	Culture experiment	DIC manipulation 6 weeks 5 $p\mathrm{CO_2}$ levels (620 – 3130 µatm)	[graph: decreasing line]	Reduced calcification at elevated $p\mathrm{CO_2}$ De-calcification started at 930 µatm
Haynert et al. (2012)	Foraminiferal assemblage	*In situ* investigation	Assemblage study at different natural $p\mathrm{CO_2}$ levels (1200 – 3300 µatm)	[graph: flat line]	Seasonal community shifts No dynamic response between population density/ diversity and pore water $p\mathrm{CO_2}$

Study	Species	Type of investigation	Methods	Response to $p\mathrm{CO_2}$	Response parameter
Haynert et al. (2014)	Foraminiferal assemblage	Laboratory experiment on natural assemblage	DIC manipulation 6 months 4 $p\mathrm{CO_2}$ levels (430 – 3247 µatm)	(flat line)	Living assemblage largely unaffected by $p\mathrm{CO_2}$ treatment Post-mortem dissolution of *Ammonia aomoriensis*
Hikami et al. (2011)	*Calcarina gaudichaudii*	Culture experiment	DIC manipulation 6 weeks, 4 $p\mathrm{CO_2}$ levels (250 – 910 µatm)	(increasing line)	Net calcification increased with $p\mathrm{CO_2}$
	Amphisorus kudakajimensis			(decreasing line)	Reduced net calcification with increased $p\mathrm{CO_2}$
	Amphisorus hemprichii		Constant carbonate ion concentration	N/A	Constant calcification under constant $[\mathrm{CO_2^{3-}}]$
Keul et al. (2013a)	*Ammonia* sp.	Culture experiment	TA manipulation (4 treatments) DIC and TA manipulation (4 treatments) 8 – 14 weeks	(decreasing line)	Decrease in SNW and growth rate with a decrease in $[\mathrm{CO_2^{3-}}]$
Khanna et al. (2013)	*Haynesina germanica*	Culture experiment	DIC manipulation 36 weeks 3 $p\mathrm{CO_2}$ levels (380 – 1000 ppm)	(decreasing line)	Evidence of shell dissolution and a significant reduction in, and deformation of, ornament-ation at high $p\mathrm{CO_2}$
Kuroyanagi et al. (2009)	*Marginopora kudakajimensis*	Culture experiment	TA manipulation 10 weeks 4 pH levels (7.7 – 8.3)	(decreasing line)	Weight, shell size and growth rates decrease with decreasing pH Growth rate between pH 7.9 and 8.2 not significantly different

Study	Species	Type of investigation	Methods	Response to pCO_2	Response parameter
Lombard et al. (2010)	*Globigerinoides sacculifer* *Orbulina universa*	Culture experiment	TA manipulation 4 – 7 day incubation	(decreasing trend graph)	Reduced shell weight and calcification rate under low carbonate ion concentration
Manno et al. (2012)	*Neo- globoquadrina pachyderma*	Culture experiment	2 pH and 2 temperature treatments 6 day incubation	(decreasing trend graph)	Decrease in net calcification rate in low pH treatment
McIntyre- Wressnig et al. (2013)	*Amphistegina gibbosa*	Culture experiment	DIC manipulation 6 weeks 3 pCO_2 levels (410 – 2000 µatm)	(flat trend graph)	No effect on survival or growth at higher pCO_2
Moy et al. (2009)	*Globigerina bulloides*	Palaeo and *In situ* investigation	Comparison of core tops with sediment trap data Comparison of down core weights with Vostok pCO_2	(decreasing trend graph)	30 – 35% decrease in shell weights between sediment trap and core tops Link between down-core shell weight decrease and high pCO_2 for last 50 000 years
Naik et al. (2010)	*Globigerinoides sacculifer*	Palaeo	Comparison of down core weights and Vostok pCO_2 and Mg/Ca – temperature	(decreasing trend graph)	Link between down-core shell weight decrease and high pCO_2 values
Pettit et al. (2013)	Foraminiferal assemblage	Shallow water CO_2 seeps	Assemblage study at different natural pH levels (7.55 – 7.88)	(flat trend graph)	Living calcareous foraminifera in low pH conditions, but impoverished species assemblage
Reymond et al. (2013)	*Marginorpora rossi*	Culture experiment	DIC manipulation 5 weeks 3 pH levels (7.6 – 8.1)	(decreasing trend graph)	Reduced growth under lower pH Interactive impact of eutrophication
Ricketts et al. (2009)	Foraminiferal assemblage	*In situ* experiment	DIC manipulation *in situ* at 3.6 km water depth 1 month 2 pCO_2 treatments	(decreasing trend graph)	Decrease in the total number of foraminifera and species richness with high pCO_2

Study	Species	Type of investigation	Methods	Response to pCO_2	Response parameter
Russell et al. (2004)	*Orbulina universa*	Culture experiment	TA manipulation ($[CO_3^{2-}]$ = 110-470 µmol kg^{-1})	(decreasing line)	Shell weights increased with an increase in carbonate ion concentration
Schmidt et al. (2014)	*Heterostegina depressa*	Culture experiment	DIC manipulation 7 weeks 2 pCO_2 levels (490 and 790 µatm)	(decreasing line)	Reduced growth and decreased chlorophyll *a* content in high pCO_2
	Marginopora vertebralis			(flat line)	No effect on survivorship and increase in chlorophyll *a* content in high pCO_2
Sinutok et al. (2011)	*Marginopora vertebralis*	Culture experiment	DIC manipulation 4 weeks 4 pH levels (7.4 – 8.1)	(decreasing line)	Reduced calcification under elevated pCO_2
Sinutok et al. (2014)	*Marginopora vertebralis*	Culture experiment	DIC manipulation 5 weeks 2 pCO_2 levels (400 and 1000 µatm)	(decreasing line)	Reduction in chlorophyll *a* concentration at high pCO_2 Reduction in calcification rate at high pCO_2 and temperature Temperature more impact than CO_2
Spero et al. (1997)	*Orbulina universa*	Culture experiment	TA manipulation pH levels (7.87 – 8.97)	(decreasing line)	Specimens grown in high $[CO_3^{2-}]$ 37% heavier than those in ambient sea water
Uthicke and Fabricius (2012)	*Marginopora vertebralis*	Culture experiment	DIC manipulation	(decreasing line)	Reduced calcification at high pCO_2
		Shallow water CO_2 seeps	Different natural pH levels (6.6 – 8.1)	(decreasing line)	Absent in field at pCO_2 of >700 µatm
Uthicke et al. (2013)	Foraminiferal assemblage	Shallow water CO_2 seeps	Assemblage study at different natural pH levels (7.52 – 8.08)	(decreasing line)	Foraminifera absent at pH <7.9 Non-calcifying taxa declined less steeply

Study	Species	Type of investigation	Methods	Response to pCO$_2$	Response parameter
Vogel and Uthicke (2012)	*Amphistegina radiata* *Heterostegina depressa*	Culture experiment	DIC manipulation 6 weeks 4 pCO$_2$ treatments (470 – 1925 µatm)	——	Growth rates not affected by pCO$_2$
	Marginopora vertebralis			/	Significant increase in calcification rates at high pCO$_2$

1.3.1 Palaeoceanographic investigations

Foraminifera have been used extensively in palaeoceanographic investigations; this is because of their excellent fossil record (Murray, 1991a). Palaeoceanographic investigations are extremely useful to ocean acidification research. Although the current rate of change in ocean pH is thought to be much faster than that which has occurred in the past (Hönisch et al., 2012), the examination of past changes to ocean chemistry can provide an indication as to how biological organisms may respond to future changes (Pelejero et al., 2010). The lack of a major extinction of certain foraminifera during periods of ocean acidification in the geological record cannot, however, be taken as evidence that modern calcifiers are insensitive to anthropogenic acidification because of differences in the rate of change (Penman et al., 2014).

Barker and Elderfield (2002) examined the shell weights of the planktonic foraminifera *Globigerina bulloides* across glacial-interglacial Termination 1 and found a response related to the carbonate ion concentration of seawater. Shell weights were found to be heavier during glacial times when carbonate ion concentration was higher. Moy et al. (2009) and Naik et al. (2010) also found evidence for a link between lower atmospheric CO_2 and heavier shell weights. It can, therefore, be proposed that future increases in atmospheric CO_2 may lead to a reduction in shell weight of planktonic foraminifera. Barker and Elderfield (2002) suggested that the shell weight of planktonic foraminifera could be used as a carbonate saturation and atmospheric CO_2 proxy. Keul et al.

(2013a) found that CO_3^{2-} concentration, which determines saturation state if Ca^{2+} concentration remains constant, was the parameter affecting size normalised weights and growth rates in foraminifera.

Other studies, however, have found that controls on shell weight are complicated (de Villiers, 2003, de Villiers, 2004, Gonzalez-Mora et al., 2008). Gonzalez-Mora et al. (2008) examined three species of planktonic foraminifera across stadials and interstadials (250 – 160 Ka) and found water temperature to be the main control on shell weight for *G. bulloides*, *Globigerinoides ruber* and *Neogloboquadrina pachyderma.* Gonzalez-Mora et al. (2008) pointed out that water temperature can also affect the carbonate ion concentration in seawater. The colder waters of glacial times enabled more CO_2 to dissolve, resulting in a reduction in the carbonate ion concentration and saturation state. De Villiers (2004) found that planktonic foraminiferal calcification rates were not related to calcite saturation state. They were, instead related to optimum growth conditions. Heaviest shell weights were observed where growth conditions were considered to be optimal for each species. De Villiers (2004) argued that calcification and carbonate saturation relationships should be evaluated in a broader environmental context.

Sediment trap data can be used to examine how planktonic foraminifera have been affected by ocean acidification over time. Moy et al. (2009) compared the shell weights of the modern planktonic foraminifera *G. bulloides*, collected from sediment traps in the Southern Ocean, with shells preserved in underlying sediments of Holocene age. Modern shell weights were 30 – 35% lower than those from the sediments. This suggests reduced calcification in modern foraminifera.

De Moel et al. (2009) examined the impact of ocean acidification on planktonic foraminifera in the Arabian Sea. They examined calcification and shell weight of the foraminifera *G. ruber* in the surface sediment. Light, thin-walled tests were found to be

younger (based on ^{14}C and $\delta^{13}C$ measurements) than the heavier, thicker-walled tests. Seasonal upwelling also seemed to result in lighter tests, with heavier tests occurring during non-upwelling periods. This could be due to increased CO_2 during upwelling periods.

Foraminifera have also been used in the development of palaeo proxies (Lea, 1999). The boron isotopic composition of foraminiferal tests can be used as a palaeo-proxy for ocean pH. The relative proportions of the two main species of boron (boric acid and the borate ion) are known to change according to the pH of the seawater and this is reflected in the isotopic ratio of foraminiferal tests. It is important to determine the ocean carbonate system in the past, as this is essential for understanding past changes in ocean acidification, pCO$_2$ and climate (Rae et al., 2011). In addition, if more information is known about ocean acidification events in the past, these can be used to inform what may happen in the future (Hönisch et al., 2012).

1.3.2 Culture experiments

Initial investigations into the impact of ocean acidification on foraminifera were based in laboratories (e.g. Spero et al., 1997, Bijma et al., 1999; 2002). Laboratory work has the advantage of allowing environmental conditions to be carefully controlled and they can be replicated easily. Although they provide a valuable and important way to examine the impacts on individual species, long-term interactions between organisms are harder to investigate in laboratory settings and it is more difficult to simulate natural conditions, for example, in temperature, pressure, light and CO_2. Stress associated with being in captivity may alter responses (Widdicombe et al., 2010) and the artificial environment may introduce culture effects. Laboratory experiments are usually conducted over short timescales, this may stress the individuals through rapid perturbation of chemical conditions and do not allow for the possibility of acclimatisation to be considered (Thomsen et al., 2010). A criticism of some laboratory investigations is that the pH values used are far lower than expected to occur in the future, even with highest

emission scenarios (Barry et al., 2010a). Laboratory investigations also pose a risk of pseudo-replication (Havenhand et al., 2010). This might occur when replicate samples for each treatment level are taken from the same aquarium. Any patterns may be a result of differences in conditions between aquaria and cannot be attributed solely to treatment. Ideally, there will be replicate aquaria at each of the chosen treatment levels. Although laboratory investigations often allow for easier replication than *in situ* investigations, replication can nevertheless be costly and time consuming.

Some early laboratory investigations into the impact of ocean acidification focused on planktonic foraminifera (Spero et al., 1997, Bijma et al., 1999; 2002). These investigations revealed shell weight to be dependent upon the carbonate chemistry of the ambient seawater. Spero et al. (1997) found that shell masses of *Orbulina universa* grown in high CO_3^{2-} concentrations were 37% heavier than those grown in ambient seawater due to higher calcification rates. This finding was supported by a subsequent experiment, which reported an increase in *O. universa* shell weights with an increase in carbonate ion concentration (Russell et al., 2004). Lombard et al. (2010) examined the effect of carbonate ion concentration on calcification rates of *O. universa* and *Globigerinoides sacculifer*. Both species exhibited reduced calcification rates under low carbonate ion concentrations (Lombard et al., 2010) supporting the findings of Spero et al. (1997) and Russell et al. (2004). Examination of another planktonic species *N. pachyderma* also revealed a decrease in size and weight as pH reduced (Manno et al., 2012).

In one of the first laboratory experiments that examined the effects of low pH on benthic foraminifera, hydrochloric acid was used to lower the pH (Le Cadre et al., 2003). At a pH of 7.0 pseudopodial emission by *Ammonia beccarii* was reduced or stopped and decalcification of the test occurred. This investigation, however, was not examining the impact of ocean acidification and the associated alterations in carbonate

chemistry, but was examining the impact of low pH, mainly due to industrial pollution, on benthic foraminifera (Le Cadre et al., 2003).

An early investigation into the effects of ocean acidification on benthic foraminifera examined allogromiids. Allogromiids have organic walls, not calcium carbonate tests. Bernhard et al. (2009b) examined the response of the allogromiid *Allogromia laticollaris* to elevated CO_2 concentrations. The CO_2 concentrations used (15 000, 30 000, 60 000, 90 000 and 200 000 ppm) were far higher than predicted future surface ocean values, but were designed to reflect the range of conditions that the benthos is expected to experience if deep-sea CO_2 sequestration becomes a reality (Bernhard et al., 2009b). Injection of CO_2 into the deep-sea could potentially slow climate change by removing CO_2 from emission sources and pumping it as a liquid to sea-floor depressions, where it could dissipate over millennial time scales (Marchetti, 1977, Haugan and Drange, 1992, Yamasaki, 2003, Brewer et al., 2005). The duration of the experiments conducted by Bernhard et al. (2009b) were only ten to 14 days. Survival of the allogromiids was determined in two ways: 1) measurement of adenosine triphosphate (ATP), which indicates cell energy and 2) pseudopodial presence/absence. Although the mean ATP was statistically lower in the treatment with the highest CO_2 compared to the control, substantial proportions of the population survived and reproduction by some individuals even occurred. The short duration of the experiments means that negative effects, occurring over longer time-scales, cannot be ruled out, but suggests that allogromiids may be resistant to ocean acidification. The problem with such work is that it is not known how the organisms that *A. laticollaris* interacts with will be affected by ocean acidification. If their predators benefit, then they could be severely impacted, but if not, they may thrive.

Although there are some contrasting results, most calcareous benthic foraminifera appear to be negatively affected by ocean acidification. Kuroyanagi et al. (2009) revealed that growth rate and shell weight in *Marginopora kudakajimensis* decreased

as pH decreased. Kuroyanagi et al. (2009) predicted that at a pH of approximately 7.7, the calcification of *M. kudakajimensis* would decline so steeply that it would probably preclude their survival. Shell weights of *Ammonia tepida* decreased with decreasing CO_3^{2-} concentration (Dissard et al., 2010a). Calcification, however, still occurred despite calcium carbonate undersaturation and no dissolution of the live foraminifera was observed. Shell weights also decreased with increasing temperature (Dissard et al., 2010a). *Elphidium williamsoni* had significantly thinner chamber walls when cultured at low pH (7.6) for eight weeks (Allison et al., 2010).

Fujita et al. (2011) subjected three foraminiferal species (*Baculogypsina sphaerulata*, *Calcarina gaudichaudii* and *Amphisorus hemprichii*) to elevated pCO$_2$ for 12 weeks. Response to increased pCO$_2$ appeared to be related to test type. Net calcification of the porcelaneous species, *A. hemprichii*, decreased at higher pCO$_2$, whereas the two hyaline species *B. sphaerulata* and *C. gaudichaudii* showed an increase in net calcification at intermediate pCO$_2$ (~ 770 µatm) and a decrease in net calcification at higher pCO$_2$ (~ 970 µatm) (Fujita et al., 2011). Hikami et al. (2011), however, found that calcification of *C. gaudichaudii* increased under the highest pCO$_2$ conditions of 907 µatm. This difference could be due to the slightly lower levels of pCO$_2$ (907 µatm compared to 970 µatm), or, more likely, the shorter duration of the experiment (six weeks compared to 12 weeks). It may be that *C. gaudichaudii* are able to compensate for reduced pH by increasing their calcification rate for short time periods, after which it becomes too energetically expensive and their calcification rate reduces.

Under elevated pCO$_2$ there was reduced calcification of *Marginorpora vertebralis* (Sinutok et al., 2011). Haynert et al. (2011) examined the benthic foraminifera *Ammonia aomoriensis.* Shell dissolution was evident at pCO$_2$ levels of ~ 929 µatm and there was reduced calcification at elevated pCO$_2$. Keul et al. (2013a) found that carbonate ion concentration was the parameter that affected growth rate and size normalised weights in the benthic foraminifera *Ammonia*. In a laboratory experiment,

shell dissolution in the shallow water calcified foraminifera *Haynesina germanica* occurred at 1000 ppm CO_2 and their calcareous ornamentation used for feeding was reduced and deformed (Khanna et al., 2013).

Following a six month experiment, where a natural sediment foraminiferal community was exposed to different pCO_2 levels, living assemblages were largely unaffected by the pCO_2 treatment applied (Haynert et al., 2014). The response of foraminifera kept within their natural sediment habitat was different from those isolated from the sediment and suggests that although negative effects of ocean acidification may be apparent in isolated species, the impact of ocean acidification on natural assemblages may not be as severe (Haynert et al., 2014).

The response of foraminifera to ocean acidification is made more complex if algal symbionts are present, as these may modify internal carbonate chemistry (Bijma et al., 1999). Through examining changes in pH in the diffusion boundary layer (DBL) around symbiont-bearing foraminifera, Glas et al. (2012) found that pH elevation due to photosynthesis of the symbionts was insufficient to compensate for ambient seawater decreases. Increased pCO_2 did not affect chlorophyll *a* content in the symbiont-bearing species *M. vertebralis*, *Heterostegina depressa* or *Amphistegina radiata* (Vogel and Uthicke, 2012). Overall, *M. vertebralis*, *H. depressa* and *A. radiata* did not show negative effects in exposures up to 1925 µatm pCO_2 in a six week experiment and *M. vertebralis* showed increased calcification rates (Vogel and Uthicke, 2012), suggesting that, in the short-term, the symbiont-bearing foraminifera may have benefited from elevated CO_2. Symbiont-bearing *Amphistegina gibbosa* had areas of dissolution on the test surface after exposure to 2000 ppm CO_2 for six weeks, even though calcite saturation state remained above one (McIntyre-Wressnig et al., 2013). Fitness and survival (measured by adenosine triphosphate (ATP) content), however, did not appear to be directly affected by elevated pCO_2.

The impact of ocean acidification on large benthic foraminifera with algal symbionts has been examined in combination with other environmental stressors such as increased temperature or eutrophication. The effects of environmental change are likely to be underestimated when the effects of temperature or $p\text{CO}_2$ are examined in isolation (Schmidt et al., 2014). The growth rate of *Marginorpora rossi* was inhibited by the interaction of ocean acidification and eutrophication (Reymond et al., 2013) and there appeared to be a threshold value of pH 7.6, after which there was a decline in calcification. Sinutok et al. (2014) examined photosynthesis and calcification in the symbiont-bearing *M. vertebralis*. They found that both photosynthesis and calcification were reduced under elevated temperature and ocean acidification scenarios, with a reduction in calcification of >0.1% per day at $p\text{CO}_2$ of 1000 µatm when cultured at 28°C and 32°C. Schmidt et al. (2014) also examined the combined effects of ocean acidification and temperature on *M. vertebralis* and another symbiont-bearing coral reef foraminifera; *H. depressa*. The strongest stress responses were observed when both stressors acted in combination, although the mortality of *M. vertebralis* has been shown to increase with increased temperatures alone (Uthicke et al., 2012). Schmidt et al. (2014) reported an increase in chlorophyll *a* content of *M. vertebralis* with increasing $p\text{CO}_2$, whereas Sinutok et al. (2014) observed a decrease in Chlorophyll *a*. This difference could be due to the $p\text{CO}_2$ levels (~790 µatm compared to 1000 µatm) or the length of the experiments (five weeks compared to seven weeks). A recent meta-analysis (18 publications, 84 individual experiments) on the effects of ocean warming and acidification on large benthic foraminifera (which host algal symbionts) found a general negative trend on holobiont growth (Doo et al., 2014). The only exception to this trend was found for hyaline species that host diatom symbionts.

Laboratory investigations have also examined the calcification process of foraminifera in relation to ocean acidification (de Nooijer et al., 2014). Foraminifera are thought to promote calcification by elevating their intracellular pH (de Nooijer et al., 2008; 2009a; 2009b). A reduction in pH would, therefore, be expected to increase the amount of

energy required to elevate intracellular pH to levels required for calcification (de Nooijer et al., 2009b). Alternatively, if the organisms are unable to elevate intracellular pH to the required levels for calcification, it is expected that there will be reduced carbonate concentrations for precipitation. Either of these two scenarios is likely to lead to a reduction in calcification. This view might be too simplistic and the methods used by de Nooijer et al. (2008; 2009b) are likely to lead to reduced resolution and unreliable pH measurements (Bentov et al., 2009). Bentov et al. (2009) observed that Ca^{2+} was supplied to the site of calcification in the benthic foraminifera *Amphistegina lobifera*, through the transport of seawater in vacuoles rather than through membrane ion transporters. The seawater vacuoles underwent alkalisation (to a pH of 8.7) during their intracellular passage which elevated CO_3^{2-} concentration. The energy expenditure of the foraminifera, needed to reach the required pH and maintain the same calcification rate, would still be determined by the initial saturation state of the seawater in the vacuole. Wolf-Gladrow et al. (1999) hypothesise that for foraminifera with high calcification rates, such as the planktonic foraminifera *G. sacculifer*, the use of bicarbonate or an internal carbon pool must be required because the supply of carbonate ions would be insufficient.

1.3.3 *In situ* experiments

There are several problems associated with conducting *in situ* pCO_2 perturbation experiments, such as funding and logistics. Carbon dioxide perturbation experiments, however, have been conducted to examine the impact of CO_2 injection into the deep-sea, a potential mitigation strategy to cope with increasing atmospheric carbon dioxide (Marchetti, 1977, Parson and Keith, 1998, Yamasaki, 2003). Free-Ocean-Carbon Dioxide-Enrichment (FOCE) experiments can be used at shallower depths as an alternative approach to examine ecosystem processes (Arnold et al., 2012). FOCE experiments are analogous to terrestrial free-air-carbon-enrichment experiments. They allow the manipulation of pH under otherwise natural conditions. One of the major

limitations of FOCE experiments, however, is that they do not allow migration of fauna into or out of the experimental area.

Bernhard et al. (2009a) examined the survivorship of benthic foraminifera to deep-sea CO_2 release. They placed PVC cylinders with a diameter of 40.6 cm on the sea floor of Monterey Bay at a water depth of 3100 and 3500 m. Seven cylinders were arranged in an approximately 20 m diameter circle. Liquid CO_2 was injected into each cylinder. The target pH decrease within the experimental area was 0.2 units. The experiment was left for approximately five weeks after which CellTracker Green was injected directly into emplaced pushcores to distinguish between live and dead foraminifera. Foraminifera within the top 1 cm of pushcores were analysed. The survivorship of thecate and agglutinated foraminifera did not appear to be impacted by direct exposure to injected CO_2. The survivorship of calcareous foraminifera, however, was significantly lower in direct exposure treatments compared to controls. This is similar to the findings of Ricketts et al. (2009) who, after conducting a similar experiment, also reported an increase in mortality and dissolution of calcareous foraminifera in treatment cores. This shows that calcareous foraminifera are likely to be more severely affected by CO_2 injection and, potentially, future ocean acidification than thecate and agglutinated forms. The resources required to perform a small scale perturbation experiment such as this are extensive.

1.3.4 Sampling of natural environmental settings

Planktonic foraminifera can be examined through the use of Continuous Plankton Recorder (CPR) survey data or plankton nets. The CPR survey is operated by the Sir Alister Hardy Foundation for Ocean Science (SAHFOS) and the survey data offers a long-term (~ 80 year) database on the abundance of plankton in relation to environmental factors such as temperature, nutrients and pH (Richardson et al., 2006, Beare et al., 2013). The survey routinely identifies 500 plankton taxa, most to species

level, and the methodology has remained virtually unchanged since 1931 (Richardson et al., 2006).

Beer et al. (2010) measured planktonic foraminiferal shell weight across a range of carbonate ion concentrations in the Arabian Sea and found that carbonate ion concentration did not exert a dominant control on foraminiferal test weight. Beaugrand et al. (2013) reported that planktonic foraminifera, along with other calcifying plankton, showed an abrupt shift in the North Atlantic around 1996 when there was an abrupt shift in temperature. They concluded that in the North Atlantic, oceanic temperature, rather than ocean acidification, was the primary driver of calcifying plankton. Beare et al. (2013) found an increase in the abundance of planktonic foraminifera in the North Sea since 1950. This shows that the foraminifera were tolerant of changes in pH that have occurred since the 1950s.

Benthic foraminifera have also been examined in natural environmental settings. Haynert et al. (2012) examined benthic foraminiferal assemblages in Flensburg Fjord, south western Baltic Sea. They found that foraminifera were able to tolerate high levels of $p\text{CO}_2$ provided that sediment pore water remained supersaturated with respect to calcite. This suggests that calcite saturation state is the carbonate chemistry parameter which has the most important control on benthic foraminiferal assemblages. In opposition to what has been found for many laboratory experiments, these investigations in natural environmental settings suggest that foraminifera will not be negatively affected by ocean acidification, or that other environmental factors exert a dominant control over abundance.

1.3.5 Shallow water CO_2 seeps

Natural gradients in CO_2, caused by shallow water gas seeps, provide a new and useful approach to ocean acidification research (Hall-Spencer et al., 2008). Some of these seeps release gas (and water) at ambient seawater salinity, temperature and

alkalinity and lack toxic sulphur compounds (Boatta et al., 2013). These seeps can be used as natural laboratories to study the effects of ocean acidification on marine organisms (Figure 1.2). The seeps can be present on time scales of hundreds to thousands of years, avoiding some of the problems of short-term, rapid perturbation experiments (Hall-Spencer et al., 2008). They have widespread environmental relevance as multi-species interactions and ecosystem feedbacks can be examined.

Figure 1.2: The CO_2 seep study site off Vulcano, Italy. Photograph taken by J. Hall-Spencer.

Although natural CO_2 gradients can be very useful in examining ecosystem effects of ocean acidification, they are not ideal predictors. Recruitment of organisms can occur from areas unaffected by high pCO_2 due to the close spatial proximity of populations that are not affected by the seeps (Barry et al., 2010b). Smith and Buddemeier (1992) discuss that it is often not possible to separate the effects of climate change, natural environmental variability and anthropogenic alterations to ecosystems. Many of these seep sites have very variable pH profiles (Kerrison et al., 2011). There is, however, a high degree of natural pH variability within the marine environment (Raven et al., 2005), so study locations need to be chosen carefully to mimic likely future conditions.

The nature of the seeps means that there is limited control over the experimental conditions and great care is needed to avoid confounding factors since seeps often emit toxic compounds such as H_2S or have high levels of heavy metals (Boatta et al., 2013, Vizzini et al., 2013).

Shallow water CO_2 seeps were first used by Hall-Spencer et al. (2008) to examine the impact of ocean acidification on benthic ecosystems. This pioneering *in situ* investigation used natural CO_2 gradients off the Island of Ischia, Italy in a location that is not affected by elevated sulphur or metals. Carbon dioxide emissions from these seeps alter local ocean carbonate chemistry where pH varied along a gradient from ambient (8.1 – 8.2) to low pH (lowest mean pH was 6.57) (Kerrison et al., 2011). One important aspect of these seeps is that the water is emitted at ambient seawater temperature, salinity and Total Alkalinity.

Hall-Spencer et al. (2008) found that at ambient pH (8.1 - 8.2) typical rocky shore communities were present with many calcareous organisms such as coralline algae, sponges, polychaetes, crustaceans, molluscs and echinoderms. At lowered pH (mean 7.8 – 7.9) there was a 30% reduction in the number of species and communities shifted to those dominated by non-calcareous organisms such as fleshy algae. Organisms with aragonite skeletons were absent at a mean saturation state of Ω_{Arag} <2.5. Coralline algal cover fell from >60% cover outside the seep areas to zero closer to the seeps. Non-calcareous algal cover increased from almost zero outside the seep area to >60% within it, with an abundance of large Phaeophytes.

The strength of using the seeps is that they allow us to see which organisms are tolerant of long-term exposure to elevated CO_2 levels and how those tolerant species interact to form communities (Hall-Spencer et al., 2008). The seeps are most useful if they are used as an indication of what may happen to coastal ecosystems with future ocean acidification rather than a direct analogue. The CO_2 seeps are too small and

transient to mimic the effects of global ocean acidification, but as they show long-term responses of coastal ecosystems to increased CO_2 levels at a variety of locations worldwide, they supplement predictions based on laboratory experiments (Johnson et al., 2012).

Further investigations have now examined benthic foraminifera along shallow water CO_2 gradients worldwide (Cigliano et al., 2010, Dias et al., 2010, Fabricius et al., 2011, Uthicke and Fabricius, 2012, Uthicke et al., 2013). Dias et al. (2010) were the first to examine the impact of ocean acidification on benthic foraminifera using shallow water CO_2 seeps. They found that species diversity fell from 24 species at sites with ambient pH (~ 8.17) to just four species at sites with low pH (~ 7.6). This reduction in species diversity reflected those recorded for the larger calcified benthic organisms (Hall-Spencer et al., 2008). Together with a reduction in the number of species recorded, there was a change in the community from one dominated by calcareous foraminifera to one dominated by agglutinated forms. The results from this study suggested that foraminifera are sensitive to the effects of ocean acidification over the range of pH occurring at the CO_2 seeps.

By placing artificial collectors along the pH gradient at Ischia, Cigliano et al. (2010) were able to examine the settlement of benthic invertebrates and microfauna. Increased levels of CO_2 had significant adverse impacts on the settlement of a wide variety of meroplankton. As pCO_2 increased from normal (336 – 341 ppm), to elevated levels (886 – 5148 ppm), serpulid polychaetes, gastropods, bivalves and calcareous foraminifera showed significant reductions in recruitment. There was a significant decrease in species richness and the number of individuals of benthic foraminifera in the low pH conditions.

Other shallow water CO_2 seeps have since been used to examine the impact of ocean acidification on benthic foraminifera. Fabricius et al. (2011) were the first to examine

foraminifera from shallow water CO_2 seeps offshore Papua New Guinea. Sediment at high pCO$_2$ sites (up to 953 ppm) was found to be almost free of calcareous biota (including benthic foraminifera) and even sites with lower pCO$_2$ (up to 494 ppm) contained many pitted or eroded tests of foraminifera. This finding is surprising given the relatively high pH and suggests that the pH may fall well below the mean for certain periods. In a subsequent investigation, Uthicke et al. (2013) found steep declines in foraminiferal densities and diversity as pCO$_2$ increased near to the seeps. At sites with a pH ~7.9 foraminifera were almost absent. The decline in non-calcifying taxa was less steep, but at pH ~7.9 they were also absent (Uthicke et al., 2013). The symbiont-bearing benthic foraminifera, *M. vertebralis,* was absent from three seep sites with pH below ~7.9, but present in densities of over 1000 m^{-2} at reference sites off Papua New Guinea (Uthicke and Fabricius, 2012).

1.4 Summary

Ocean acidification is a rapidly developing area of research and the response of foraminifera to elevated CO_2 is variable, although most work indicates that calcified benthic species are highly vulnerable. It is apparent that there will be non-uniform responses due to differences in test types and life modes. Investigations carried out in laboratory experiments often contrasts with studies in natural environmental settings. Current evidence suggests that calcareous foraminifera will be negatively impacted by ocean acidification through reduced calcification and increased dissolution, whereas allogromiids and agglutinated foraminifera will be less severely affected and may even be at a competitive advantage due to ocean acidification. This raises serious concerns for the survival of calcareous benthic foraminifera as the oceans continue to acidify. The loss of calcareous foraminifera may have dramatic consequences for inorganic carbon cycling. The short duration of many of the laboratory experiments makes it hard to scale-up responses over the longer-term and species interactions cannot be examined. In order to increase our understanding of the impact of ocean acidification on benthic foraminifera, laboratory investigations can be augmented with *in situ*

investigations (Murray and Bowser, 2000, Havenhand et al., 2010). Although this may be time consuming and expensive, it will provide multiple strands of evidence which will equip society with a more robust set of data on which to base predictions about biological, ecosystem and societal impacts if the biosphere is disrupted by ocean acidification.

1.5 Thesis aims

This thesis describes experiments that use shallow water CO_2 gradients to investigate the effects of ocean acidification on shallow water benthic foraminiferal assemblages. The examination of benthic foraminiferal assemblages in response to elevated CO_2 has been relatively overlooked and the use of shallow water CO_2 seeps allows species interactions to be examined. This thesis aims to examine how shallow water, natural benthic foraminiferal assemblages respond to increased pCO_2. As the boron isotopic composition of foraminiferal tests is used as a palaeo-proxy for ocean pH, the boron isotopic composition of benthic foraminifera collected across shallow water CO_2 gradients were also examined.

Chapter 2 describes benthic foraminiferal assemblages along a CO_2 gradient in the northern Gulf of California, Mexico.

The aims of this chapter were to examine the community composition of living benthic foraminifera and the preservation of living and dead benthic foraminifera along a natural gradient of CO_2 in the northern Gulf of California. It was hypothesised that there would be an overall reduction in the number of species and a reduction in calcifying species in the areas with lower pH conditions. It was also expected that there would be test dissolution in the lowest pH conditions.

Chapter 3 describes foraminiferal assemblages along shallow water CO_2 gradients in the Mediterranean Sea.

The aims of this study were to examine foraminiferal assemblages along shallow water CO_2 gradients in the Mediterranean Sea and to determine if the calcifying macroalgae *Padina pavonica* was capable of providing a refugia for epiphytic foraminifera along gradients of overlying seawater acidification. It was hypothesised that there would be a reduction in the number of species and a reduction in calcifying taxa of benthic foraminifera across shallow water CO_2 gradients off Ischia and Vulcano, Italy. It was also hypothesised that *Padina pavonica* would provide refugia for calcareous epiphytic foraminifera along gradients of overlying seawater acidification.

Chapter 4 describes an experiment to examine the settlement of benthic foraminifera on artificial collectors along a shallow water CO_2 gradient in the Mediterranean Sea. The aims of this chapter were to examine the impact of ocean acidification on the settlement of benthic foraminifera and to see if the results of a previous investigation (Cigliano et al., 2010) were reproducible at other shallow water CO_2 seeps. It was hypothesised that there would be a change in the assemblage of benthic foraminifera found on artificial collectors placed along a gradient of overlying seawater acidification and a reduction in calcifying taxa in the more acidified sites.

Chapter 5 examines the major and trace element concentrations and boron isotopic composition of benthic foraminifera collected across shallow water CO_2 gradients. The aim of this chapter was to examine the major and trace element composition and the boron isotopic composition of benthic foraminifera, collected along natural CO_2 gradients. It was hypothesised that as seawater pH decreased, $\delta^{11}B_{carb}$ of benthic foraminiferal tests would also decrease. It was also hypothesised that there would be a $\delta^{11}B$ offset between species, as others have shown (Sanyal et al., 2001).

Chapter 2: Benthic foraminiferal assemblages along a CO$_2$ gradient in the northern Gulf of California, Mexico

Abstract

It is expected that ocean acidification will alter the assemblage composition of benthic foraminifera, but, to date, foraminiferal assemblages from natural situations have only been examined in the Mediterranean Sea and off Papua New Guinea. Here, benthic foraminiferal assemblages were examined along a natural gradient in pH (7.88 – 7.55) in the northern Gulf of California at water depths 74 – 207 m. It was hypothesised that there would be an overall reduction in the number of species and a reduction in calcifying species in lower pH. It was also expected that there would be test dissolution in the lowest pH conditions. Living calcareous benthic foraminifera were found in the lowest pH conditions (7.55), although there was an impoverished species assemblage. There was no statistically significant correlation between the number of species or individuals in the living assemblage and pH. There was a reduction in the number of species in the dead assemblage as pH decreased, but no significant change in the number of individuals. There was also evidence of post-mortem test dissolution. The reason for the presence of some living calcareous foraminifera in the lowest pH conditions are not clear, but may be due to the greater availability of food, more stable temperatures or a more stable saturation state in the northern Gulf of California compared to previously examined shallow water CO_2 seeps in the Mediterranean Sea and off Papua New Guinea.

2.1 Introduction

The examination of benthic foraminiferal assemblages in relation to ocean acidification from natural gradients in CO_2 is a new area of research. As benthic foraminifera are an important constituent of many benthic ecosystems it is key to determine if they are likely to be impacted by ocean acidification. Living assemblages of benthic foraminifera have previously been examined in relation to ocean acidification around natural CO_2 seeps adjacent to the Island of Ischia, Italy (Dias et al., 2010) and at CO_2 seeps off Papua New Guinea (Fabricius et al., 2011, Uthicke et al., 2013). No previous

41

investigations have been conducted around newly discovered CO_2 seeps in the northern Gulf of California in the Wagner and Consag basins (Canet et al., 2010). If similar patterns in the response of living foraminiferal assemblages are found around different CO_2 seeps then this provides more support to previous findings.

Previous studies of benthic foraminifera around shallow water CO_2 seeps have found a change in the community from one dominated by calcareous forms, in ambient pH conditions, to one dominated by agglutinated forms in lowered pH conditions (Dias et al., 2010). Examination of benthic foraminifera off Ischia revealed that along a gradient from normal pH (mean pH ~8.14) to acidified areas (mean pH ~7.6), the number of species fell from 24 to just four species of benthic foraminifera (Dias et al., 2010). The reduction in the number of species near to the CO_2 seeps mirrored those found for larger benthic calcifying organisms (Hall-Spencer et al., 2008). In addition, Fabricius et al. (2011), who examined calcareous biota at CO_2 seeps off Papua New Guinea, found that sediment at high $p$$CO_2$ sites (up to 953 ppm) was almost free of calcareous biota (including benthic foraminifera) and sites with lower $p$$CO_2$ (~ 444 ppm) contained many pitted or eroded tests of foraminifera. A subsequent study, at CO_2 seeps around Papua New Guinea, also found steep declines in foraminiferal densities and diversity as $p$$CO_2$ increased near to the seeps (Uthicke et al., 2013). At sites with a pH ~7.9 foraminifera were almost absent. The decline in non-calcifying taxa was less steep, but at pH ~7.9 they were also absent (Uthicke et al., 2013). It is thought that symbiont-bearing foraminifera may be less susceptible to ocean acidification because photosynthesis of the symbionts elevates pH in the diffusive boundary layer (Uthicke et al., 2013). The symbiont-bearing benthic foraminifera, *Marginopora vertebralis*, however, was absent from three seep sites with pH below ~7.9, but present in densities of over 1000 m^{-2} at control sites off Papua New Guinea (Uthicke and Fabricius, 2012).

The study of benthic foraminifera around CO_2 seeps can also be used to predict what may happen as sediments are exposed to calcium carbonate undersaturation as the

aragonite and calcite saturation horizons shoal. The level of saturation with respect to aragonite and calcite decreases as depth increases (Sabine et al., 2002). The limit between supersaturation, in surface waters, and undersaturation, at depth, is known as the saturation horizon (Orr et al., 2005). The depth of the saturation horizons varies spatially (Bindoff et al., 2007). These saturation horizons are important as they determine the preservation of calcium carbonate on the sea floor and the amount buried within sediments (Sabine et al., 2002). They also determine the distribution of certain organisms such as deep-sea corals (Guinotte et al., 2006, Tittensor et al., 2010, Jackson et al., 2014).

Increasing carbon dioxide levels are causing the aragonite saturation horizon to shoal in all ocean basins (Feely and Chen, 1982, Sabine et al., 2002, Sarma et al., 2002, Olafsson et al., 2009). This means that the limit between undersaturated and supersaturated waters is becoming shallower, leaving a greater proportion of the water column subject to undersaturated conditions. Olafsson et al. (2009) calculated that the aragonite saturation horizon in the Iceland Sea was shoaling at 4 myr^{-1}, exposing another 800 km^2 of seafloor to undersaturated conditions each year. The surface waters of the Southern Ocean are expected to become undersaturated with respect to aragonite by the year 2050 (Caldeira and Wickett, 2005, Orr et al., 2005). The saturation horizon has varied significantly in the geologic past (Van Andel, 1975, Coxall et al., 2005, Zachos et al., 2005, Hönisch et al., 2012), but rates of change have been much slower than those experienced today.

It is important to investigate the impact on the benthos as it undergoes a rapid transition from saturated to undersaturated conditions (Olafsson et al., 2009). Examining foraminiferal assemblages around natural CO_2 seeps can help to increase our understanding. If dead benthic foraminifera dissolve due to exposure to undersaturated conditions then fewer will eventually be buried within sediments.

2.1.1 Study area

The tectonically active Wagner and Consag basins are situated near the northernmost end of the Gulf of California (Figure 2.1) (Aragón-Arreola and Martín-Barajas, 2007, Canet et al., 2010). They are up to ~ 225 m deep, making them the shallowest active basins in the Gulf of California (Canet et al., 2010). These pull-apart basins, formed by oblique rifting, were formed during the middle to late Pliocene (Aragón-Arreola and Martín-Barajas, 2007) and are controlled and connected by the Wagner Fault (Aragón-Arreola and Martín-Barajas, 2007).

During acoustic surveys in 2007 and 2010, over 300 large, diffuse seafloor gas seeps were discovered. These seeps were concentrated along the eastern edge of the Wagner and Consag basins, along the Wagner Fault (Canet et al., 2010, Prol-Ledesma et al., 2013). These seeps alter the carbonate chemistry of the overlying seawater making the area suitable for the examinination of the long-term effects of ocean acidification (Prol-Ledesma et al., 2013). Over approximately 42 000 km^2, 20 – 50 m of sea water near to the bottom has a reduced pH of 7.55 – 7.98 (Prol-Ledesma et al., 2013). This reduced pH predominates over mixing caused by strong currents. Although the vent gases are thought to contain some methane (Canet et al., 2010), CO_2 predominates in the whole area (Prol-Ledesma et al., 2013).

The Gulf of California is a narrow, marginal sea. High, but variable nutrient levels are characteristic in the northern part due to upwelling (Halfar et al., 2004). The area is characterised by high rates of primary productivity, approximately two to three times greater than the open Atlantic at similar latitudes (Zeitzschel, 1969). Strong winds and tides create a well-mixed water column which persists for most of the year (Zeitzschel, 1969). This strong mixing means that temperatures at depth in the basin are almost constant.

Of the larger benthic fauna examined around the seeps in the Wagner and Consag basins, polychaetes were found to be the dominant infaunal taxa (Canet et al., 2010). Epifaunal taxa consisted of sponges, hydrozoans, gastropods, bivalves and some scleractinian corals (Canet et al., 2010). The coarser sediments had a rich fauna including decapods, amphipods and echinoderms.

The aims of this study were to examine the community composition of living benthic foraminifera and the preservation of living and dead benthic foraminifera along a natural gradient in CO_2 in the northern Gulf of California. It was hypothesised that there would be an overall reduction in the number of species and a reduction in calcifying species in the areas with lower pH conditions. It was also expected that there would be test dissolution in the lowest pH conditions as this has been shown in other studies of the effects of ocean acidification on calcareous foraminifera (e.g. Le Cadre et al., 2003, Moy et al., 2009).

2.2 Methods

2.2.1 Sample collection

Samples were collected during the WAG-02 cruise aboard R/V *El Puma* by colleagues from the National Autonomous University of Mexico. Sample stations were chosen to represent the variations in pH due to the presence of seeps (Figure 2.1). Sediment samples were collected using a Smith McIntyre grab which had an area of 0.1 m^2 (Wigley and McIntyre, 1964, Canet et al., 2010, Prol-Ledesma et al., 2013). Once aboard the research vessel, subsamples were obtained with 60 ml syringes with an area of 7.06 cm^2. They were sliced every 2 cm to sample the different strata. The sediment was stained with rose Bengal (1 g/L) and preserved in 4% buffered formalin for up to three months, until the samples were processed.

Figure 2.1: Map of the Gulf of California showing the study area. The locations from which benthic foraminifera were examined are marked with stars. The number next to the star refers to the station number. Circles represent the position of CO_2 seeps in the area.

Ideally, to avoid pseudo replication (Schönfeld et al., 2012), replicate samples would have been obtained from separate deployments of the Smith McIntyre grab. This, however, was not possible. No replicate cores were taken from the same deployment. This was in part because the samples were used by other members of the research cruise in other analyses. The lack of replicates is problematic due to patchiness in the distribution of foraminifera (Buzas, 1970, Bernstein et al., 1978, Griveaud et al., 2010). The samples can still be used to indicate patterns in foraminiferal assemblages.

Rosette mounted CTD casts (General Oceanics, Mark III WOOCE) were taken at every sampling station to monitor environmental parameters. Seawater samples were collected 10 m above the bottom to avoid physical damage to the CTD. To test if seawater samples collected a few centimetres above the seabed differed from ones collected 10 m above the bottom, near-bottom water samples were taken at two sampling stations. A Niskin bottle was connected to an ROV arm with a hand-made

system. Seawater bottom samples collected using the ROV did not differ from seawater collected 10 m above the seabed using the rosette.

2.2.2 Carbonate chemistry

The carbonate chemistry parameters were measured and calculated by Dr Riccardo Rodolfo-Metalpa. Seawater samples for the measurement of carbonate chemistry parameters were immediately collected from the recovered rosette in glass bottles. The pH_T (in total scale) was measured using a meter (Metrohm pH mobile) accurate to 0.01 pH units and calibrated using TRIS/HCl and 2-aminopyridine/HCl buffer solutions (Dickson et al., 2007). Seawater samples were then passed through Whatman® glass microfiber filters (grade GF/F), treated with 0.05 ml of 50% $HgCl_2$ (Merck, Analar) and stored in the dark at 4°C pending analysis. Three replicate 20 ml sub-samples were analysed at 25°C using a titration system composed of a pH-meter with a Metrohm (UK, Ltd.) pH electrode and a 1 ml automatic burette (Metrohm, UK, Ltd.). pH was measured at 0.02 ml increments of 0.1 M HCl. Total alkalinity (TA) was calculated from the Gran function applied to pH from 4.2 to 3.0, as mEq l^{-1} from the slope of the curve pH *vs*. HCl volume. Titrations of total alkalinity standards, provided by A.G. Dickson Laboratory (batch 99 and 102; Scripps Institution of Oceanography), were within 0.7 µmol kg^{-1} of the nominal value. Parameters of the carbonate system (pCO_2, CO_3^{2-}, HCO_3^-, DIC and saturation state of calcite (Ω_{Calc}) and aragonite (Ω_{Arag})) were calculated from pH_T, mean TA, temperature and salinity using the free-access CO2SYS package (Lewis and Wallace, 1998) with the constants of Mehrbach et al. (1973). pH is expressed on the total scale at the depth at which the sample was collected. Total Alkalinity was virtually constant at all sites, therefore its mean was used in the calculation of the carbonate chemistry parameters (TA = 2359.01 µmolkg^{-1}). Means of pH_T were calculated from hydrogen ion concentrations of each measurement and then re-converted back to pH (Dickson et al., 2007).

2.2.3 Sample processing

Only the core top (0 – 2 cm) samples were analysed as previous work in a nearby location has found that the majority of living foraminifera are found in the upper 1 to 2 cm (Lesen, 2005) and this is the segment most suitable for finding live foraminifera (Corliss, 1985, Alve and Murray, 1997). Although some living infaunal taxa are likely to have been present in the deeper intervals, these were not examined as there are likely to have been very few living individuals. Barmawidjaja et al. (1992) found that more than 71% of all living foraminifera were found in the upper 2 cm of sediment in the northern Adriatic Sea, a cold water/temperate site. Standard micropalaeontological techniques were employed with samples washed on a 63 µm sieve (Murray, 2006). The retained fraction was left to air dry for up to 72 hours. Once dry, the sediment was transferred to labelled plastic weighing vials. Prior to analysis, sediment samples were split using an Otto sediment splitter. Split samples were placed onto a brass picking tray and analysed under a stereo-binocular microscope. Individual foraminifera were dry picked using a fine paint brush and placed onto micropalaeontological slides. The foraminifera were identified, in the first instance, using (Loeblich and Tappan, 1964, 1987) and identified to species level, where possible, using Bandy (1953, 1961), Brenner (1962), McGann (2002) and Lesen (2005). The smallest split fraction was examined first. If at least 300 individuals were found within the fraction, no additional fractions were examined from this sample as 300 individuals are believed to be statistically representative of the whole sample (Pielou, 1966, Murray, 1991a). If fewer than 300 individuals were found then an additional fraction was examined. This continued until at least 300 individuals had been found, or the whole sample had been examined, whichever came first.

The rose Bengal stain was used to determine living individuals; it adsorbs onto proteins and stains the cytoplasm magenta (Walton, 1952). Individuals were determined to have been live at the time of collection if they were stained dark magenta in at least half of their chambers (Bernhard et al., 2006). This excluded counting individuals with just a

faint red dot in one chamber. As rose Bengal is a non-vital stain, it also has the potential to stain remaining protoplasm or bacteria, thereby overestimating the number of living individuals (Bernhard et al., 2006). Murray and Bowser (2000), however, argue that most foraminifera die through processes that do not leave protoplasm-filled tests, such as predation. It can also be difficult to see the staining in opaque specimens including many species of agglutinated foraminifera (Murray, 1991a, Bernhard, 2000) and some foraminifera with living cytoplasm may not take up the stain if the aperture is blocked (Bernhard et al., 2006). If used carefully, however, rose Bengal can be accurate in environments where there are unlikely to be large numbers of dead foraminifera that still contain protoplasm (Walton, 1952, Murray and Bowser, 2000, Figueira et al., 2012) and it is inexpensive and relatively easy to use on a research cruise, where there may be limited facilities. It is often not practical to examine large amounts of sediment immediately after collection as would be required with vital stains, which may be more appropriate for laboratory experiments (Murray and Bowser, 2000).

The number of foraminifera per gram of sediment examined were calculated using the following equation:

$$\frac{\text{Number of individuals}}{\text{Weight of sediment examined (g)}}$$

2.2.4 SEM

In order to assess their preservation, some foraminifera were imaged using a JEOL JSM 6610 LV scanning electron microscope (SEM) with a digital imaging system. Individuals were mounted on aluminium SEM stubs and sputter coated in an Emitech K550 gold sputter coater.

2.2.5 Statistical analysis

The Shannon-Wiener diversity index, Pielou's evenness index and the Fisher Alpha index were calculated. The Fisher Alpha index was used in addition to Shannon-

Wiener diversity as this is the most commonly used index in foraminiferal studies (Murray, 2006) allowing comparisons to be made with other formainiferal studies. Correlations between the number of species, the number of individuals, Shannon-Wiener diversity index and environmental parameters were tested using Spearman's rank order correlation. Spearman's rank order correlation measures the strength of a relationship between two variables (Field, 2005). It was used because it is non-parametric and the data failed the Shapiro-Wilk test for normality. As a means of testing whether there was a difference between stations with higher and lower pH, a Mann-Whitney rank sum test was conducted on the data. The stations were separated into two groups: those with a low pH (7.55, 7.63, 7.66, 7.69 and 7.75 (n=5)) and those with a high pH (7.83, 7.83, 7.86 and 7.88 (n=4)). The Mann-Whitney rank sum test is a non-parametric test that looks for differences between two independent samples (Field, 2005). SigmaPlot v.12.0 and PRIMER v.6. were used to perform these analyses. Further statistical analysis was not undertaken due to limited sample numbers and sample size.

The Fisher Alpha index examines the relationship between the number of individuals and the number of species in each sample. It takes rarer species into account and assumes that the number of individuals of each species follows a logarithmic series (Peet, 1974, Murray, 1991a). The Shannon-Wiener index is based on information theory and takes into account the distribution of individuals between species as well as the number of species. Pielou's evenness index is a measure of how dominant species are within the sample. If all species within the sample have the same number of individuals then the evenness index will equal one. If one species dominates the assemblage then the index will approach zero. The Shannon-Wiener index and Pielou's evenness index are co-dependent.

2.3 Results

2.3.1 Environmental parameters

The water chemistry data are based on single point measurements. Salinity ranged from 35.2 to 35.5 and total alkalinity ranged from 2331 – 2386 µmol kg^{-1}. The pH$_T$ of the bottom waters ranged from 7.55 – 7.88, indicative of prevailing low pH conditions due to the presence of the CO_2 seeps. The seawater at all stations was oversaturated with respect to calcite (Ω_{Calc}), the lowest Ω_{Calc} value being 1.47 (Table 2.1). The aragonite saturation state (Ω_{Arag}) ranged from 0.95 – 2.37, with undersaturation only at the station with the lowest pH.

Table 2.1: The range in carbonate chemistry parameters from sample stations in the northern Gulf of California. Measurements are single point measurements, with no replicates. Measurements were taken in July and August 2010.

Station	Depth (m)	Temp (°C)	Salinity (‰)	pH$_T$	Ω_{Calc}	pCO$_2$ (µatm)	TA (µmol kg^{-1})	Living calcareous foraminifera	Dead calcareous foraminifera
31	80	20.6	35.4	7.88	3.60	674	2330.55	X	✓
27	88	20.4	35.4	7.86	3.46	675	-	X	✓
25	80	23.3	35.5	7.83	3.61	730	2386.43	✓	✓
9	74	22.4	35.5	7.83	3.50	735	2363.00	✓	✓
29	105	20.0	35.4	7.75	2.69	917	2382.21	✓	✓
12	195	14.0	35.2	7.69	1.90	1021	-	✓	✓
1	160	15.1	35.2	7.66	1.84	1130	2330.55	X	✓
2	160	17.3	35.4	7.63	1.89	1222	2383.26	✓	✓
19	207	15.2	35.2	7.55	1.47	1466	2330.55	✓	✓

2.3.2 Living assemblages

There were low numbers of living (stained) individuals present in all samples and three of the samples had no living individuals. Two of the samples with no living individuals were stations with the highest pH (7.86 and 7.88 units). Of the living individuals found, the most abundant species were *Nonionella basispinata* (37.7%), *Epistominella bradyana* (27.0%) and *Bulimina marginata* (11.3%) (Table 2.2).

Table 2.2: A list of the species of benthic foraminifera found within the stained (living) assemblage from the Gulf of California. Numbers per gram of sediment are reported along with the percentage contribution. Numbers per gram were calculated from the dry sediment weight of the >63 µm fraction.

	pH																	
	7.55		7.63		7.66		7.69		7.75		7.83		7.83		7.86		7.88	
Species	Live per g	%	Live per g	%	Live per g	%	Live per g	%	Live per g	%	Live per g	%	Live per g	%	Live per g	%	Live per g	%
Ammonia sp. 1	-	-	-	-	-	-	-	-	12	38	-	-	-	-	-	-	-	-
Bolivina (inflated sutures)	-	-	50	18	-	-	-	-	2	6	-	-	-	-	-	-	-	-
Bolivina acutula	-	-	-	-	-	-	-	-	-	-	5	100	2	17	-	-	-	-
Bolivina sp. 2	-	-	25	9	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bulimina denudata	-	-	-	-	-	-	-	-	-	-	-	-	2	17	-	-	-	-
Bulimina marginata	-	-	50	18	-	-	-	-	4	13	-	-	-	-	-	-	-	-
Elphidium excavatum	-	-	-	-	-	-	1	50	4	13	-	-	-	-	-	-	-	-
Epistomella bradyana	-	-	125	45	-	-	-	-	4	13	-	-	-	-	-	-	-	-
Hanzawaia nitidula	-	-	-	-	-	-	1	50	2	6	-	-	2	17	-	-	-	-
Nonionella basispinata	155	100	25	9	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Reophax sp. 2	-	-	-	-	-	-	-	-	-	-	-	-	2	17	-	-	-	-
Indeterminate calcareous sp.11	-	-	-	-	-	-	-	-	-	-	-	-	2	17	-	-	-	-
Indeterminate aggultinate sp. 10	-	-	-	-	-	-	-	-	2	6	-	-	-	-	-	-	-	-
Indeteminate aggultinate sp. 7	-	-	-	-	-	-	-	-	-	-	-	-	2	17	-	-	-	-
Indeteminate aggultinate sp. 8	-	-	-	-	-	-	-	-	2	6	-	-	-	-	-	-	-	-

Although some calcareous benthic foraminifera were present within the samples, the living assemblage was impoverished. The percentage of living individuals out of the total species assemblage (living and dead) varied from zero to 20%, with the highest percentage of living individuals occurring in the lowest pH sample. In addition to calcareous foraminifera, four species of agglutinated foraminifera were found within the living assemblage. These were found in two of the sample stations (Station 25 and 29). Dry picking may have resulted in the loss of some of the more fragile agglutinated taxa (Murray and Bowser, 2000), but these taxa were not the focus of this investigation. Dias et al. (2010), who also used the method of dry picking, found that the proportion of agglutinated taxa increased as pH decreased, as did Uthicke et al. (2013). There were no clear shifts in the assemblage from calcareous to agglutinated forms as pH reduced. There were low proportions of agglutinated taxa, regardless of pH. There were no living miliolid taxa in any of the samples. Given the low numbers of living individuals, this is not surprising. Rose Bengal staining in some miliolid taxa can be hard to see and often requires breaking the specimen (Murray, 1991a, Bernhard, 2000).

A total of 15 species were found in the living assemblage and up to eight species were found per sample. In those samples that did have living individuals, the Shannon-Wiener diversity index ranged from 0.00 – 1.84, suggesting a low diversity in all samples. There were no clear patterns between the number of species or the number of individuals per gram and pH, calcite saturation state, water temperature and water depth (Figure 2.2). The low number of living specimens, however, made it difficult to fully assess the statistical significance of any relationships. Spearman's rank order correlation revealed that there was no significant correlation between the number of species and pH (r = -0.247, p = 0.491, n = 9), Ω_{Calc} (r = 0.085, p = 0.809, n = 9), water temperature (r = 0.094, p = 0.775, n = 9) or water depth (r = 0.077, p = 0.809, n = 9). There was no significant correlation between the number of individuals per gram and pH (r = -0.610, p = 0.067, n = 9), Ω_{Calc} (r = -0.305, p = 0.407, n = 9), water temperature (r = -0.034, p = 0.913, n = 9) or water depth (r = 0.316, p = 0.381, n = 9).

The results of the Mann-Whitney rank sum test revealed that there was no significant difference in the number of species (U = 6.500, *p* = 0.413) or the number of individuals per gram (U = 5.000, *p* = 0.286) between the low (n = 5) and high (n = 4) pH groups.
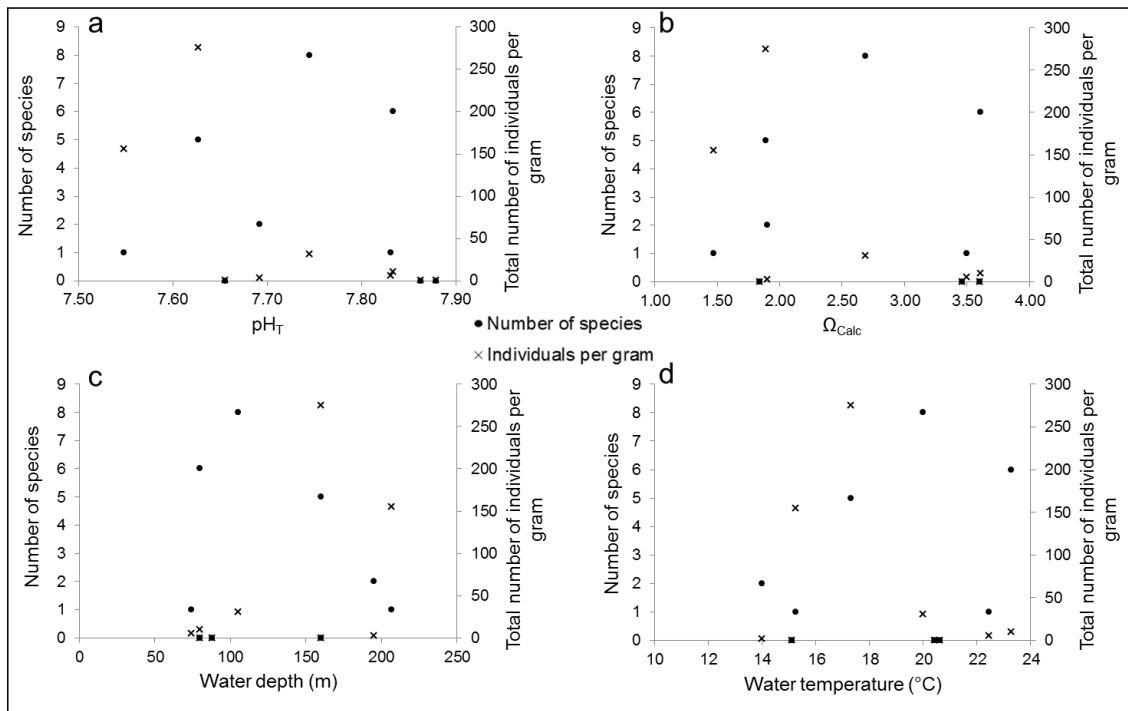
Figure 2.2: The number of species and the number of individuals per gram for the living (stained) assemblage plotted against: a) pH_T, b) calcite saturation state, c) water depth and d) water temperature.

2.3.3 Dead assemblages

Dead (unstained) benthic foraminifera present in the top 2 cm of the sediment contained a selection of mainly calcareous taxa (including *E. bradyana* (53.6%)*, B. marginata* (13.5%)*, Eponides* sp. (7.4%), *Elphidium excavatum* (6.4%) and *N. basispinata* (3.8%)) (Table 2.3). These species are considered to constitute a normal composition for these water depths with the exception of *E. excavatum* which is typically a shallow water species occurring in water depths between zero and 50 m (Murray, 1991a). The presence of *E. excavatum* suggests possible down-slope transportation in the area.

Table 2.3: A list of the species of benthic foraminifera found within the unstained (dead) assemblage from the Gulf of California. Numbers per gram of sediment are reported along with the percentage contribution. Numbers per gram were calculated from the dry sediment weight of the >63 μm fraction.

	pH																	
	7.55		7.63		7.66		7.69		7.75		7.83		7.83		7.86		7.88	
Species	Dead per g	%	Dead per g	%	Dead per g	%	Dead per g	%	Dead per g	%	Dead per g	%	Dead per g	%	Dead per g	%	Dead per g	%
Ammonia sp.1	-	-	-	-	16	15.8	1	0.9	242	49.0	5	0.3	0	0.0	64	1.8	6	5.7
Bolivina (inflated sutures)	-	-	150	2.5	2	1.8	-	-	-	-	-	-	-	-	-	-	1	0.5
Bolivina acuminata	-	-	100	1.7	11	11.4	5	4.5	2	0.4	40	2.7	2	0.8	43	1.2	1	0.5
Bolivina acutula	-	-	100	1.7	-	-	3	2.7	-	-	111	7.4	18	8.3	64	1.8	2	1.5
Bolivina sp. 1	-	-	25	0.4	2	1.8	-	-	-	-	20	1.4	-	-	32	0.9	-	-
Bolivina sp. 2	-	-	-	-	-	-	-	-	-	-	10	0.7	-	-	-	-	-	-
Bolivina sp. 3	-	-	-	-	-	-	-	-	-	-	25	1.7	-	-	-	-	-	-
Bolivina sp. 4	70	11.6	-	-	-	-	1	0.9	2	0.4	5	0.3	-	-	32	0.9	-	-
Bolivina sp. 5	-	-	-	-	-	-	-	-	4	0.8	-	-	-	-	-	-	-	-
Buccella tenerrima	-	-	-	-	-	-	-	-	-	-	5	0.3	-	-	74	2.2	-	-
Bulimina denudata	-	-	-	-	-	-	-	-	2	0.4	-	-	-	-	21	0.6	-	-
Bulimina marginata	-	-	925	15.4	10	9.6	3	2.7	36	7.4	106	7.1	97	44.4	521	15.1	10	9.3
Bulimina sp. 1	-	-	-	-	1	0.9	-	-	-	-	5	0.3	-	-	-	-	-	-
Bulimina sp. 2	-	-	25	0.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cancris auriculus	28	4.7	-	-	-	-	1	0.9	8	1.6	20	1.4	8	3.8	21	0.6	1	0.5
Cassidulina sp. 1	28	4.7	-	-	-	-	1	0.9	2	0.4	-	-	-	-	11	0.3	-	-
Cassidulina sp. 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	43	1.2	-	-
Elphidium excavatum	14	2.3	100	1.7	33	33.3	9	8.1	115	23.3	20	1.4	20	9.0	500	14.5	3	3.1
Epistomella bradyana	296	48.8	3750	62.5	16	15.8	72	67.6	54	10.9	975	65.2	39	18.0	1511	43.7	65	60.3
Elphidium sp. 1	-	-	-	-	-	-	1	0.9	-	-	-	-	-	-	-	-	-	-
Eponides sp. 1	42	7.0	700	11.7	6	6.1	8	7.2	13	2.7	121	8.1	11	5.3	21	0.6	17	16.0
Eponides sp. 2	-	-	-	-	-	-	-	-	-	-	-	-	2	0.8	-	-	-	-
Hanzawaia nitidula	14	2.3	-	-	-	-	2	1.8	6	1.2	5	0.3	-	-	106	3.1	-	-
Lagena sp. 1	-	-	-	-	-	-	-	-	-	-	5	0.3	2	0.8	-	-	-	-
Lagena sp. 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0.5
Lagena sp. 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0.5
Lenticulina sp. 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	11	0.3	-	-
Loxostomum pseudobeyrichi	-	-	-	-	-	-	-	-	-	-	-	-	2	0.8	-	-	-	-
Nonionella basispinata	99	16.3	25	0.4	2	1.8	-	-	6	1.2	5	0.3	10	4.5	330	9.5	-	-
Nonionella sp. 1	14	2.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Reophax sp. 1	-	-	-	-	-	-	-	-	-	-	5	0.3	3	1.5	-	-	1	0.5
Reophax sp. 2	-	-	-	-	-	-	-	-	-	-	-	-	3	1.5	-	-	-	-

Table 2.3 continued:

	pH																	
	7.55		7.63		7.66		7.69		7.75		7.83		7.83		7.86		7.88	
Species	Dead per g	%	Dead per g	%	Dead per g	%	Dead per g	%	Dead per g	%	Dead per g	%	Dead per g	%	Dead per g	%	Dead per g	%
Textularia sp. 1	-	-	25	0.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Textularia sp. 2	-	-	25	0.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Textularia sp. 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	11	0.3	-	-
Trochammina sp. 1	-	-	25	0.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trochammina sp. 2	-	-	25	0.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trochammina sp. 3	-	-	-	-	-	-	1	0.9	-	-	-	-	-	-	-	-	-	-
Trochammina sp. 4	-	-	-	-	-	-	-	-	2	0.4	-	-	-	-	-	-	-	-
Trochammina sp. 5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0.5
Uvigerina excellens	-	-	-	-	2	1.8	-	-	-	-	5	0.3	-	-	43	1.2	1	0.5
Veleroninoides sp. 1	-	-	-	-	-	-	-	-	-	-	-	-	2	0.8	-	-	-	-
Indeterminate calcareous sp. 1	-	-	-	-	1	0.9	-	-	-	-	-	-	-	-	-	-	-	-
Indeterminate calcareous sp. 2	-	-	-	-	1	0.9	-	-	-	-	-	-	-	-	-	-	-	-
Indeterminate calcareous sp. 3	-	-	-	-	-	-	-	-	-	-	5	0.3	-	-	-	-	-	-
Indeterminate calcareous sp. 4	-	-	-	-	-	-	-	-	-	-	5	0.3	-	-	-	-	-	-
Indeterminate calcareous sp. 6	-	-	-	-	-	-	-	-	-	-	5	0.3	-	-	-	-	-	-
Indeterminate calcareous sp. 7	-	-	-	-	-	-	-	-	-	-	-	-	10	4.5	-	-	-	-
Indeterminate calcareous sp. 8	-	-	-	-	-	-	-	-	-	-	-	-	3	1.5	-	-	-	-
Indeterminate calcareous sp. 9	-	-	-	-	-	-	-	-	-	-	-	-	2	0.8	-	-	-	-
Indeterminate calcareous sp. 10	-	-	-	-	-	-	-	-	-	-	-	-	2	0.8	-	-	-	-
Indeterminate calcareous sp. 12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	11	0.3	-	-
Indeterminate calcareous sp. 13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0.5
Indeterminate calcareous sp. 14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0.5
Indeterminate aggultinate sp. 1	-	-	-	-	1	0.9	-	-	-	-	5	0.3	-	-	-	-	-	-
Indeterminate aggultinate sp. 2	-	-	-	-	-	-	-	-	-	-	5	0.3	-	-	-	-	-	-
Indeterminate aggultinate sp. 3	-	-	-	-	-	-	-	-	-	-	-	-	2	0.8	-	-	-	-
Indeterminate aggultinate sp. 6	-	-	-	-	-	-	-	-	-	-	-	-	2	0.8	-	-	-	-
Indeterminate aggultinate sp. 8	-	-	-	-	-	-	-	-	6	1.2	-	-	-	-	-	-	-	-
Indeterminate aggultinate sp. 9	-	-	-	-	-	-	-	-	2	0.4	-	-	-	-	-	-	-	-
Indeterminate aggultinate sp. 11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	1.5

Low numbers of agglutinated taxa were found in all samples, with the exception of the station with the lowest pH which had no agglutinated taxa. As mentioned above, dry picking may have resulted in the loss of some of the more fragile agglutinated taxa (Murray and Bowser, 2000). There were no miliolid taxa in the assemblage, thus the assemblage consisted almost entirely of hyaline taxa.

The highest number of species for any one sample was 24 and the lowest was nine. The number of species appeared to increase with an increase in pH, calcite saturation state and water temperature and decrease as water depth increased (Figure 2.3). These patterns were statistically significant and Spearman's rank order correlation revealed that the number of species increased as pH ($r = 0.79$, $p = 0.009$, $n = 9$), Ω_{Calc} ($r = 0.84$, $p = 0.002$, $n = 9$) and water temperature ($r = 0.88$, $p = <0.001$, $n = 9$) increased and decreased as water depth increased ($r = -0.96$, $p = <0.001$, $n = 9$). There was no significant correlation between the number of individuals per gram and pH ($r = -0.067$, $p = 0.844$, $n = 9$), Ω_{Calc} ($r = 0.000$, $p = 0.983$, $n = 9$), water temperature ($r = 0.317$, $p = 0.381$, $n = 9$) or water depth ($r = -0.143$, $p = 0.676$, $n = 9$). These results suggest that there was a reduction in the number of species in the dead assemblage as pH decreased, but no statistically significant change in the number of individuals.

The results of the Mann-Whitney rank sum test revealed there to be a significant difference in the number of species ($U = 0.000$, $p = 0.016$) and the Fisher Alpha index ($U = 0.000$, $p = 0.016$) between the low ($n = 5$) and high ($n = 4$) pH groups. There was no statistically significant difference in the number of individuals per gram of sediment ($U = 8.000$, $p = 0.730$) or the Shannon-Wiener diversity index ($U = 7.000$, $p = 0.556$) between the two groups (low ($n = 5$) and high ($n = 4$) pH).
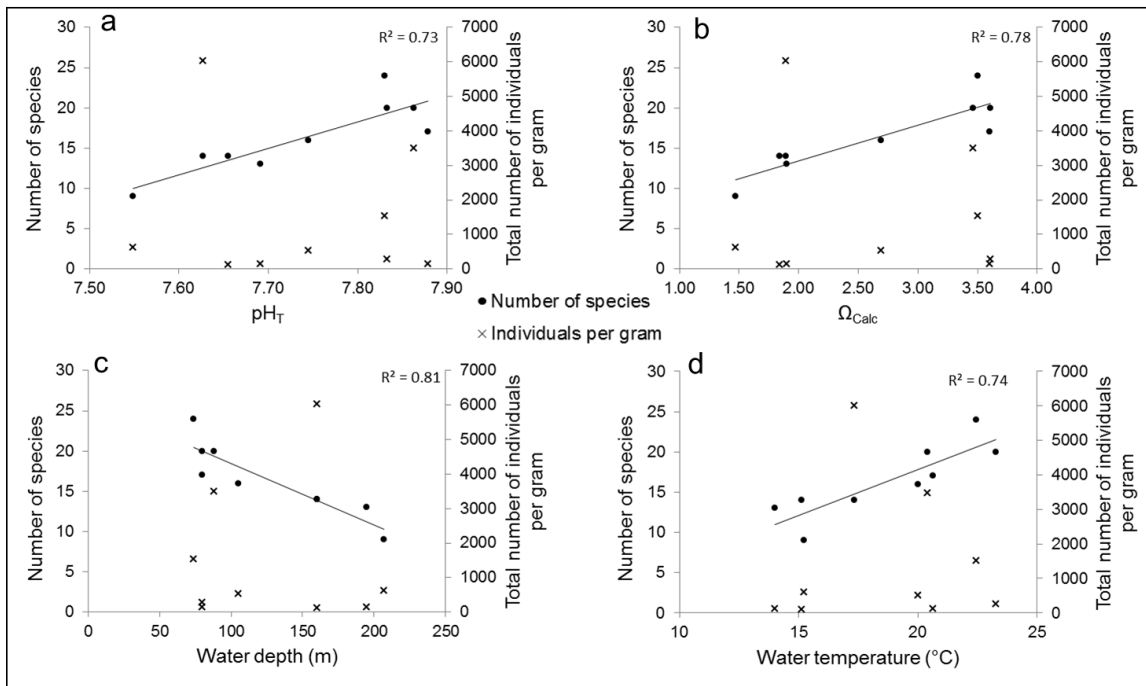
Figure 2.3: The number of species and the number of individuals per gram for the dead assemblage plotted against: a) pH_T, b) calcite saturation state, c) water depth and d) water temperature. A linear trend line has been plotted on the graph for the data points for the number of species along with the R^2 value.

2.3.4 SEM

A total of 40 individuals (stained and unstained) belonging to six species were examined under the SEM. The wall detail was examined under a higher magnification (up to x 8000) in 26 of these individuals. The wall detail of the final chamber was examined if possible, as this is often the thinnest and, therefore, often shows damage quicker than the older, thicker-walled, chambers (Hansen, 1999). Some individuals had their last chamber broken off, in which case the penultimate chamber was examined. Some dissolution was determined to have occurred if the foraminifera test showed etching, pitting, fragmentation or enlarged pores.

Unfortunately, the low number of stained individuals limited the number that could be viewed under the SEM. Out of the 40 individuals examined under the SEM, ten were stained. Some of the stained foraminifera (30%) showed signs of dissolution, suggesting that they were able to live in the low pH conditions, but their tests were beginning to dissolve. Alternatively, the dissolution could be early post-mortem. Most of

58

the dead individuals (60%) showed signs of dissolution and many of these showed severe signs of dissolution, with extensive pitting and fragmentation.

Although there were signs of dissolution in some of the foraminifera, there were no deformities (such as abnormally shaped chambers) in any of the individuals examined under the SEM (Figure 2.4 and Figure i). This was also true for samples examined from Ischia studied by Dias et al. (2010). Circular borings with a distinct profile were found in a few of the individuals examined (Figure 2.5), which are thought to be caused by gastropods belonging to the families Naticidae and Muricidae (Maddocks, 1988). These are not, however, a dissolution feature and indicate that the specimen was almost certainly living when it was bored by the gastropods.
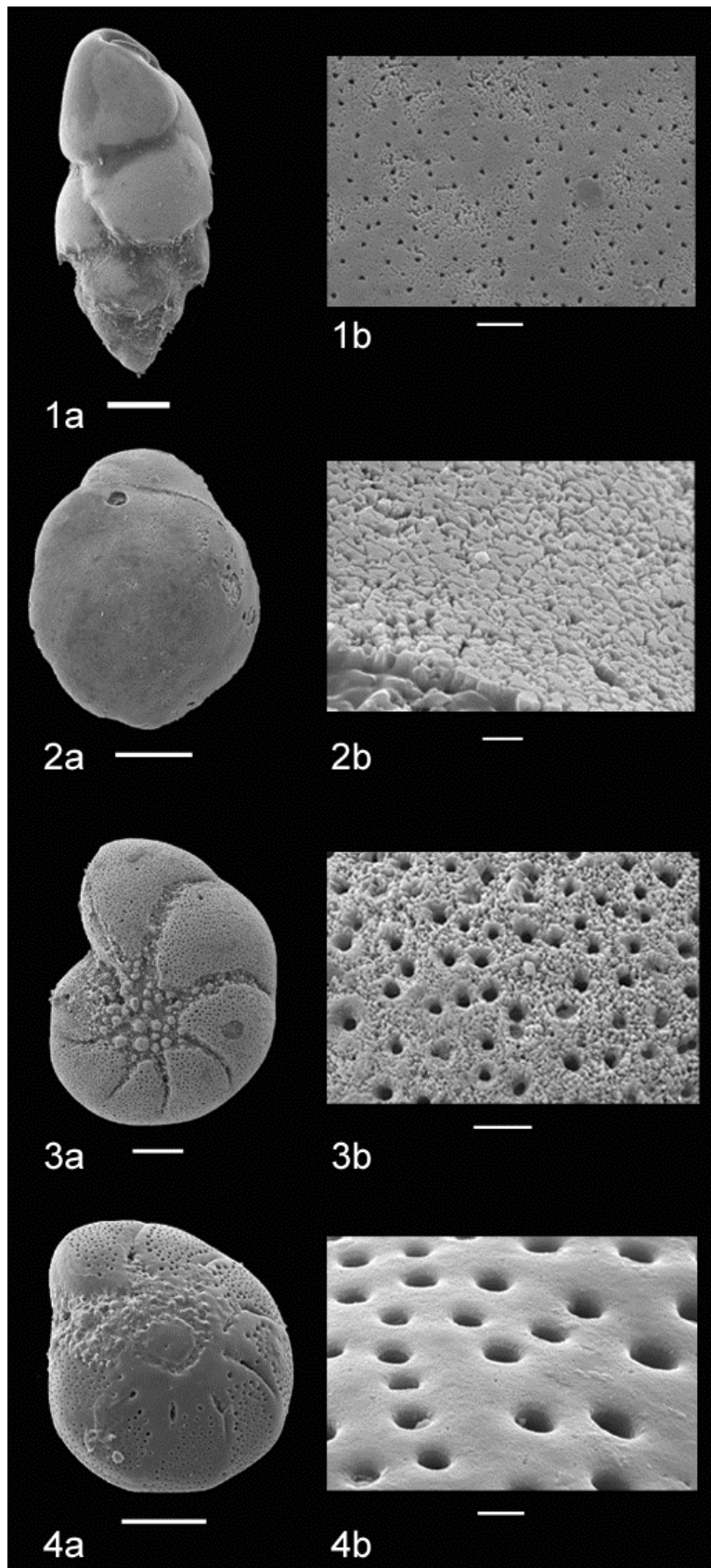
Figure 2.4: Scanning electron microscope images of some rose Bengal stained benthic foraminifera with surface preservation shown. 1a) *Bulimina marginata* from station 29 (pH 7.75), b) wall detail; 2a) *Epistominella bradyana* from station 29 (pH 7.75), b) wall detail; 3a) *Elphidium excavatum* from station 29 (pH 7.75), b) wall detail; 4a) *E. excavatum* from station 12 (pH 7.69), b) wall detail. The scale bars for 1a), 2a), 3a) and 4a) are 50 μm, and the scale bars for 1b), 2b) and 4b) are 2 μm; the scale bar for 3b) is 5 μm. The figure was compiled by C. Smart.

Figure 2.5: A SEM image of *Epistominella bradyana* from station 9 (pH 7.83) with evidence of boring features. The roundness and evenness of the features suggest a biological origin, thought to be caused by gastropods belonging to the families Naticidae and Muricidae. This specimen did not pick up the rose Bengal stain. The scale bar is 50 μm. The figure was compiled by C. Smart.

2.4 Discussion

Calcifying benthic foraminifera were present in pH conditions ranging from 7.55 to 7.88 near CO_2 seeps in the northern Gulf of California. Although there were low numbers of species, calcareous foraminifera dominated the species composition at the lowest pH stations. The hypothesis that there would be a reduction in the number of species and the number of calcifying species for the living assemblage in the lower pH conditions was not supported. This was perhaps due to the low number of living foraminifera. The dead assemblage did show a significant reduction in the number of species as pH reduced. The hypothesis that there would be evidence of test dissolution in calcareous benthic foraminifera was supported with 30% of the stained individuals that were examined under the SEM showing some signs of dissolution.

The range in pH between the stations was 0.33 units. Although this is less than the total decrease in pH (0.77 units) that is predicted to occur due to anthropogenic emissions (Caldeira and Wickett, 2003), it still represents a large range in the concentration of hydrogen ions. The range in pH (0.33 units) is greater than the mean

pH reductions (<0.05 units) measured in bottom waters during an experiment assessing the impact of injected carbon dioxide on deep-sea benthic foraminifera (Bernhard et al., 2009a) and is similar to those expected along a dilution gradient, under carbon sequestration options (Bernhard et al., 2009a). The highest pH recorded was 7.88, this is below the value of 8.1, considered to be a global average for surface waters (Raven et al., 2005, Guinotte and Fabry, 2008), but it is expected that infaunal benthic foraminifera will experience lower pH conditions within the sediment pore water. At the locations from which benthic foraminifera were examined, pore water pH ranged from 6.06 – 7.34 (Ruth Esther Villanueva-Estrada, unpublished data).

Elphidium excavatum, which is a shallow water species was found within the species assemblage. *Elphidium excavatum* typically lives in water depths between zero and 50 m (Murray, 1991a). The presence of this species suggests possible down-slope transportation in the area. Only a very small percentage of *E. excavatum* (0.6%) were stained. It is possible that the majority of the individuals of this species had been washed into the sample sites and were not living in the vicinity of the seeps. Of the two stained *E. excavatum* examined under the SEM, neither showed signs of dissolution.

Stained calcareous species also included a mixture of infaunal and epifaunal taxa such as *N. basispinata* (infaunal), *B. marginata* (infaunal) and *E. bradyana* (epifaunal) which are usually found at these water depths (Murray, 1991a). There were no miliolid taxa within either the living or the dead assemblage. It is expected that miliolid taxa will be less resilient to ocean acidification than hyaline taxa because they have high-Mg calcite (Bentov and Erez, 2006). It could be that miliolid taxa were absent from the assemblage due to the low pH conditions. Bandy (1961), who examined foraminifera from different depths and locations in the Gulf of California, reported high percentage contributions of miliolid taxa in water depths of 18 – 37 m and 37 – 73 m (41% and 52%, respectively). In water depths similar to the ones in this study (74 – 207 m), however, lower percentages of miliolid taxa were reported (23% in water depths 73 –

152 m and 5% in water depths 152 – 244 m) (Bandy, 1961) and shelf sea assemblages typically have low proportions of porcelaneous taxa (Murray, 2006).

It is perhaps surprising that more agglutinated foraminifera were not found within the species assemblage, although Bandy (1961) also reported low percentage contributions of agglutinated foraminifera. In the lowest pH conditions examined around CO_2 seeps at Ischia, Italy, Dias et al. (2010) found that agglutinated foraminifera dominated the assemblage, although there was a low species diversity.

There were no stained *Eponides* species within the samples, although these dominated the dead assemblage. This suggests that these species were unable to survive in the low pH conditions or that they were not actually autochthonous and had been transported in from elsewhere. *Eponides* have been reported to dominate species assemblages in low oxygen environments (0.25 – 1.1 ml l^{-1} O_2) (Douglas and Heitman, 1979). Pore waters in anoxic sediments can sometimes be alkaline as a result of the sulphate-reducing bacteria (Murray, 1991a), which involves the reduction of CO_2 and the formation of hydrocarbons (Wright and Colling, 1995). This may explain why living *Eponides* species, which are typically tolerant of low oxygen, alkaline conditions, were not found in low pH conditions. Other taxa found within the living assemblage, however, such as *Bolivina* and *Bulimina* are also tolerant of low oxygen environments (Douglas and Heitman, 1979).

Dias et al. (2010) found a dramatic reduction in benthic foraminiferal diversity and abundance near to CO_2 seeps around the Island of Ischia, Italy. In contrast to the findings from this study, no living calcareous foraminifera were found below pH ~ 7.6 at Ischia (Dias et al., 2010) or below pH ~ 7.9 at Papua New Guinea (Fabricius et al., 2011, Uthicke and Fabricius, 2012, Uthicke et al., 2013). Although there were some living calcareous benthic foraminifera in this study, they were present in low numbers. Fabricius et al. (2011) and Uthicke et al. (2013) however, did not distinguish between

living and dead individuals. If the total assemblage (living and dead individuals) is considered from the northern Gulf of California samples, then disparity with Fabricius et al. (2011) seems to be even greater, as there was no reduction in the number of calcareous benthic foraminifera in the lowest pH sites.

Some living benthic foraminifera may be able to survive the low pH conditions in the northern Gulf of California due to the supply of nutrients and the availability of food. The northern Gulf of California is characterised by abundant nutrients which are carried to the surface through upwelling and tidal mixing (Zeitzschel, 1969, Halfar et al., 2004, Halfar et al., 2006). A plentiful supply of food for foraminifera in the northern Gulf of California may enable them to persist in the stress of a low pH environment. *Bolivina* and *Nonionella*, two living taxa found in this investigation, are typically found in organic rich environments (Kennett et al., 2000, Rathburn et al., 2000). In the high nutrient conditions of Kiel Fjord, calcifying invertebrates were found to dominate the macrobenthic community despite low pH conditions (Thomsen et al., 2010). Conversely, the Mediterranean Sea is an oligotrophic area in which nutrients decrease towards the east. Low pH conditions at Ischia might be an additional stress to that of limited food supply. Bacteria on the seagrass at Ischia, which would be expected to provide a food source for some epiphytic foraminifera (Muller and Lee, 1969, Murray, 2006), are reported to have low densities (Novak, 1982, Velimirov et al., 1984, Velimirov, 1986). Limited food supply at Ischia could, therefore, be a reason for the absence of living calcareous benthic foraminifera in low pH conditions.

Rodolfo-Metalpa et al. (2010) found that increased temperature and decreased pH acted synergistically to reduce calcification in the bryozoan *Myriapora truncata* at CO_2 seeps around Ischia. Further work revealed that the effects of ocean acidification on molluscs and corals were also exacerbated by high temperatures (Rodolfo-Metalpa et al., 2011). If the same principle applies to calcareous benthic foraminifera, this could be a possible explanation for their presence in the northern Gulf of California, but not

Ischia or Papua New Guinea where seawater temperatures are higher. The shallow waters off Ischia and Papua New Guinea can warm quickly and temperatures can reach up to 30 °C at Ischia (Rodolfo-Metalpa et al., 2011). Temperature can control the major distribution patterns of shallow water foraminifera (Murray, 2006) and the higher seawater temperatures at Ischia may provide an additional stress to the benthic foraminifera. The extent of the change in temperature, above or below the mean, may have more importance than the absolute temperature. The deeper environment and strong mixing in the northern Gulf of California means that temperature fluctuations are likely to be less severe than those in the shallow waters around Ischia and Papua New Guinea.

Variability in the calcite saturation state could also affect the ability of calcareous benthic foraminifera to survive in low pH conditions in the northern Gulf of California. The variability in Ω_{Calc} in the northern Gulf of California is unknown, but there is a high degree of variability at Ischia (Hall-Spencer et al., 2008, Kerrison et al., 2011). The emissions from the seeps in the Wagner Basin are continuous and the pH recorded in this study roughly corresponds to pH values measured during the previous WAG-01 cruise (Prol-Ledesma, personal communication). It is, therefore, expected that the saturation state will be fairly stable. In addition, the lack of strong temperature variations in the northern Gulf of California could mean that there is little variation in Ω_{Calc}. It may be that foraminifera cannot survive in the variable saturation states at Ischia, and possibly Papua New Guinea, but more stable saturation states in the northern Gulf of California allow some to survive.

In many organisms, calcium carbonate saturation is thought to exert the strongest control on calcification. As the saturation state reduces, the energetic cost of creating and maintaining biogenic calcium carbonate is expected to increase (Riebesell et al., 2000, Orr et al., 2005, Guinotte and Fabry, 2008, de Nooijer et al., 2009a; 2009b). Differences in the saturation state were proposed as a possible explanation for

differences in the abundance of the coccolithophore *Emiliania huxleyi* between the Baltic Sea and the Black Sea (Tyrrell, 2008). *Emiliania huxleyi* were found to be abundant in the Black Sea, but absent from the Baltic Sea, which is surprising given their abundance in nearby waters at similar latitudes. Although differences in environmental factors, such as salinity or silicate concentration, could be an explanation for their absence, undersaturation with respect to aragonite and calcite in the Baltic Sea, but not the Black Sea, could also be an explanation. It is possible, however, that calcification rates in foraminifera are not related to calcite saturation state (de Villiers, 2004). Heaviest foraminiferal shell weights were found not to have a straightforward relationship with saturation state; instead calcification appeared to be a function of the complex interplay of environmental parameters (de Villiers, 2004). This argues against the role that saturation state can play in determining the resilience of benthic foraminifera to ocean acidification. Keul et al. (2013a), however, argue that CO_3^{2-} concentration, which will determine saturation state if Ca^{2+} concentration remains constant, is the parameter affecting size normalised weights and growth rates in foraminifera.

Foraminifera are thought to promote calcification by elevating their intracellular pH (de Nooijer et al., 2008; 2009a; 2009b). A reduction in pH would, therefore, be expected to increase the amount of energy required to elevate intracellular pH to levels required for calcification. If more energy is required for calcification then food availability may become a limiting factor for calcification. Alternatively, if the organisms are unable to elevate intracellular pH to the required levels, it is expected that there will be reduced carbonate concentrations for precipitation (de Nooijer et al., 2009b). Either of these two scenarios is likely to lead to a reduction in calcification. This view might be too simplistic and the methods used by de Nooijer et al. (2008, 2009b) are likely to lead to reduced resolution and unreliable pH measurements (Bentov et al., 2009). Bentov et al. (2009) observed that Ca^{2+} was supplied to the site of calcification in *Amphistegina lobifera*, a benthic foraminifera, through the transport of seawater in vacuoles rather

than through membrane ion transporters. The seawater vacuoles underwent alkalisation (to a pH of 8.7) during their intracellular passage which elevated CO_3^{2-} concentration. The energy expenditure of the foraminifera, needed to reach the required pH and maintain the same calcification rate, would still be determined by the initial saturation state of the seawater in the vacuole. The need for increased energy expenditure, in order to maintain the same rate of calcification, may explain why some of the stained foraminifera examined under the SEM showed signs of dissolution. Although the foraminifera were still living in the low pH sites, they may not have had the required energy to maintain their calcification rate.

It is also likely that calcareous benthic foraminifera experience increased dissolution of their tests when exposed to corrosive waters. Rodolfo-Metalpa et al. (2011) found that when corals and molluscs were transplanted near to CO_2 seeps in the Mediterranean, they were still able to calcify and grow faster, but dissolution rates of their calcium carbonate skeletons increased. This may also be the case for benthic foraminifera in the northern Gulf of California. It may be that in some low pH environments, although the foraminifera are still able to produce their calcium carbonate tests, they have thinner or smaller tests and reduced shell weights. This was found in a study of planktonic foraminifera in the Southern Ocean (Moy et al., 2009). When comparing sediment trap data with sediments of pre-industrial age, Moy et al. (2009) found approximately a 30 – 35% reduction in the shell weight of *Globigerina bulloides*.

The sample stations were not ideal for studying the effects of a shoaling saturation horizon on benthic foraminifera. The calcite saturation state was above one at all sampling stations and there was aragonite undersaturation at one sample station only. Many of the dead benthic foraminifera were, however, showing signs of dissolution, even though they were collected from environments that were supersaturated with respect to calcium carbonate. This suggests that if saturation states lower in the future and carbonate sediments experience undersaturation due to shoaling of the saturation

horizon, benthic foraminifera will experience dissolution before they can be buried within sediment.

Foraminifera must have survived in high CO_2 environments that occurred during the Cretaceous–Paleogene "greenhouse" world where atmospheric pCO_2 was very much higher (estimates have ranged from ~1000 ppm up to ~ 4000 ppm), but with calcareous foraminifera apparently thriving (Walker et al., 1981, Berner et al., 1983, Berner, 1990, Caldeira and Rampino, 1991, Andrews et al., 1995, Bice and Norris, 2002, Royer et al., 2004, Tyrrell and Zeebe, 2004, Kintisch, 2006). Concentrations of CO_3^{2-} are thought to have almost quadrupled since the Cretaceous and pH has increased, whilst the calcium carbonate saturation state has varied very little (Tyrrell and Zeebe, 2004). This suggests that foraminifera were able to survive in the low pH conditions (~ 0.6 - 0.7 pH units lower than present day) of the Cretaceous because the calcite saturation state remained high (Stanley and Hardie, 1998). It is possible that the saturation state was decoupled from pH and remained high (Ridgwell and Schmidt, 2010, Hönisch et al., 2012). In events involving a geologically rapid release of CO_2, such as is occurring in the present day, the balances between sources (weathering) and sinks ($CaCO_3$ burial) of calcium will not respond fast enough to regulate the calcium carbonate saturation state. This will result in a coupled decline of both pH and saturation state (Ridgwell and Schmidt, 2010).

2.5 Conclusions

Examination of calcareous benthic foraminifera collected from around natural CO_2 seeps in the northern Gulf of California has revealed that they show some resilience to low pH conditions. Living calcareous benthic foraminifera were present in pH conditions as low as 7.55 units, albeit in low numbers. Although these results have some similarities to the findings from other shallow water CO_2 seeps at Papua New Guinea (Fabricius et al., 2011, Uthicke et al., 2013) and Ischia (Dias et al., 2010), calcareous species were not found in the lowest pH conditions examined at Papua New Guinea

(pH ~7.9 units) or Ischia (pH ~7.6 units). Differences in nutrient concentrations and carbonate saturation state variability in the northern Gulf of California may be possible explanations for the discrepancies. There was some evidence of dissolution in individuals examined under the SEM. This could be a result of the foraminifera not being able to meet the increased energy requirements to maintain the same calcification rate under low pH conditions and/or a result of increased dissolution of their tests.

The measurement of shell weights would be an interesting follow-up study. Although the foraminifera are still able to produce their calcium carbonate tests in low pH conditions, they may have thinner or smaller tests and reduced shell weights. Benthic foraminifera, however, are difficult to accurately weigh. The presence of organic matter and sediment on the tests can complicate this process and the presence of borings on some of the specimens may lead to misleading results.

It would also be interesting to examine the carbonate shell chemistry of some of the specimens in order to investigate whether the Mg/Ca ratios of the foraminiferal tests vary in the different pH conditions. Carbonate shell chemistry, however, varies within a species (Allison and Austin, 2008) and even within an individual across different chambers (Allison and Austin, 2008); it is therefore difficult to assign a high or low Mg/Ca 'type' to a particular species.

Chapter 3: Changes in foraminiferal assemblages along CO$_2$ gradients in the Mediterranean Sea

Abstract

It is expected that ocean acidification will alter the assemblage composition of benthic foraminifera and previous investigations of foraminiferal assemblages across shallow water CO_2 seeps in the Mediterranean Sea have revealed a reduction in calcareous taxa with an increase in pCO_2. The aim of this chapter was to assess if this reduction was found across different shallow water CO_2 gradients in the Mediterranean Sea and if photosynthetic activity of macroalgae would be able to protect epiphytic foraminifera from the adverse effects of ocean acidification. Benthic foraminifera were unusually rare in the sediment at Vulcano, even at ambient levels of CO_2 (~ 423 µatm), meaning that infaunal and epifaunal foraminifera could not be examined across the CO_2 gradient. This was thought to be a result of the inhospitable nature of the sediment. There was a reduction in the number of species of epiphytic foraminifera found on the small macroalga, *Padina pavonica,* near to CO_2 seeps and the community assemblage changed from one dominated by calcareous forms (*Pileolina patelliformis*, *Rosalina globularis*, *Elphidium* spp.) at reference sites (mean pH 8.19) to predominately agglutinated forms (*Daitrona* sp.) near to the seeps (mean pH 7.71). As the same pattern has been found in sediments along calcium carbonate saturation gradients, this raises serious concerns for the survival of shallow water, calcareous benthic foraminifera as the oceans continue to acidify. Although small stands of macroalgae did not provide a refuge for calcareous foraminifera, higher biomass stands of algae or seagrass meadows may be able to mitigate the effects of ocean acidification at local scales.

3.1 Introduction

Ocean acidification has the potential to impact calcareous foraminifera. Laboratory investigations have shown that the shell weight of planktonic foraminifera declines as seawater carbonate saturation falls (Bijma et al., 1999). Spero et al. (1997) found that *Orbulina universa* shell weight increased by 37% when grown at high *vs.* background calcium carbonate concentration. Bernhard et al. (2009a; 2009b) showed that deep-

sea calcareous foraminifera are killed by direct exposure to injected CO_2, whereas thecate and agglutinated species survive. Acidification of seawater with 1000 ppm CO_2 caused shell dissolution in the shallow water calcified foraminifera *Haynesina germanica* and their calcareous ornamentation used for feeding was reduced and deformed (Khanna et al., 2013). Analysis of data from the Continuous Plankton Recorder (CPR) has revealed that planktonic foraminifera have survived the rate of seawater acidification that has occurred in the North Sea since the 1950s (Beare et al., 2013). In the Southern Ocean, Moy et al. (2009) suspect that ocean acidification is the reason why shells of a modern planktonic foraminifera (*Globigerina bulloides*), collected from sediment traps, are ~ 30 – 35% lighter than those preserved in underlying Holocene sediments when atmospheric levels of CO_2 were lower.

Studies of benthic foraminifera around shallow water CO_2 seeps have shown steep reductions in species richness; calcified foraminifera appear to be intolerant of chronic exposure to acidified waters in coastal sediments of the Mediterranean Sea (Dias et al., 2010) and off Papua New Guinea (Fabricius et al., 2011, Uthicke et al., 2013). Dias et al. (2010) examined foraminiferal assemblages across a natural gradient in CO_2 caused by shallow water gas seeps off the Island of Ischia, Italy. They found that species diversity fell from 24 species in sites with normal pH (~ 8.17) to just four species in sites with low pH (~ 7.6). This reduction in species diversity reflected that recorded for larger benthic organisms such as cnidarians, molluscs, crustaceans and echinoderms (Hall-Spencer et al., 2008). As well as a reduction in the number of species recorded, the community changed from one dominated by calcareous foraminifera at mean pH 8.17 to one dominated by agglutinated forms at mean pH 7.6. The results from this study suggest that foraminifera are sensitive to the effects of ocean acidification over the range of pH occurring at the CO_2 seeps. At seeps off Papua New Guinea, sediment at high pCO_2 sites (up to 953 ppm) lacked most calcareous biota (including benthic foraminifera) and even sites with lower pCO_2 (up to 494 ppm) contained many pitted or eroded tests of foraminifera (Fabricius et al., 2011).

Near to the CO_2 seeps with mean pH below ~7.9, tests of the symbiont-bearing foraminifera *Marginopora vertebralis* were absent from seagrass blades, but were present in densities of over 1000 m^{-2} at nearby reference sites off Papua New Guinea (Uthicke and Fabricius, 2012). There were steep declines in foraminiferal density and diversity as pCO$_2$ increased near to the seeps (Uthicke et al., 2013). At sites with a mean pH ~7.9 foraminifera were almost absent. The decline in non-calcifying taxa (*Textularia* spp., *Septotextularia* sp. and *Clavulina* spp.) was less steep, but at pH ~7.9 they were also absent (Uthicke et al., 2013).

These findings raise serious concerns for the survival of shallow water benthic foraminifera as the oceans continue to acidify. It is, therefore, important to investigate whether macroalgae or seagrasses are capable of protecting foraminifera against the effects of ocean acidification. Foraminifera play an important role in the Earth's CO_2/CO_3^{2-} budget (Lee and Anderson, 1991, Langer et al., 1997), thus their response to ocean acidification may have important consequences for inorganic carbon cycling. A major foraminiferal die-off may act as a negative feedback on atmospheric CO_2 levels and lead to a reduction in globally precipitated calcium carbonate (Dissard et al., 2010a). The foraminifera may serve as indicator species (Ferrari et al., 2011), reflecting responses of other small calcified animals, such as larval bivalves.

Seaweeds and seagrasses can thrive in waters with naturally high pCO$_2$ (Martin et al., 2008, Porzio et al., 2011) and have the potential to provide an ecologically important and cost effective means for improving seawater conditions for sensitive organisms by consuming dissolved CO_2 and raising local seawater pH (Gao and McKinley, 1994, Manzello et al., 2012, Chung et al., 2013, Cornwall et al., 2014, Hendriks et al., 2014). An increase in pH of up to 0.38 units is possible within seagrass meadows (Unsworth et al., 2012). The primary production of those seagrasses and macroalgae which are carbon limited increases as CO_2 levels rise (Connell and Russell, 2010, Manzello et al., 2012), which has led to a drive by the United Nations to conserve such habitats. A blue

carbon project has been established in South Korea, where natural and made-made marine communities are being used to remove CO_2 in coastal regions. The higher the biomass of these plant communities, the more CO_2 is drawn down (Chung et al., 2013).

Photosynthesis by macroalgae utilises the dissolved inorganic carbon (DIC) pool, usually in the form of CO_2 (Gao and McKinley, 1994). The surfaces of organisms have a micro-layer (known as a diffusion boundary layer, DBL) that usually differs from the surrounding seawater chemistry (Hurd et al., 2011). Within the DBL, the chemistry is altered by metabolic processes and in macroalgae, photosynthesis increases the pH in the daytime (De Beer and Larkum, 2001, Hurd et al., 2009; 2011, Cornwall et al., 2013). These DBLs are on the scale of micrometres to millimetres, but within macroalgal canopies, where there is reduced flow, larger concentration gradients can develop (up to 68 mm) (Cornwall et al., 2013). Although macroalgae raise pH during the day, at night, pH in the DBL may drop to ~ 7.8, under slow flows (Cornwall et al., 2013). This means that epiphytes can experience a large range in pH on a daily basis which is likely to be greater than the mean changes in pH expected over the next hundred years as a result of ocean acidification (Cornwall et al., 2013) and may negate the potential benefit that macroalgae may have on epiphytes.

In laboratory experiments, it has been found that algal epiphytes can resist levels of acidification associated with pCO$_2$ values of 1193±166 µatm (Saderne and Wahl, 2013). *In situ* experiments show that seagrass photosynthesis can enhance calcification of the calcareous red algae *Hydrolithon* sp. 5.8-fold (Semesi et al., 2009a). There are limits, however, to the role that marine plants can play in buffering the effects of ocean acidification. Martin et al. (2008) found a dramatic reduction in calcareous epiphytes on seagrass blades as pH reduced below a mean pH 7.7, near to CO_2 seeps off Ischia in the Mediterranean Sea.

Shallow water CO_2 seeps are useful for examining the ability of macroalgae to protect epiphytes from the effects of ocean acidification (Hall-Spencer et al., 2008, Boatta et al., 2013). They allow species interactions, such as that between macroalgae and epiphytes, to be examined (Martin et al., 2008, Porzio et al., 2011, Johnson et al., 2012).

There have been many previous studies on epiphytic foraminifera (e.g. Langer, 1993, Bresler and Yanko, 1995, Ribes et al., 2000, Semeniuk, 2000, 2001, Debenay and Payri, 2010, Mateu-Vicens et al., 2010, Mateu-Vicens et al., 2014), but few in relation to ocean acidification. Langer (1993) examined epiphytic foraminifera from algae and seagrasses around the Island of Vulcano, Italy. There were four different phytal microhabitats dominated by species with distinct test morphologies. The diversity of epiphytic assemblages was controlled by the temporal availability of phytal substrates and the shape of the substrate (Langer, 1993). This suggests that the temporal availability of the phytal substrate will determine the degree of protection against ocean acidification that the macroalgae can provide. Epiphytic foraminifera have also been examined following a submarine gas eruption which created acidified conditions. Panieri (2006) examined the effect of a submarine gas eruption on benthic foraminiferal assemblages off the Island of Panarea, Italy. Ten days after the gas eruption, agglutinated foraminifera dominated the sediment samples and most calcareous taxa were affected by severe dissolution. In samples consisting of *Posidonia oceanica* leaves and roots, hyaline forms dominated the assemblage. Panieri (2006) speculated that hyaline species may have been able to survive in the seagrass meadows due to a greater variety of habitats (i.e. seagrass leaves, roots or sediment within the meadow). Photosynthesis of the seagrass may have provided some protection for epiphytic foraminifera against the acidic conditions, although this possibility was not examined by Panieri (2006). In addition, epiphytic foraminifera living higher-up on the *P. oceanica* leaves may be subjected to less concentrated hydrothermal fluids and those living in the seagrass roots will be less exposed to the fluids (Panieri, 2006).

In this chapter, the study of Dias et al. (2010) was extended by examining benthic foraminifera across a natural CO_2 gradient off Ischia, Italy. As a means of extending this study, the ability of a seagrass species endemic to the Mediterranean Sea (*Posidonia oceanica*) to create local ocean acidification sanctuaries by providing refugia for calcification was examined. *Posidonia oceanica* is a long-living, slow growing seagrass species that is endemic to the Mediterranean Sea (Marbá et al., 1996). It forms large meadows, with individual clones spanning up to 15 km (Arnaud-Haond et al., 2012) and it covers about 2% of the seafloor in the Mediterranean (Gobert et al., 2006). It is ecologically important, providing a habitat for many species (Duarte, 2002, Marbà et al., 2014). The formation of meadows means that it may be capable of buffering water chemistry over large areas, including sediment within the seagrass meadows. *Posidonia oceanica* occurs across a CO_2 gradient off Ischia (Hall-Spencer et al., 2008, Martin et al., 2008) and epibionts have previously been examined along this gradient, but epiphytic foraminifera were not investigated (Martin et al., 2008). Martin et al. (2008) found that the total mass of epiphytic calcium carbonate was 90% lower at a mean pH of 7.7 compared to a mean pH of 8.2. Hall-Spencer et al. (2008) found that *P. oceanica* production and shoot density increased as CO_2 levels increased and were highest at a mean pH of 7.6. This pattern appears to be identical in the tropics, Russell et al. (2013) examined two seagrass species across a CO_2 gradient caused by seeps off Papua New Guinea where they also found elevated primary production and biomass of *Cymodocea serrulata* and *Halophila ovalis* as pCO_2 increased.

The impact of ocean acidification on sediment dwelling benthic foraminifera across a shallow water CO_2 gradient off Vulcano was also examined to assess whether a common Mediterranean seaweed (*Padina pavonica*) can protect epiphytic foraminifera from the effects of ocean acidification. *Padina pavonica* was chosen since many heterokont algae, and *Padina* spp. in particular, are resilient to the effects of ocean

acidification (Porzio et al., 2011, Johnson et al., 2012), although *P. pavonica* has been observed to reduce and change its mineralisation (from aragonite to less soluble calcium sulphates) with increasing pCO$_2$ (Goffredo et al., 2014). *Padina* is abundant and hosted calcified foraminifera epiphytes at the study site (Langer, 1993). It was important to examine epiphytes from the same species of macroalgae as algal structural features are known to have a large influence on the composition and diversity of epiphytic assemblages (Langer, 1993).

In this chapter, the hypothesis that there will be a reduction in the number of species and a reduction in calcifying taxa of benthic foraminifera across a shallow water CO$_2$ gradient off Vulcano, Italy was tested. The hypothesis that the seagrass *P. oceanica* collected along a CO$_2$ gradient off Ischia, Italy and the brown seaweed *P. pavonica* collected along a CO$_2$ gradient off Vulcano, Italy provide refugia for benthic foraminifera along gradients of overlying seawater acidification was also tested.

3.2 Ischia

3.2.1 Study site

Ischia is an island in the Tyrrhenian Sea, *ca.* 30 km from Naples (Figure 3.1). It was the first shallow water CO$_2$ seep site to be used as an analogue for ocean acidfication (Hall-Spencer et al., 2008) and its use as a suitable study site has since been established by further investigations (Martin et al., 2008, Cigliano et al., 2010, Dias et al., 2010, Rodolfo-Metalpa et al., 2010; 2011, Porzio et al., 2011, Kroeker et al., 2013a). The seeps occur on the northern and southern side of Castello d'Aragonese, with a bridge linking this area to the main island (Figure 3.1). Large beds of *P. oceanica* occur on the southern side of Castello d'Aragonese (Hall-Spencer et al. 2008).
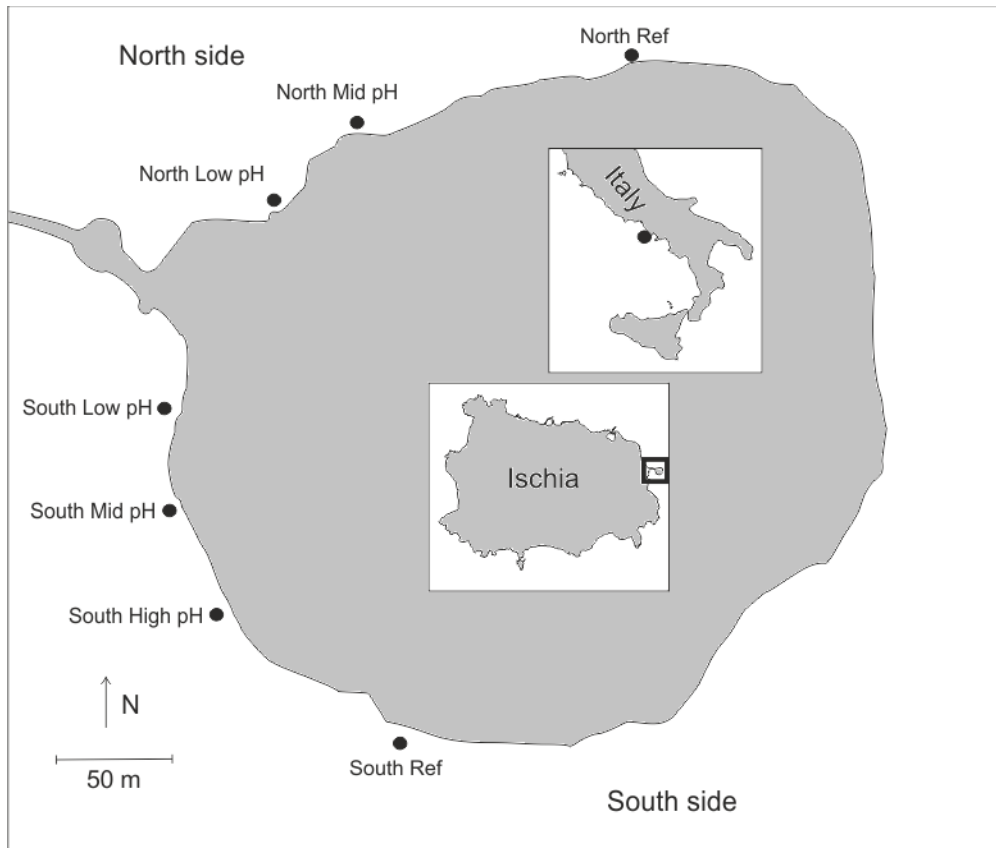
Figure 3.1: Study area off Ischia, Italy with approximate location of sample sites marked by black circles. Inserts show Italy with positioning of Ischia marked by a black circle and Ischia with sample area marked by a black square. Redrawn from Dias et al. (2010).

3.2.2 Methods

Seagrass samples were collected along a CO_2 gradient at Ischia by Dr Riccardo Rodolfo-Metalpa in November 2011. Samples were collected at 2 – 20 m water depth. Samples were collected at four sites along the gradient on the south side of Castello d'Aragonese and at three sites along the gradient on the north side of the Castello. At each site, four different types of sample were collected: 1) surface sediment between *P. oceanica*; 2) *P. oceanica* leaves; 3) *P. oceanica* rhizomes and 4) surface sediment outside the seagrass meadows (Figure 3.2). Only one sample of each type was collected at each sample site. The experiment had been designed as a pilot investigation with additional sampling planned for April 2012, but for logistical reasons this additional sampling was not undertaken.
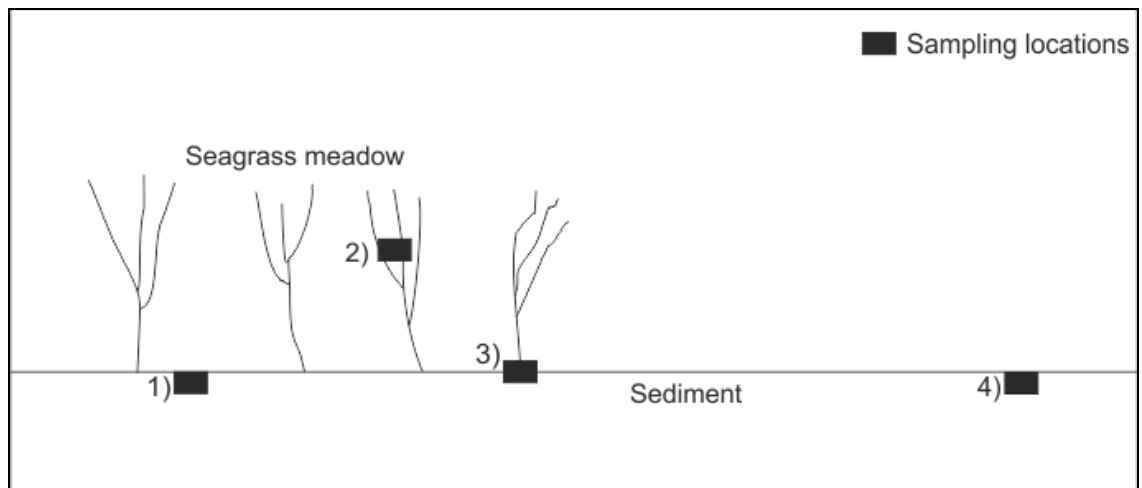
Figure 3.2: Four different habitat types from which samples for the analysis of benthic foraminifera were collected at Ischia. 1) Sediment between seagrass (*Posidonia oceanica*) plants; 2) *P. oceanica* leaves; 3) *P. oceanica* rhizomes and 4) Sediment outside the seagrass meadow. Not to scale.

Posidonia oceanica leaves were collected by cutting the leaves just above the rhizome base to avoid contaminating epiphytic fauna by sediment dwelling species (Langer, 1993). Approximately five leaves were collected per sample. The leaves were placed into pre-labelled plastic sampling bags underwater. Samples were then transferred to plastic sampling pots and preserved in 4% buffered formalin. Rhizome samples were collected by digging into the sediment around the rhizome with a stainless steel spoon. The rhizome was then cut and a small amount of it pulled out of the sediment and placed into pre-labelled plastic sampling bags underwater (Langer, 1993).

Sediment samples were collected using a 10 cm diameter PVC ring which was placed in the top 3 cm of sediment. Sediment contained within the ring was removed using a stainless-steel spoon and placed into pre-labelled plastic sampling bags underwater. Samples were then transferred to plastic sample containers and preserved in 4% buffered formalin.

Once in the laboratory, the seagrass leaves and rhizomes were washed over a 63 µm sieve. The retained fraction was stained with rose Bengal (1 gL^{-1}) for three hours (Sadri et al., 2011). The samples were re-washed over a 63 µm sieve and placed in a drying

oven at 40 °C for 72 hours. Once dry, the samples were examined under a stereo-binocular microscope and epiphytic foraminifera were dry-picked from the samples using a fine paint brush and placed onto micropalaeontological slides. Foraminifera were identified to species level where possible using Cimerman and Langer (1991) and Milker and Schmiedl (2012) and counted. Individuals stained dark magenta in at least half of their chambers were determined to have been live at the time of collection (Bernhard et al., 2006). After washing over a sieve, the *P. oceanica* leaves and rhizomes were also examined and any epiphytic foraminifera remaining were removed and placed onto micropalaeontological slides. After examination, the leaves and rhizomes were air dried and the weight recorded. Weight rather than surface area was measured as there was concern that measuring the surface area may have resulted in the loss of some epiphytic foraminifera.

Sediment samples were processed in a similar way. Samples were washed over a 63 µm sieve. The retained fraction was stained with rose Bengal (1 gL^{-1}) for three hours (Sadri et al., 2011) to distinguish between live and dead individuals. The samples were re-washed over a 63 µm sieve and placed in a drying oven at 40 °C for 72 hours. Once dry, the samples were placed into plastic weighing vials and weighed. Approximately 15 grams of sediment from each sample was examined under a stereo-binocular microscope and benthic foraminifera were dry-picked using a fine paint brush and placed onto micropalaeontological slides. Foraminifera were identified and counted. Criteria for determining living individuals were the same as those used for the epiphytic foraminifera.

Untransformed foraminifera community assemblage data were used to calculate Shannon-Wiener diversity, Fisher Alpha and Pielou's evenness indices using PRIMER v.6. Further statistical analysis was not undertaken due to the limited sample numbers and sample size (Table 3.1).

Table 3.1: Sample names, location and number of replicates for samples collected off Ischia in November 2011. The numbers in brackets after the sample location refer to the habitat type shown in Figure 3.2.

Sample Name	Location	Number of replicates
North Ref sed. in	North side of Castello d'Aragonese Reference site Sediment within seagrass meadow (1)	1
North Ref sed. out	North side of Castello d'Aragonese Reference site Sediment outside seagrass meadow (4)	1
North Mid pH sed. in	North side of Castello d'Aragonese Mid pH site Sediment within seagrass meadow (1)	1
North Mid pH sed. out	North side of Castello d'Aragonese Mid pH site Sediment outside seagrass meadow (4)	1
North Low pH sed. in	North side of Castello d'Aragonese Low pH site Sediment within seagrass meadow (1)	1
North Low pH sed. out	North side of Castello d'Aragonese Low pH site Sediment outside seagrass meadow (4)	1
South Ref sed. in	South side of Castello d'Aragonese Reference site Sediment within seagrass meadow (1)	1
South Ref sed. out	South side of Castello d'Aragonese Reference site Sediment outside seagrass meadow (4)	1
South High pH sed. in	South side of Castello d'Aragonese High pH site Sediment within seagrass meadow (1)	1
South Mid pH sed. in	South side of Castello d'Aragonese Mid pH site Sediment within seagrass meadow (1)	1
South Mid pH sed. out	South side of Castello d'Aragonese Mid pH site Sediment outside seagrass meadow (4)	1
South Low pH sed. out	South side of Castello d'Aragonese Low pH site Sediment outside seagrass meadow (4)	1
North Ref leaves	North side of Castello d'Aragonese Reference site *Posidonia oceanica* leaves (2)	1
North Ref rhizomes	North side of Castello d'Aragonese Reference site *Posidonia oceanica* rhizomes (3)	1
North Mid pH leaves	North side of Castello d'Aragonese Mid pH site *Posidonia oceanica* leaves (2)	1
North Mid pH rhizomes	North side of Castello d'Aragonese Mid pH site *Posidonia oceanica* rhizomes (3)	1
North Low pH leaves and rhizomes	North side of Castello d'Aragonese Low pH site *Posidonia oceanica* leaves and rhizomes (2 and 3)	1
South ref leaves	South side of Castello d'Aragonese Reference site *Posidonia oceanica* leaves (2)	1
South ref rhizomes	South side of Castello d'Aragonese Reference site *Posidonia oceanica* rhizomes (3)	2

Sample Name	Location	Number of replicates
South High pH leaves	South side of Castello d'Aragonese High pH site *Posidonia oceanica* leaves (2)	1
South High pH rhizomes	South side of Castello d'Aragonese High pH site *Posidonia oceanica* rhizomes (3)	1
South Mid pH leaves	South side of Castello d'Aragonese Mid pH site *Posidonia oceanica* leaves (2)	1
South Mid pH rhizomes	South side of Castello d'Aragonese Mid pH site *Posidonia oceanica* rhizomes (3)	1
South Low pH leaves	South side of Castello d'Aragonese Low pH site *Posidonia oceanica* leaves (2)	1
South Low pH rhizomes	South side of Castello d'Aragonese Low pH site *Posidonia oceanica* rhizomes (3)	1

3.2.3 Results

Only one stained individual, belonging to the species *Lobatula lobatula,* was found from the Mid pH sample site, outside of the *P. oceanica* meadow in the sediment samples from the north side of Castello d'Aragonese. All following information refers to the dead (unstained) assemblage. Foraminifera were found in five out of the six sediment samples examined from the north side of Castello d'Aragonese with a total of 1094 individuals counted (Table 3.2). The number of species of foraminifera found within the sediment decreased along a carbon saturation gradient from reference sites to low pH sites (Figure 3.3). The number of species ranged from zero to 50. The number of individuals per 15 grams of sediment (15 g was the weight of sample examined) ranged from zero in sediment collected in the low pH areas outside of the seagrass meadow to 773 in sediment collected from the reference site outside of the seagrass meadow. There was a general trend for the number of species and individuals to decrease across the gradient.

Table 3.2: A list of the species of benthic foraminifera found within the unstained (dead) assemblage from sediment samples from Ischia, North side of Castello d'Aragonese. Numbers per 15 grams of sediment are reported along with the percentage contribution. Numbers per 15 grams were calculated from the dry sediment weight of the >63 µm fraction.

Species	North Ref sed. in		North Ref sed. out		North Mid pH sed. in		North Mid pH sed. out		North Low pH sed. in		North Low pH sed. out	
	Per 15 g	%	Per 15 g	%	Per 15 g	%	Per 15 g	%	Per 15 g	%	Per 15 g	%
Ammoglobigerina globigeriniformis	0	0	1	0	10	2	1	5	6	86	0	0
Ammonia sp. 2	0	0	0	0	2	0	0	0	0	0	0	0
Bolivina difformis	0	0	3	0	0	0	0	0	0	0	0	0
Bolivina pseudoplicata	1	4	14	2	0	0	1	5	0	0	0	0
Bolivina striatula	1	4	9	1	3	1	1	5	0	0	0	0
Bulimina sp. 3	0	0	1	0	0	0	0	0	0	0	0	0
Cornuspira involvens	0	0	15	2	8	2	0	0	0	0	0	0
Cribrostomoides subglobosum	0	0	1	0	2	0	0	0	0	0	0	0
Cymbaloporetta sp. 1	0	0	7	1	0	0	0	0	0	0	0	0
Eggerelloides sp. 1	0	0	0	0	0	0	0	0	1	14	0	0
Elphidium aculeatum	0	0	28	4	49	12	6	32	0	0	0	0
Elphidium margaritaceum	0	0	3	0	5	1	0	0	0	0	0	0
Elphidium sp. 4	0	0	9	1	13	3	0	0	0	0	0	0
Elphidium sp. 5	0	0	23	3	27	7	0	0	0	0	0	0
Elphidium sp. 6	0	0	0	0	0	0	2	11	0	0	0	0
Elphidium sp. 5 of Cimerman and Langer	0	0	3	0	10	2	0	0	0	0	0	0
Fursenkoina acuta	0	0	2	0	0	0	0	0	0	0	0	0
Haplophragmoides canariensis	0	0	1	0	0	0	0	0	0	0	0	0
Haynesina depressula	0	0	34	4	6	2	0	0	0	0	0	0
Lobatula lobatula	1	4	26	3	14	4	1	5	0	0	0	0
Affinetrina gualtieriana	0	0	0	0	8	2	0	0	0	0	0	0
Massilina gualtieriana	0	0	2	0	2	0	0	0	0	0	0	0
Miliolinella subrotunda	1	4	17	2	11	3	0	0	0	0	0	0
Nubeculina divaricata	1	4	0	0	0	0	0	0	0	0	0	0
Peneroplis pertusus	0	0	120	15	11	3	1	5	0	0	0	0
Peneroplis planatus	1	4	21	3	0	0	0	0	0	0	0	0
Pileolina patelliformis	0	0	29	4	16	4	3	16	0	0	0	0
Pileolina patelliformis with float chamber	0	0	2	0	0	0	0	0	0	0	0	0
Planorbulina mediterranensis	6	24	16	2	21	5	0	0	0	0	0	0
Pseudotriloculina laevigata	1	4	10	1	2	0	0	0	0	0	0	0
Pseudotriloculina rotunda	0	0	0	0	2	0	0	0	0	0	0	0
Quinqueloculina auberiana	0	0	13	2	6	2	0	0	0	0	0	0
Quinqueloculina bosciana	0	0	8	1	5	1	0	0	0	0	0	0
Quinqueloculina stelligera	0	0	10	1	8	2	0	0	0	0	0	0
Rosalina globularis	7	28	204	26	99	25	1	5	0	0	0	0
Rosalina sp. 1	0	0	9	1	6	2	0	0	0	0	0	0
Sigmoilina costata	2	8	22	3	2	0	0	0	0	0	0	0
Spiroloculina excavata	0	0	0	0	2	0	0	0	0	0	0	0
Spiroloculina ornata	0	0	15	2	5	1	0	0	0	0	0	0
Textularia sp. 4	0	0	1	0	0	0	0	0	0	0	0	0
Textularia sp. 5	0	0	0	0	2	0	0	0	0	0	0	0
Triloculina adriatica	1	4	0	0	0	0	0	0	0	0	0	0
Triloculinella dilatata	0	0	8	1	2	0	0	0	0	0	0	0
Triloculina marioni	0	0	1	0	2	0	0	0	0	0	0	0
Triloculina plicata	1	4	3	0	3	1	0	0	0	0	0	0
Trochammina inflata	0	0	1	0	2	0	1	5	0	0	0	0
Vertebralina striata	0	0	47	6	8	2	0	0	0	0	0	0
Indeterminate agglutinated sp. 1	0	0	2	0	0	0	0	0	0	0	0	0
Indeterminate agglutinated sp. 2	1	4	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 4	0	0	8	1	3	1	0	0	0	0	0	0
Indeterminate porcelaneous sp. 5	0	0	7	1	11	3	0	0	0	0	0	0
Indeterminate porcelaneous sp. 9	0	0	4	1	2	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 10	0	0	1	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 11	0	0	1	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 13	0	0	3	0	5	1	0	0	0	0	0	0
Indeterminate porcelaneous sp. 28	0	0	1	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 31	0	0	4	1	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 39	0	0	2	0	2	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 40	0	0	4	1	3	1	0	0	0	0	0	0
Indeterminate porcelaneous sp. 41	0	0	1	0	2	0	1	5	0	0	0	0
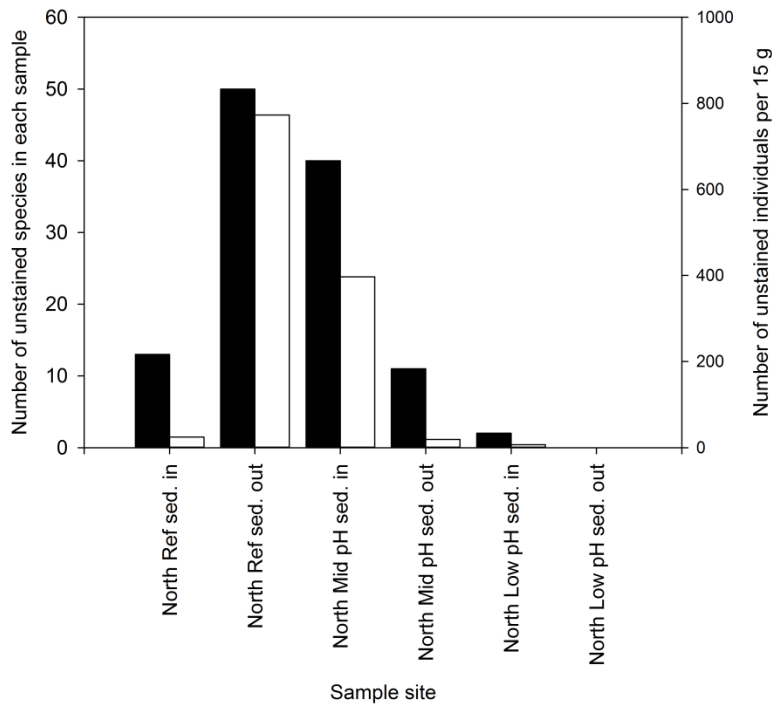
Figure 3.3: Number of species and individuals of unstained foraminifera found in each sample (15 grams of sediment) collected from the north side of Castello d'Aragonese, Ischia in November 2011. Black bars: number of species, white bars: number of individuals.

The low number of samples, and few individuals, made it difficult to assess any patterns in epiphytic foraminifera, or those attached to *P. oceanica* rhizomes, from the north side of Castello d'Aragonese. There were only four individuals (all belonging to the species *Rosalina globularis)* that were stained dark magenta in at least half of their chambers and, therefore, presumed to be living at the time of collection. No further analysis, therefore, was carried out on the stained assemblage and all following data refer to the unstained (dead) assemblage. A total of 63 foraminifera were found in the unstained assemblage on the leaves and rhizomes form the north side of Castello d'Aragonese (Table 3.3). There did not appear to be a decrease in the number of species across the calcium carbonate saturation gradient. The number of species found ranged from one to four. Due to the extremely low number of individuals, no further analysis was conducted on the data.

Table 3.3: A list of the species of benthic foraminifera found within the dead (unstained) assemblage from *Posidonia oceanica* samples from Ischia, North side of Castello d'Aragonese. Numbers per 2 grams of dry leaf are reported along with the percentage contribution.

Species	North Ref leaves		North Ref roots		North Mid pH leaves		North Mid pH roots		North Low pH roots and leaves	
	Per 2 g	%	Per 2 g	%	Per 2 g	%	Per 2 g	%	Per 2 g	%
Ammoglobigerina globigeriniformis	0	0	0	0	0	0	1	50	7	64
Cribrostomoides subglobosum	0	0	0	0	0	0	1	50	0	0
Haplophragmoides canariensis	0	0	0	0	0	0	0	0	2	18
Lobatula lobatula	4	12	0	0	0	0	0	0	0	0
Planorbulina mediterranensis	1	2	0	0	0	0	0	0	0	0
Reophax sp. 3	0	0	0	0	0	0	0	0	1	9
Rosalina globularis	32	86	0	0	8	100	0	0	0	0
Trochammina inflata	0	0	0	0	0	0	0	0	1	9

When collecting sediment from the south side of Castello d'Aragonese, no sediment from outside the seagrass meadow could be collected from the high pH site because of the hard, rocky bottom which was colonized by macroalgae and from the low pH site, no sediment was collected from within the seagrass meadow. Only two individuals were stained dark magenta in at least half of their chambers from the sediment samples. All following information, therefore, refers to the unstained (dead) assemblage. Foraminifera belonging to the unstained assemblage were found in all six of the sediment samples examined with a total of 611 individuals counted (Table 3.4). The number of species ranged from three to 49, with the highest occurring in the reference site from sediment collected within the seagrass meadow. The lowest number of species also occurred in the reference site but, this time, the sediment was collected outside the seagrass meadow. The number of individuals per 15 grams of sediment (15 g was the weight of sample examined) ranged from six to 268, with the highest number of individuals occurring in the reference site from sediment collected within the seagrass meadow. There did not appear to be any clear pattern in the number of species or individuals across the CO_2 gradient (Figure 3.4).

Table 3.4: A list of the species of benthic foraminifera found within the dead (unstained) assemblage from sediment samples from Ischia, South side of Castello d'Aragonese. Numbers per 15 grams of sediment are reported along with the percentage contribution. Numbers per 15 grams were calculated from the dry sediment weight of the >63 µm fraction.

Species	South Ref sed. in Per 15 g	%	South Ref sed. out Per 15 g	%	South High pH sed. in Per 15 g	%	South High pH sed. out Per 15 g	%	South Mid pH sed. in Per 15 g	%	South Low pH sed. out Per 15 g	%
Adelosina longirostra	7	3	0	0	0	0	0	0	4	5	1	1
Afinetrina gualtieriana	1	0	0	0	0	0	0	0	0	0	0	0
Ammoglobigerina globigeriniformis	3	1	0	0	3	5	0	0	2	3	169	86
Ammonia sp. 2	7	3	3	50	5	7	0	0	1	1	0	0
Amphistegina lobifera	0	0	1	17	0	0	0	0	0	0	0	0
Cyclocibicides vermiculata	2	1	0	0	0	0	0	0	0	0	1	0
Eggerelloides sp. 1	0	0	0	0	2	4	0	0	1	1	1	1
Elphidium aculeatum	9	3	0	0	9	15	3	18	30	41	10	5
Elphidium macellum	0	0	0	0	1	2	0	0	0	0	1	1
Elphidium margaritaceum	0	0	0	0	3	5	0	0	0	0	0	0
Elphidium sp. 4	3	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 5	5	2	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 7	2	1	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 8	3	1	0	0	0	0	1	6	0	0	0	0
Elphidium sp. 5 of Cimerman and Langer	0	0	0	0	0	0	0	0	0	0	0	0
Haynesina depressula	1	0	0	0	0	0	0	0	5	7	0	0
Haynesina sp. 1 of Cimerman and Langer	9	3	0	0	0	0	0	0	1	1	3	2
Lobatula lobatula	32	12	2	33	15	24	5	29	5	7	3	2
Massilina gualtieriana	2	1	0	0	0	0	0	0	1	1	0	0
Miliolinella subrotunda	3	1	0	0	0	0	0	0	1	1	0	0
Miliolinella webbiana	1	0	0	0	0	0	0	0	0	0	0	0
Peneroplis pertusus	11	4	0	0	3	5	0	6	2	3	0	0
Peneroplis planatus	4	2	0	0	0	0	1	7	5	7	0	0
Pileolina patelliformis	2	1	0	0	0	0	0	0	0	0	0	0
Planorbulina mediterranensis	13	5	0	0	1	2	0	0	2	3	0	0
Pseudotriloculina laevigata	5	2	0	0	1	2	0	0	1	1	0	0
Pseudotriloculina rotunda	2	1	0	0	0	0	0	0	0	0	0	0
Quinqueloculina annectens	1	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina auberiana	6	2	0	0	3	5	0	0	1	1	0	0
Quinqueloculina bosciana	8	3	0	0	0	0	0	0	0	0	0	0
Quinqueloculina disparilis	1	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina stelligera	2	1	0	0	0	0	1	0	0	0	0	0
Rosalina globularis	68	26	0	0	7	11	4	24	5	7	3	2
Sigmoilina costata	2	1	0	0	0	0	0	0	0	0	0	0
Siphonaperta agglutinans	1	0	0	0	0	0	0	0	0	0	9	5
Siphonaperta sp. 1	0	0	0	0	0	0	0	0	0	0	0	0
Spiroloculina ornata	3	1	0	0	0	0	0	0	0	0	0	0
Triloculinella dilatata	1	0	0	0	0	0	1	6	0	0	0	0
Triloculina marioni	0	0	0	0	1	6	1	6	1	1	0	0
Triloculina sp. 1	1	1	0	0	0	0	1	6	0	0	0	0
Triloculina sp. 2	2	1	0	0	0	0	0	0	0	0	0	0
Trochammina inflata	1	1	0	0	3	5	0	6	0	0	0	0
Vertebralina striata	2	1	0	0	2	4	1	0	2	3	0	0
Wellmanellinella striata	1	0	0	0	0	0	0	0	1	1	0	0
Indeterminate porcelaneous sp. 5	2	1	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 8	3	1	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 9	1	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 13	4	2	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 31	5	2	0	0	0	0	0	0	1	1	0	0
Indeterminate porcelaneous sp. 39	2	1	0	0	0	0	0	0	1	1	0	0
Indeterminate porcelaneous sp. 40	1	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 41	9	3	0	0	0	0	0	0	2	3	0	0
Indeterminate porcelaneous sp. 42	7	3	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 43	1	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 44	2	1	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 45	1	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 46	1	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 47	0	0	0	0	0	0	0	0	1	1	0	0
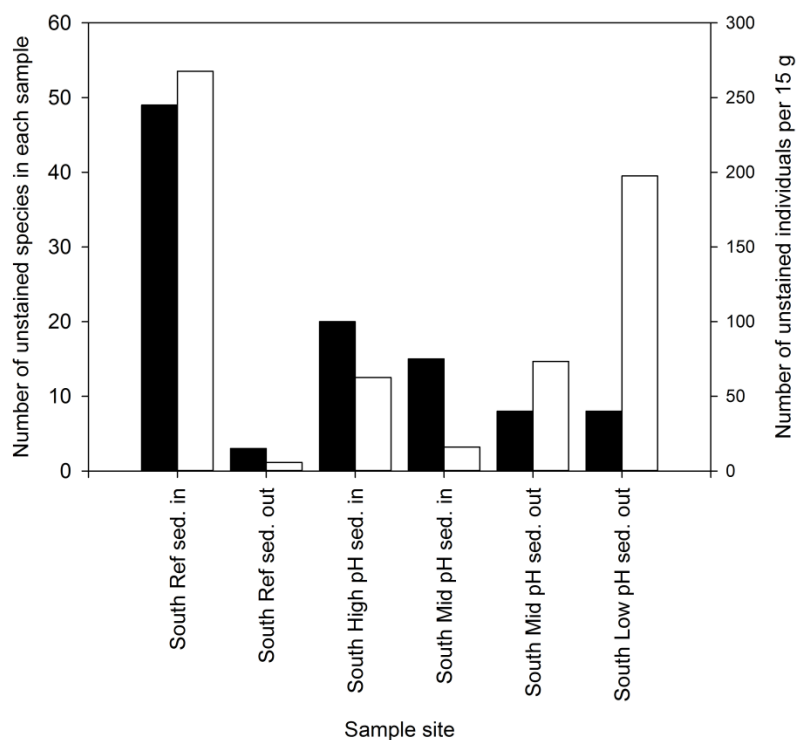
Figure 3.4: Number of species and individuals of dead foraminifera found in each sample (15 grams of sediment) collected from the south side of Castello d'Aragonese, Ischia in November 2011. Black bars: number of species, white bars: number of individuals.

The low number of individuals made it difficult to assess any patterns in epiphytic foraminifera, or those attached to *P. oceanica* rhizomes, from the south side of Castello d'Aragonese. None of the individuals were stained dark magenta in at least half of their chambers. A total of 17 individuals belonging to the unstained assemblage were found on the leaves and rhizomes. Due to the extremely low number of individuals, no further analysis was conducted on the data.

3.2.4 Discussion

There were too few samples and individuals collected from Ischia to draw any firm conclusions about whether the seagrass *P. oceanica* collected along a CO_2 gradient provided refugia for benthic foraminifera along gradients of overlying seawater acidification. More samples would be required to test this hypothesis. The time of year that the sampling took place (November) could be a cause of the low number of individuals because many species of foraminifera are known to have a peak in

abundance in summer (e.g. Schmiedl et al., 2000, Fontanier et al., 2003, Sadri et al., 2011), following the spring bloom. The experiment had been designed as a pilot investigation with additional sampling planned for April 2012, when it was anticipated that there would be higher abundances of foraminifera. Due to logistical reasons, this additional sampling could not be undertaken. Foraminifera have previously been examined from sediment along a CO_2 gradient off Ischia, where there was a large reduction in the number of calcareous foraminifera in the lowest pH areas (Dias et al., 2010), but it was not possible to examine if *P. oceanica* was capable of protecting calcareous foraminifera from the adverse effects of ocean acidification. Semesi et al. (2009a) found that seagrass photosynthesis increased the pH in experimental cylinders from 8.3 – 8.4 to 8.6 – 8.9. This increased pH was thought to be responsible for enhanced calcification of the calcareous red algae *Hydrolithon* sp. 5.8-fold and *Mesophyllum* sp. and *Halimeda renschii* 1.6-fold. Martin et al. (2008), however, found that increased photosynthesis of seagrass did not protect calcareous epiphytes from the adverse effects of ocean acidification; the total mass of epiphytic calcium carbonate on *P. oceanica* leaves was 90% lower at a mean pH of 7.7 compared to a mean pH of 8.2.

3.3 Vulcano

3.3.1 Study site

Vulcano is situated *ca.* 25 km north-east of Sicily, in the Mediterranean Sea (Figure 3.5). It forms part of the Aeolian Island chain and is an active volcano formed by the collision of the African and Eurasian plates (Barberi et al., 1974). Shallow submarine gas seeps occur in Levante Bay (Figure 3.5 and 3.6) and create acidified waters along the northern shore that form a CO_2 gradient over a distance of approximately 200 m (Boatta et al., 2013). A gradient from the present day ocean surface mean pH of ~ 8.1 to a minimum pH of ~ 7.4 occurs along a shallow water (0 – 5 water depth) rocky shore (Kerfahi et al., 2014). This site is considered well suited for ocean acidification research since it releases gases at ambient seawater temperature and there are gradients in

CO_2 that lack confounding gradients in alkalinity, salinity or in chemicals such as H_2S (Boatta et al., 2013), although sediment close to the seeps is contaminated with H_2S and heavy metals (Vizzini et al., 2013).
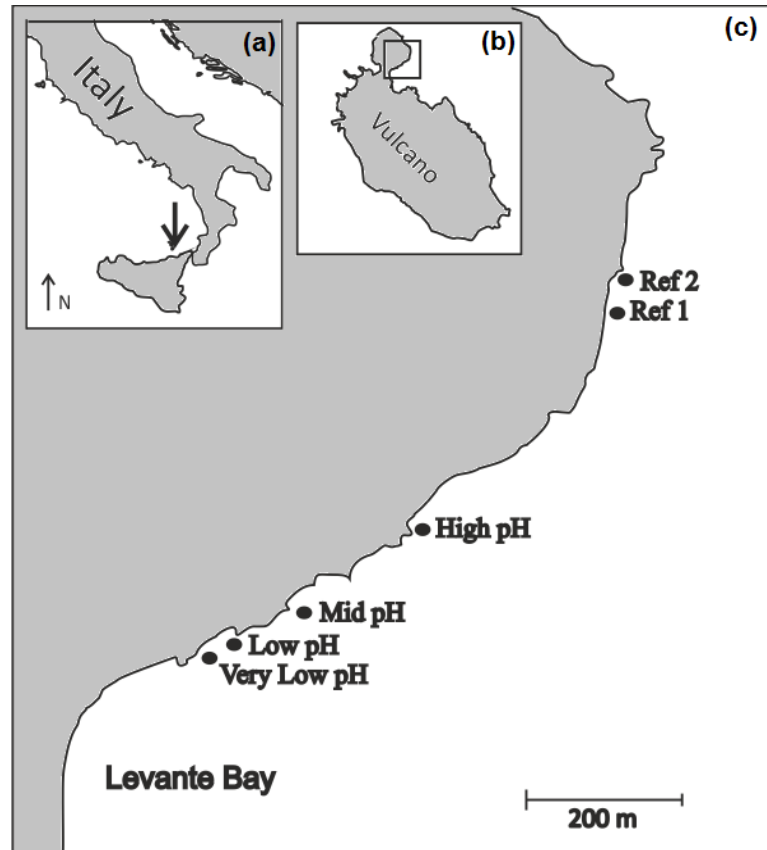
Figure 3.5: Study area at Vulcano. (a) Italy with arrow marking Vulcano, part of the Aeolian Island chain, northeast Sicily, (b) Vulcano, (c) Location of sample sites. Very Low pH was at 38°25′9″ N, 14°57′38″E, Ref 2 was at 38°25′20″N, 14°58′3″E.
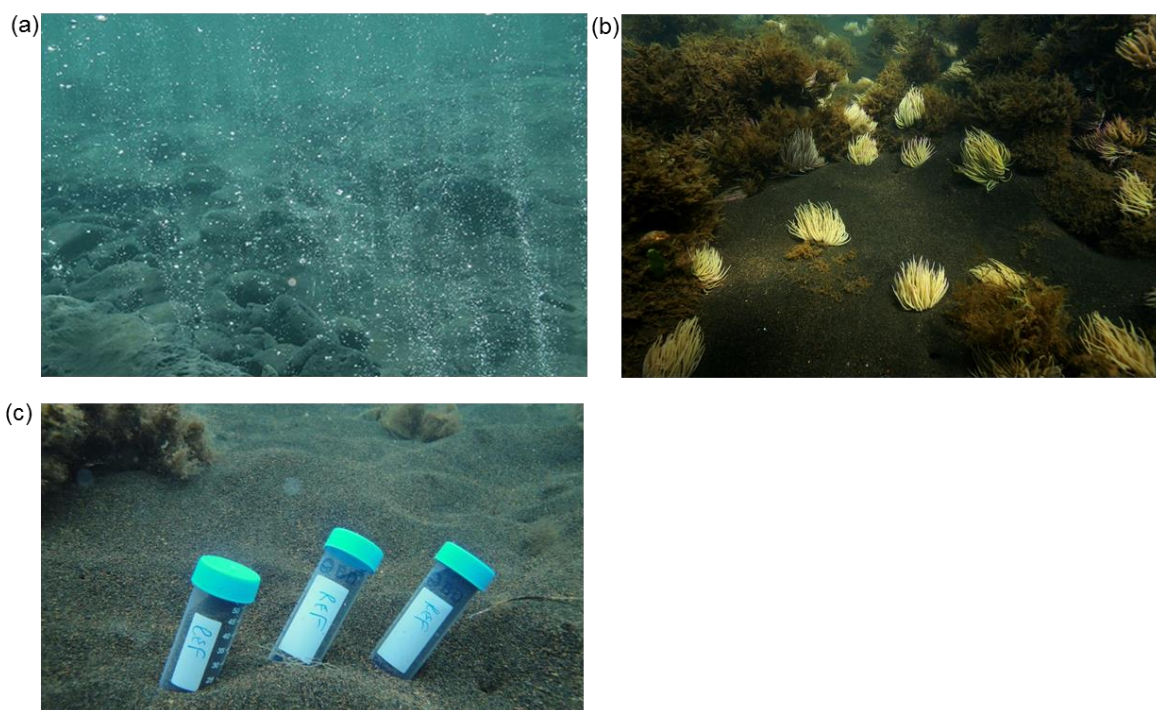
Figure 3.6: The study site off the Island of Vulcano, Sicily. (a) CO_2 seeps in Levante Bay at 1 m water depth, (b) the seabed at 1 m water depth at the Low pH site, (c) the seabed at 3 m water depth at Ref 2 with some sediment samples collected for the examination of benthic foraminiferal assemblages. Photographs taken by J. Hall-Spencer.

3.3.2 Methods

3.3.2.1 Carbonate chemistry

Carbonate chemistry conditions along the CO_2 gradient at Vulcano have been reported by others (e.g. Johnson et al., 2012, Boatta et al., 2013, Johnson et al., 2013, Vizzini et al., 2013, Kerfahi et al., 2014, Milazzo et al., 2014), but additional measurements were made between May 2011 and May 2013. At each of the sample sites, pH, temperature and salinity were recorded using a calibrated YSI (556 MPS) pH (National Bureau of Standards (NBS) scale) meter. National Bureau of Standards calibration buffers were used to calibrate the pH meter before each use. The NBS scale can lack precision (approximately 0.05 pH units) (Dickson et al., 2007), but due to the large fluctuations in pH across the gradient this lack of precision was considered acceptable for this study (Johnson et al., 2013).

Samples for total alkalinity (TA) were also collected at each sample site in order that the remaining carbonate chemistry parameters could be calculated (Hoppe et al., 2010). Three replicates were collected from each site. Seawater was collected in a 150 ml glass bottle with a screw-cap lid. After rinsing three times in seawater, the bottles were filled. Upon collection, 1.5 ml of seawater was removed from the sample using an automated pipette to allow sufficient room for expansion and 30 µl (0.02% volume ratio) of mercuric chloride (saturated solution 7 g per 100 ml) was added to the sample to prevent further biological activity. Upon collection of total alkalinity samples in May 2013, no glass bottles were available. For these samples, seawater was collected in 50 ml Falcon^{TM} tubes and 10 µl (0.02% volume ratio) of mercuric chloride was added to the sample. The bottles were sealed and wrapped in parafilm and stored in the dark until analysis using an AS-ALK2 Total Alkalinity Titrator (Apollo SciTech Inc., Georgia, USA), calibrated using Total Alkalinity standards (Dickson Laboratory, batch 121, Scripps Institution of Oceanography, California, USA). The pH, TA, salinity and temperature were used to calculate the remaining carbonate chemistry parameters using CO2SYS (Lewis and Wallace, 1998). The dissociation constants for carbonic acid supplied by Mehrbach et al. (1973) refitted by Dickson and Millero (1987) and the KSO_4 dissociation constant of Dickson (1990) were used. As recommended by Kerrison et al. (2011), pH data were transformed to hydrogen ion concentrations before analysis. This prevents underestimation of variability and overestimation of the mean. Following calculation of the means and statistical testing, they were re-converted to pH for graphical representation and easy comparison with other studies (Dickson et al., 2007, Kerrison et al., 2011).

3.3.2.2 Sediment collection

In order to examine surface sediment benthic foraminifera, sediment samples were collected at Vulcano. In a pilot investigation, shallow water sediment samples from 0.5 m water depth were collected along the CO_2 gradient off Vulcano in April 2011. The sample sites used were Ref 1, High pH, Mid pH, Low pH and Very Low pH (Figure 3.5).

Sediment was collected using a 10 cm diameter PVC ring which was placed in the top 3 cm of sediment. Sediment contained within the ring was removed using a stainless-steel spoon and placed into pre-labelled plastic sampling bags underwater. Samples were then transferred to drip-proof plastic sample containers and preserved in 4% buffered formalin. Once in the laboratory, samples were processed in the same way as the Ischia samples, described in section 3.2.2. If no benthic foraminifera were found within one gram of sediment, the sample was labelled as barren and no additional examination took place.

Following the pilot investigation, in May 2011, SCUBA divers collected sediment samples from along the CO_2 gradient at a depth of approximately 3 m. Ideally, sediment would have been collected from greater water depths, but due to logistical constraints and the loss of the CO_2 gradient at greater water depths in the bay (Boatta et al., 2013), this was not possible. The sample sites from which sediment was collected were Ref 2, Ref 1, High pH, Mid pH, Low pH and Very Low pH (Figure 3.5). Five replicates were collected from each sample site. Samples were collected and examined using the same methods used in the pilot investigation. An additional sample was collected from each sample site for sediment size analysis.

Sediment particle size was determined at Plymouth University using a sediment settling tube (length 2.5 m, diameter 25 cm). Samples were removed from their plastic sampling bags and placed onto a plastic tray. A few drops of water were added to the samples to make them more cohesive and the sample was gently mixed using a metal spatula. A small amount of the sample was placed on the base of a Petri dish and the surface was smoothed over. Carefully, the Petri dish was placed on the surface of the water at the top of the sediment settling tube. As soon as the sediment made contact with the surface of the water, the sediment was released from the underside of the Petri dish due to surface tension. The sediment was allowed to fall through the settling tube. A mass balance recorded the cumulative weight of sediment reaching the bottom

of the settling column allowing sediment settling velocities to be determined. Settling velocity data was converted into grain size (Ferguson and Church, 2004). Grain size data was analysed using the software GRADISTAT V. 8 (Blott and Pye, 2001).

3.3.2.3 Padina pavonica *epiphyte sample collection*

Padina pavonica thalli were collected from six different sites along a CO_2 gradient (Figure 3.5 and 3.7) in September 2011 and May 2012. Thalli were collected from approximately 1 m water depth, by cutting algal blades above the sediment surface and placing them into labelled plastic sample bags underwater following the methods of Langer (1993). Five replicate thalli were collected from each site and placed in aluminium trays and left to air dry.

Figure 3.7: The brown macroalgae *Padina pavonica* at 1 m water depth off Vulcano. Photograph taken by J. Hall-Spencer.

Once in the laboratory, 2 g of dry thallus from each sample was examined under a stereo-binocular microscope. Weight rather than surface area was measured as surface area was harder to standardise between samples and there was concern that measuring the surface area may have resulted in the loss of some epiphytic foraminifera. Epiphytic foraminifera on the thalli were removed and placed on micropalaeontological slides. Foraminifera were identified to species level where possible using Cimerman and Langer (1991) and Milker and Schmiedl (2012) and counted.

To assess the number of living individuals, additional samples were collected and preserved in 4% buffered formalin. This meant that they could be stained with rose Bengal and the number of stained individuals determined. The *P. pavonica* thalli were collected using the methods described above, but rather than leaving them to air dry, they were placed in plastic sample containers and preserved in 4% buffered formalin. In September 2011, one sample was collected from each sample site. In May 2012 three replicates were collected from each sample site and preserved in formalin. The samples were processed in the same way as the *P. oceanica* samples collected from Ischia, described in section 3.2.2. The samples that had been preserved in formalin were divided into three assemblage types; 1) stained, 2) unstained (dead) and 3) attached. Individuals were determined to have been live at the time of collection and placed into the stained assemblage if they were stained dark magenta in at least half of their chambers (Bernhard et al., 2006). This excluded counting individuals with just a faint red dot in one chamber. Foraminifera that had been processed by staining with rose Bengal, but did not pick up the stain, were placed into the unstained (dead) assemblage. The attached assemblage consisted of individuals that remained on the thalli after washing and were, therefore, not processed by staining with rose Bengal. These individuals were picked directly from examining the thalli under a stereo-binocular microscope, therefore, there was no way of knowing if they were living or dead at the time of collection. Foraminifera in the attached assemblage were presumed to be living at the time of collection (Langer, 1993).

3.3.2.4 Scanning electron microscopy

To aid taxonomic identification, some of the foraminifera were examined under a JEOL JSM 5600 LV scanning electron microscope (SEM) with a digital imaging system. Individuals were mounted on aluminium SEM stubs and sputter coated in an Emitech K550 gold sputter coater. Test walls were examined at a higher magnification (x 4000) in 15 of the individuals (to assess the effects of dissolution). Dissolution was noted if

foraminiferal tests showed etching, pitting, fragmentation or enlarged pores. The final chamber was examined as this is often the thinnest and, therefore, often shows damage quicker than the older, thicker-walled, chambers (Hansen, 1999).

3.3.2.5 Statistical analysis

To test for differences between the number of species and individuals between sample sites a one-way ANOVA was conducted on the untransformed data. Where the results of ANOVA were significant a Tukey test of pairwise multiple comparison was conducted to determine which sites were responsible for the differences. Where the data failed the Shapiro-Wilk normality test, a Kruskal-Wallis one-way analysis of variance on ranks was conducted, followed by Mann-Whitney U tests between each combination of pairs to determine which sites were responsible for the differences. These tests were conducted using SigmaPlot v.12.5. Untransformed foraminiferal community assemblage data were used to calculate Shannon-Wiener diversity, Fisher Alpha and Pielou's evenness indices. The Fisher Alpha index was used in addition to Shannon diversity as this is the most commonly used index in foraminiferal studies (Murray, 2006). The data were then square root transformed to down-weight the contribution of dominant taxa. Non-metric multi-dimensional Scaling (nMDS) was used to look for similarities in species assemblage between samples. An ANOSIM test was conducted to test for similarities between sites and to determine if samples within the same site were more similar to each other than samples from different sites. ANOSIM is a non-parametric test that works by making 999 random permutations based on the data and then calculating significance levels by comparing results with the permutated results rather than assuming a particular distribution, as with parametric tests (Clarke and Warwick, 2001). A SIMPER test was used to determine discriminating species (Clarke and Warwick, 2001) using PRIMER v.6. In some cases, where there were few permutations under the ANOSIM test, Permutational Multivariate Analysis of Variance (PERMANOVA) (Anderson and Braak, 2003) was conducted. The analysis had one fixed factor of sample site and was performed on square root-transformed data with

Bray-Curtis measures of similarity. The method of permutation used was unrestricted permutation of raw data, using 9999 permutations with Monte-Carlo tests. Pairwise comparisons were performed for significant groups. This analysis was conducted using PRIMER v.6 with PERMANOVA + package.

3.3.3 Results

3.3.3.1 Carbonate chemistry

Seawater calcite saturation state ranged from a mean value of ~ 5.29 at reference sites to ~ 2.47 along a 200 m stretch of rocky shore at 0 – 5 m water depth. Mean pH_{NBS} ranged from 8.19 at the reference sites to 7.71 at the lowest pH site, which is closest to the seeps. The pH decreased across the gradient from reference sites to sample site Very Low pH (Table 3.5) and was lower and more variable, near to the seeps. These findings replicate those off Ischia (Figure 3.8). A Kruskal-Wallis one-way analysis of variance on ranks revealed a statistically significant difference in the pH between sites ($p = <0.001$, $H = 39.883$, degrees of freedom = 5). Dunn's method of pairwise multiple comparison revealed that both reference sites (Ref 2 and Ref 1) were significantly different from the Low pH and Very Low pH site at a p value of <0.05.
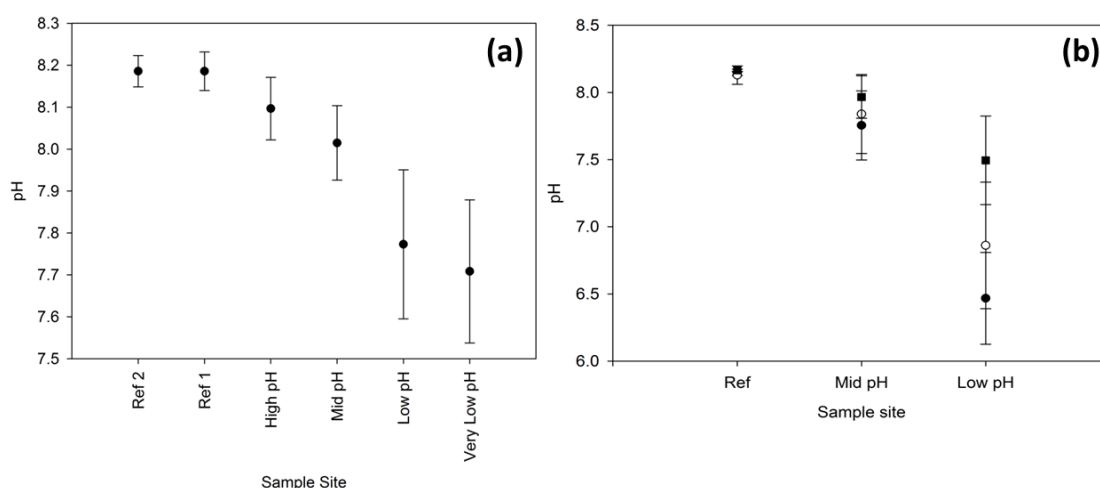
Figure 3.8: (a) Mean (± 1 SD, n = 8 – 11) pH measured across the CO_2 gradient off Vulcano, Italy between May 2011 and May 2013, (b) Mean (± 1 SD, n = 7 – 38) pH measured across the CO_2 gradient off Ischia, Italy. Black circles: sample sites on the south side of Castello d'Aragonese, open circles: sample sites on the north side of Castello d'Aragonese, black squares: sample sites within *Posidonia oceanica* meadows on the south side of Castello d'Aragonese. Data for Ischia from Hall-Spencer et al. (2008).

Table 3.5: Seawater pH and associated carbonate chemistry parameters measured between May 2011 and May 2013 at six sample sites off Vulcano, Italy. Carbonate chemistry parameters were calculated from pH_{NBS} and mean total alkalinity (TA) measurements at each site (n for each is shown below site names).

Site		pH (NBS)	TA (µmol/kgSW)	pCO_2 (µatm)	HCO_3^- (µmol/kgSW)	CO_3^{2-} (µmol/kgSW)	Ω_{Calc}	Ω_{Arag}
Ref 2 (n_{pH}=11, n_{TA}=7)	Min	8.12	2320.5	396.3	1895.7	198.7	4.66	3.10
	Median	8.17	2538.7	418.0	1973.4	216.6	5.05	3.29
	Max	8.25	2607.2	643.8	2023.3	250.8	5.90	3.95
Ref 1 (n_{pH}=10, n_{TA}=7)	Min	8.12	2368.4	395.3	1906.4	193.6	4.54	3.02
	Median	8.17	2554.6	423.1	1991.8	220.9	5.15	3.36
	Max	8.25	2645.6	682.4	2061.6	256.5	6.01	4.06
High pH (n_{pH}=8, n_{TA}=7)	Min	7.98	2368.4	444.9	2026.5	157.7	3.69	2.44
	Median	8.15	2580.3	698.6	2034.8	210.7	4.91	3.20
	Max	8.15	2702.5	1292.7	2166.5	215.7	5.12	3.49
Mid pH (n_{pH}=8, n_{TA}=6)	Min	7.90	2392.4	519.0	2042.7	136.9	3.23	2.17
	Median	8.01	2639.8	750.7	2193.1	167.0	3.92	2.60
	Max	8.18	2776.3	1317.9	2267.7	228.8	5.36	3.54
Low pH (n_{pH}=8, n_{TA}=7)	Min	7.61	2454.8	770.3	2269.9	76.0	1.77	1.15
	Median	7.78	2652.6	1676.6	2461.0	114.6	2.70	1.81
	Max	8.06	3004.1	2092.1	2552.0	193.7	4.54	3.00
Very Low pH (n_{pH}=8, n_{TA}=7)	Min	7.61	2405.5	615.3	2182.3	77.9	1.82	1.18
	Median	7.67	2663.3	1831.9	2477.0	85.9	2.01	1.34
	Max	8.11	2958.3	2658.6	2495.6	207.8	4.86	3.20

3.3.3.2 Epifaunal and infaunal benthic foraminifera

No benthic foraminifera were found in sediment samples collected during the pilot investigation in April 2011. In May 2011, after examination of at least one gram of sediment from one replicate from each sample site (with the exception of Ref 2), all of the samples were declared 'barren' (Table 3.6).

Table 3.6: The weight of sample examined and the number of foraminifera found in sediment samples collected along the CO_2 gradient at Vulcano in May 2011.

Sample site	Weight of sample examined (g)	Number of species	Number of individuals
Ref 1	2.65	0	0
High pH	1.20	0	0
Mid pH	2.21	0	0
Low pH	2.25	0	0
Very Low pH	1.98	0	0

The results of the sediment size analysis show that there was variation in the mean sediment size between sample sites. There was a general trend for the mean sediment size to increase along the gradient from the reference sites towards the CO_2 seeps (Table 3.7 and Figure 3.9). The sediment contained shards of glassy particles (Figure 3.10).

Table 3.7: Sediment size characteristics of sediment samples collected along the CO_2 gradient at Vulcano.

Sample site	Grain size with the greatest % contribution	Mean Φ	Mean sediment size
Ref 2	Medium sand	1.641	Medium sand
Ref 1	Medium sand	1.497	Medium sand
High pH	Coarse sand	1.097	Medium sand
Mid pH	Medium sand	1.210	Medium sand
Low pH	Very coarse sand	0.152	Coarse sand
Very Low pH	Coarse sand	0.606	Coarse sand
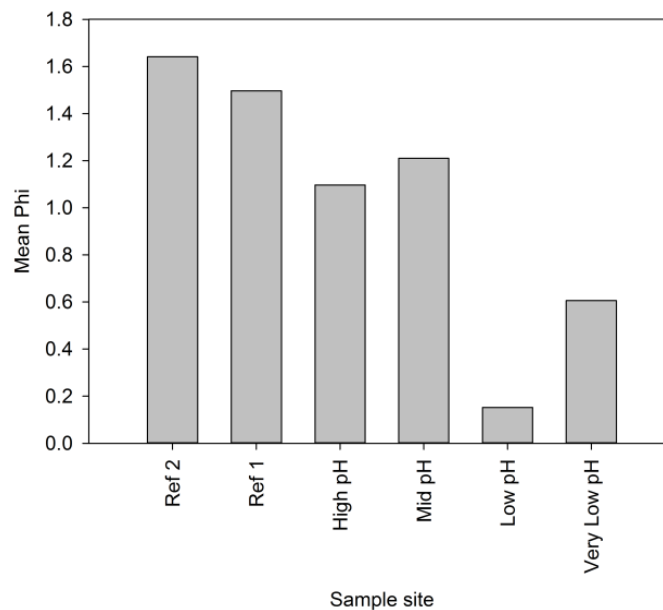
Figure 3.9: The mean Phi (Φ) size for sediment collected at different sample sites along the CO_2 gradient at Vulcano in May 2011. Note that the larger the Φ value, the finer the grain size.
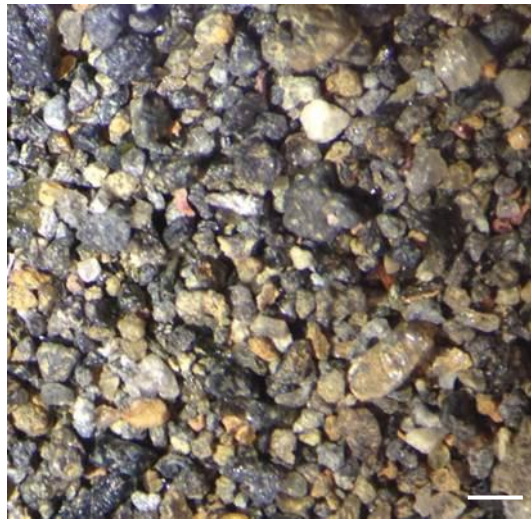
Figure 3.10: A sediment sample collected from sample site Ref 2 offshore Vulcano in May 2011. Scale bar is 1 mm.

3.3.3.3 Air dried Padina pavonica *epiphytes September 2011*

Foraminifera were found in 22 of the 30 air dried *P. pavonica* thalli samples that were collected in September 2011 with a total of 260 individuals (Table 3.8). There was a reduction in the number of species of epiphytic foraminifera along the pCO_2 gradient from reference sites (pH ~8.19) to low pH conditions (pH ~7.71) nearer to the seeps (Figure 3.11).

Table 3.8: A list of the species of benthic foraminifera found on air dried *Padina pavonica* thalli samples from Vulcano, collected in September 2011. Numbers per gram of dry thallus are reported along with the percentage contribution.

	Ref 2 A		Ref 2 B		Ref 2 C		Ref 2 D		Ref 2 E		Ref 1 A		Ref 1 B		Ref 1 C		Ref 1 D		Ref 1 E	
Species	**Per g**	**%**	**Per g**	**%**	**Per g**	**%**	**Per g**	**%**	**Per g**	**%**	**Per g**	**%**	**Per g**	**%**	**Per g**	**%**	**Per g**	**%**	**Per g**	**%**
Adelosina longirostra	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cymbaloporetta sp. 1	0	0	0	0	0	0	0	0	0	0	2	6	0	0	0	0	3	17	0	0
Elphidium aculeatum	0	0	0	0	0	0	0	0	4	14	2	6	0	0	0	0	0	0	0	0
Elphidium macellum	0	0	0	0	0	0	0	0	0	0	0	0	3	5	0	0	0	0	0	0
Elphidium sp. 5 of Cimerman and Langer	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	6
Laevipeneroplis karreri	0	0	4	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Massilina gualtieriana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	6
Miliolinella subrotunda	0	0	0	0	1	7	0	0	0	0	2	6	0	0	0	0	0	0	1	6
Patellina corrugata	3	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Peneroplis pertusus	0	0	18	13	1	13	2	50	6	24	5	13	3	5	0	0	3	17	1	6
Peneroplis planatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	20	0	0	1	6
Pileolina patelliformis	0	0	4	3	0	0	0	0	1	5	0	0	0	0	0	0	0	0	0	0
Planorbulina mediterranensis	3	7	0	0	1	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina parvula	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	17	0	0
Quinqueloculina sp. 2	3	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rosalina globularis	3	7	13	10	1	7	0	0	6	24	0	0	0	0	24	50	0	0	1	6
Rosalina sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	6
Tretomphalus bulloides	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Triloculinella dilatata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vertebralina striata	25	64	94	70	7	67	2	50	9	33	27	69	42	70	24	50	10	50	6	53
Indeterminate porcelaneous sp. 4	3	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	6
Indeterminate porcelaneous sp. 17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Species	High pH A Per g	%	High pH B Per g	%	High pH C Per g	%	High pH D Per g	%	High pH E Per g	%	Mid pH A Per g	%	Mid pH B Per g	%	Mid pH C Per g	%	Mid pH D Per g	%	Mid pH E Per g	%
Adelosina longirostra	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0
Cymbaloporetta sp. 1	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium aculeatum	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium macellum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 5 of Cimerman and Langer	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Laevipeneroplis karreri	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Massilina gualtieriana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Miliolinella subrotunda	0	0	4	50	4	10	2	2	3	4	0	0	0	0	0	0	0	0	0	0
Patellina corrugata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Peneroplis pertusus	0	0	0	0	4	10	10	10	10	12	0	0	0	0	0	0	0	0	0	0
Peneroplis planatus	0	0	0	0	0	0	0	0	3	4	0	0	0	0	0	0	0	0	0	0
Pileolina patelliformis	0	0	0	0	0	0	0	0	7	8	0	0	0	0	0	0	2	11	0	0
Planorbulina mediterranensis	0	0	0	0	0	0	0	0	0	0	3	17	0	0	0	0	0	0	0	0
Quinqueloculina parvula	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina sp. 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rosalina globularis	0	0	0	0	4	10	6	6	7	8	0	0	0	0	7	14	0	0	4	25
Rosalina sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tretomphalus bulloides	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	14	0	0	0	0
Triloculinella dilatata	0	0	0	0	0	0	4	4	0	0	3	17	0	0	0	0	0	0	0	0
Vertebralina striata	0	0	4	50	25	60	67	69	58	65	14	67	0	0	34	71	17	89	11	75
Indeterminate porcelaneous sp. 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 17	0	0	0	0	4	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 18	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0

Species	Low pH A Per g	%	Low pH B Per g	%	Low pH C Per g	%	Low pH D Per g	%	Low pH E Per g	%	Very Low pH A Per g	%	Very Low pH B Per g	%	Very Low pH C Per g	%	Very Low pH D Per g	%	Very Low pH E Per g	%
Adelosina longirostra	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cymbaloporetta sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium aculeatum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium macellum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 5 of Cimerman and Langer	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Laevipeneroplis karreri	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Massilina gualtieriana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Miliolinella subrotunda	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Patellina corrugata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Peneroplis pertusus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Peneroplis planatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pileolina patelliformis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Planorbulina mediterranensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina parvula	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina sp. 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rosalina globularis	0	0	0	0	0	0	0	0	8	100	2	100	1	100	14	100	0	0	0	0
Rosalina sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tretomphalus bulloides	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Triloculinella dilatata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vertebralina striata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure 3.11: Mean (± 1 SD, n = 5) number of species of epiphytic foraminifera found on air dried *Padina pavonica* thalli off Vulcano CO_2 seeps in September 2011. Results of Mann-Whitney U rank sum tests are depicted by letters with sites not connected by the same letter being significantly different (p < 0.05).

The number of species was low in all replicates ranging from zero to nine. A Kruskal-Wallis one-way analysis of variance on ranks revealed a statistically significant difference in the number of species between sites (p = 0.003, H = 17.913, degrees of freedom = 5). The mean number of individuals per gram ranged from zero to 47 with the highest number of individuals occurring at the high pH site (Figure 3.12). Kruskal-Wallis one-way analysis of variance on ranks revealed a statistically significant difference in the number of individuals per gram between sites (p = 0.015, H = 14.128, degrees of freedom = 5). The most abundant species was *Vertebralina striata* with a total of 164 individuals across all sites representing 65% of the total. The next most abundant species was *Peneroplis pertusus* with 26 individuals representing 10% of the total. Samples from the reference sites had the highest Shannon-Wiener diversity (1.67) and Fisher Alpha index (7.75).
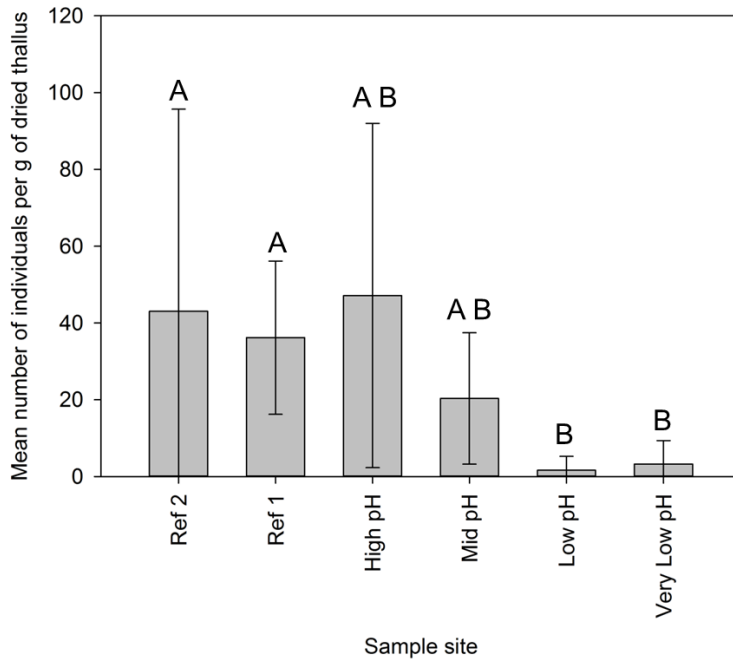
Figure 3.12: Mean (± 1 SD, n = 5) number of individuals of epiphytic foraminifera per gram of dried thallus found on air dried *Padina pavonica* thalli off Vulcano CO_2 seeps in September 2011. Results of Mann-Whitney U rank sum tests are depicted by letters with sites not connected by the same letter being significantly different ($p < 0.05$).

The high number of samples in which no foraminifera were found made it difficult to conduct multivariate statistical analysis on the data. The samples in which no foraminifera were found (High pH A, Mid pH B, Low pH A, B, C and D and Very Low pH D and E) were removed from the nMDS as they were creating outliers making the plot difficult to interpret. The foraminiferal assemblages did not appear to show any clear patterns according to the CO_2 level (Figure 3.13). Samples from reference sites and high pH sites tended to group together, in terms of similarity of assemblage and samples from the Mid pH site were grouped slightly apart. The samples from the Low pH and Very Low pH sites were grouped together, but they also showed 40% similarity with a sample from Ref 1.
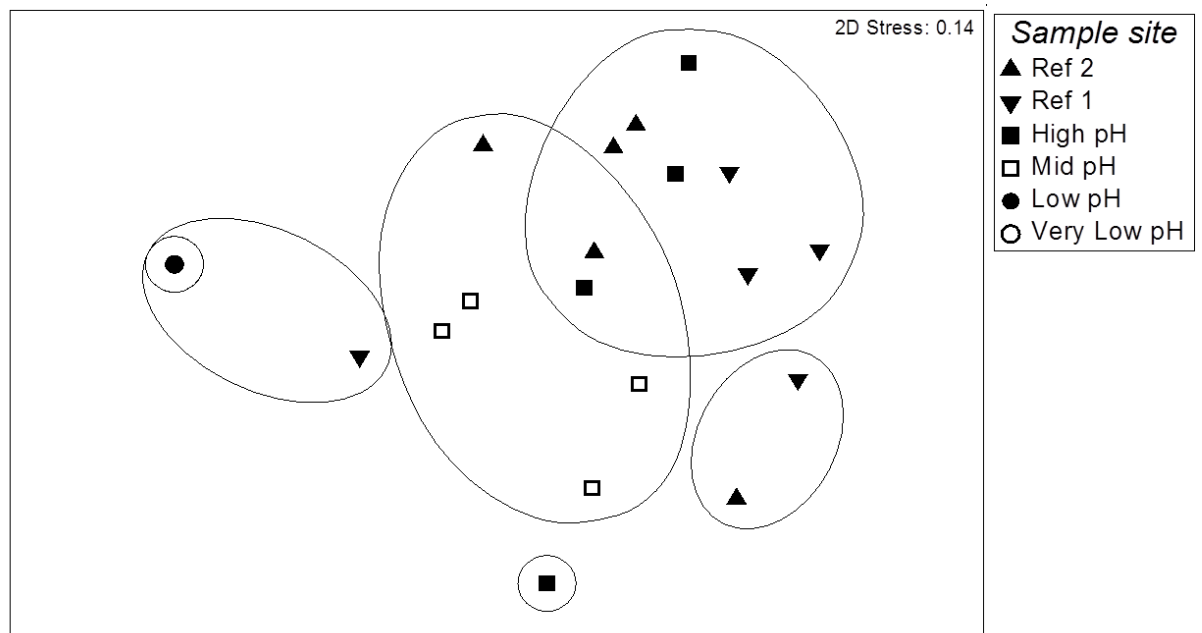
Figure 3.13: nMDS plot representing similarities between epiphytic foraminiferal assemblages found on air dried *Padina pavonica* thalli collected in September 2011 along a CO_2 gradient off Vulcano. The solid lines group samples at the 40 percent similarity level.

One-way ANOSIM test showed significant site differences in the assemblage (Global *R* statistic = 0.275, *p* = 0.001), but there were few permutations for the pairwise tests (four to 126) so PERMANOVA was conducted on the data. There were significant site differences in the assemblage (pseudo-F = 3.6996, *p* (MC) = < 0.001). Those pairwise tests with *p* (MC) values below 0.05 (significant difference) were Ref 2 – Very Low pH , Ref 1 – Very Low pH, High pH – Very Low pH and Mid pH – Very Low pH (Table 3.9). SIMPER revealed that the four taxa that contributed most to dissimilarity between sample sites were *V. striata*, *P. pertusus*, *Rosalina globularis* and *Miliolinella subrotunda*. There were no agglutinated species found in any of the samples examined.

Table 3.9: Results of pairwise tests between sample sites for epiphytic foraminiferal assemblages found on air dried *Padina pavonica* thalli collected in September 2011 along a CO_2 gradient off Vulcano. The numbers are Monte Carlo *p* values. Significant pairwise tests (< 0.05) are marked with asterisks. There is no value given for Low pH – Very Low pH because there were too few replicates in which foraminifera were present to run the test.

	Ref 2	Ref 1	High pH	Mid pH	Low pH	Very Low pH
Ref 2		0.665	0.383	0.120	0.062	0.002*
Ref 1			0.632	0.136	0.119	0.007*
High pH				0.052	0.091	0.003*
Mid pH					0.089	0.005*
Low pH						–
Very Low pH						

3.3.3.4 Formalin preserved Padina pavonica *epiphytes September 2011*

The samples collected in September 2011 that had been preserved in formalin were divided into three assemblage types; 1) stained, 2) unstained (dead) and 3) attached. Attached refers to the foraminifera that remained on the thalli after washing and were, therefore, not processed by staining with rose Bengal. There were no individuals that were stained dark magenta in at least half of their chambers in any of the samples examined. None of the foraminifera, therefore, were placed into the stained assemblage. In the unstained assemblage, foraminifera were found in four out of the six samples examined with 32 individuals counted during this study (Table 3.10). The low number of individuals in the unstained assemblage meant that no further analysis was conducted on the data. Foraminifera in the attached assemblage were found in four out of the six samples examined with a total of 90 individuals (Table 3.11). Similarly to the samples that had been air dried, there was a reduction in the number of species of epiphytic foraminifera along a carbonate saturation gradient from reference sites (Ω_{Calc} ~ 5.29) to high CO_2 conditions (Ω_{Calc} ~ 2.47) nearer to the seeps (Figure 3.14).

Table 3.10: A list of the species of benthic foraminifera found within the dead (unstained) assemblage from *Padina pavonica* thalli samples preserved in formalin from Vulcano, collected in September 2011. Numbers per 2 grams of dry thallus are reported along with the percentage contribution.

	Ref 2		Ref 1		High pH		Mid pH		Low pH		Very Low pH	
Species	Per 2 g	%	Per 2 g	%	Per 2 g	%	Per 2 g	%	Per 2 g	%	Per 2g	%
Brizalina striatula	0	0	1	6	0	0	0	0	0	0	0	0
Cornuspira involvens	0	0	1	6	0	0	0	0	0	0	0	0
Lobatula lobatula	1	33	0	0	0	0	0	0	0	0	0	0
Patellina corrugata	0	0	1	6	0	0	0	0	0	0	0	0
Peneroplis pertusus	0	0	1	6	0	0	0	0	0	0	0	0
Pileolina patelliformis	1	67	1	6	1	50	0	0	0	0	0	0
Rosalina globularis	0	0	4	50	1	50	2	100	0	0	0	0
Tortoplectella rhomboidalis	0	0	1	6	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 4	0	0	1	6	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 5	0	0	1	6	0	0	0	0	0	0	0	0

Table 3.11: A list of the species of benthic foraminifera found within the attached assemblage from *Padina pavonica* thalli samples preserved in formalin from Vulcano, collected in September 2011. Numbers per 2 grams of dry thallus are reported along with the percentage contribution.

	Ref 2		Ref 1		High pH		Mid pH		Low pH		Very Low pH	
Species	Per 2 g	%	Per 2 g	%	Per 2 g	%	Per 2 g	%	Per 2 g	%	Per 2 g	%
Affinetrina sp. 1	0	0	1	2	0	0	0	0	0	0	0	0
Pileolina patelliformis	0	0	1	4	0	0	0	0	0	0	0	0
Cymbaloporetta sp. 1	1	4	1	2	0	0	0	0	0	0	0	0
Elphidium sp. 5 of Cimerman and Langer	0	0	1	2	0	0	0	0	0	0	0	0
Triloculinella dilatata	1	4	0	0	0	0	0	0	0	0	0	0
Peneroplis pertusus	3	15	6	26	1	20	0	0	0	0	0	0
Peneroplis planatus	1	4	0	0	0	0	0	0	0	0	0	0
Planorbulina mediterranensis	1	4	0	0	0	0	0	0	0	0	0	0
Rosalina globularis	6	35	4	15	1	40	1	50	0	0	0	0
Rosalina sp. 1	0	0	1	4	0	0	0	0	0	0	0	0
Vertebralina striata	6	35	9	37	1	40	1	50	0	0	0	0
Indeterminate porcelaneous sp. 4	0	0	1	2	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 5	0	0	1	2	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 8	0	0	1	2	0	0	0	0	0	0	0	0
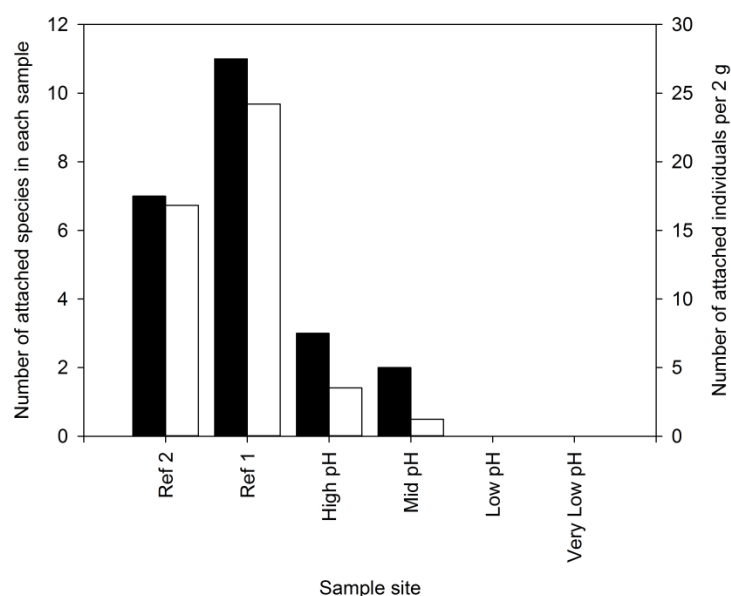
Figure 3.14: Number of species and individuals of epiphytic foraminifera found in each sample (2 grams of dried thallus) belonging to the attached assemblage collected from *Padina pavonica* thalli preserved in formalin from Vulcano CO_2 seeps in September 2011. Black bars: number of species, white bars: number of individuals.

The number of attached species found in any one sample ranged from zero to 11 with no individuals found in Low pH or Very Low pH samples. The number of attached individuals per 2 grams of dried thallus ranged from zero to 24 with the lowest number of individuals occurring at the lowest pH sites. The most abundant species was *V. striata* with 17 individuals (36.7% of attached assemblage). The next most abundant species was *R. globularis* with a total of 12 individuals (25.2% of attached assemblage). No agglutinated foraminifera were found in the samples. The lack of replication meant that further statistical analysis was not conducted on the data.

3.3.3.5 Air dried Padina pavonica *epiphytes May 2012*

Epiphytic foraminifera were found on all 30 air dried *P. pavonica* thallus samples collected in May 2012 with a total of 3851 individuals counted (Table 3.12). There was a reduction in the number of species of epiphytic foraminifera along a calcium carbonate saturation gradient from reference sites ($\Omega_{Calc} \sim 5.29$) to high CO_2 conditions ($\Omega_{Calc} \sim 2.47$) nearer to the seeps (Figure 3.15). The number of species ranged from four to 30 per replicate.

Table 3.12: A list of the species of benthic foraminifera found on air dried *Padina pavonica* thalli samples from Vulcano, collected in May 2012. Numbers per 2 grams of dry thallus are reported along with the percentage contribution.

Species	Ref 2 A		Ref 2 B		Ref 2 C		Ref 2 D		Ref 2 E		Ref 1 A		Ref 1 B		Ref 1 C		Ref 1 D		Ref 1 E	
	Per 2 g	%	Per 2 g	%	Per 2 g	%	Per 2 g	%	Per 2 g	%	Per 2 g	%	Per 2 g	%	Per 2 g	%	Per 2 g	%	Per 2 g	%
Adelosina longirostra	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Affinetrina gualtieriana	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0
Bolivina pseudoplicata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cornuspira involvens	0	0	2	2	1	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0
Daitrona sp.	0	0	0	0	0	0	0	0	2	1	1	1	0	0	1	1	13	5	9	2
Elphidium cf. *E. advenum*	0	0	0	0	0	0	0	0	0	0	0	0	3	1	0	0	0	0	0	0
Elphidium crispum	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	0
Elphidium macellum	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0
Elphidium margaritaceum	1	1	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	0	0	0
Elphidium sp. 3	1	1	1	1	0	0	1	1	0	0	0	0	0	0	0	0	2	1	4	1
Elphidium sp. 4	0	0	1	1	0	0	1	1	2	1	1	1	13	3	2	1	1	0	6	2
Elphidium sp. 5	4	3	5	4	8	6	16	9	8	4	10	7	58	13	7	4	25	10	33	9
Elphidium sp. 6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 5 of Cimerman and Langer	1	1	1	1	1	1	2	1	2	1	0	0	2	0	0	0	1	0	2	1
Elphidium sp. 7 of Cimerman and Langer	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Haynesina depressula	1	1	1	1	0	0	2	1	1	1	0	0	12	3	1	1	4	2	7	2
Lobatula lobatula	0	0	1	1	0	0	1	1	1	1	2	1	0	0	1	1	0	0	1	0
Massilina gualtieriana	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Miliolinella subrotunda	7	6	5	4	7	5	10	5	4	2	2	1	9	2	1	1	6	2	6	2
Miliolinella sp. 1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Peneroplis pertusus	8	7	3	2	8	6	14	7	11	6	10	7	7	2	40	25	8	3	5	1
Peneroplis planatus	1	1	4	3	2	2	6	3	8	4	5	3	1	0	4	3	0	0	5	1
Pileolina patelliformis	63	54	76	61	72	58	70	38	90	46	85	57	247	54	83	53	136	54	241	63
Pileolina patelliformis (plastogamous pair)	5	4	4	3	4	3	9	5	7	4	11	7	54	12	6	4	15	6	28	7
Pseudotriloculina sp.1 of Milker and Schmeidl	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina annectens	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina auberiana	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina bosciana	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	0	0	0
Quinqueloculina stelligera	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina sp. 3	0	0	0	0	0	0	0	0	3	2	0	0	0	0	0	0	0	0	0	0
Rosalina globularis	16	14	17	13	15	12	28	15	31	16	17	11	32	7	8	5	32	13	28	7
Rosalina sp. 1	0	0	1	1	0	0	0	0	3	2	1	1	0	0	1	1	3	1	0	0
Spiroloculina ornata	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Triloculinella dilatata	1	1	1	1	0	0	3	2	1	1	0	0	1	0	0	0	0	0	0	0
Vertebralina striata	1	1	1	1	0	0	1	1	2	1	0	0	3	1	0	0	0	0	2	1
Indeterminate sp. 1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
Indeterminate calcareous sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 2	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 3	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Indeterminate hyaline sp. 6	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 7	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Indeterminate hyaline sp. 9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 2	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 3	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 5	2	2	1	1	6	5	8	4	12	6	1	1	9	2	0	0	4	2	3	1
Indeterminate porcelaneous sp. 8	2	2	0	0	0	0	1	1	2	1	0	0	3	1	0	0	1	0	1	0
Indeterminate porcelaneous sp. 9	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 10	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 12	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 15	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 22	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 23	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 24	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0
Indeterminate porcelaneous sp. 25	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 26	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
Indeterminate porcelaneous sp. 28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0

Table 3.12 continued:

Species	High pH A Per 2 g	%	High pH B Per 2 g	%	High pH C Per 2 g	%	High pH D Per 2 g	%	High pH E Per 2 g	%	Mid pH A Per 2 g	%	Mid pH B Per 2 g	%	Mid pH C Per 2 g	%	Mid pH D Per 2 g	%	Mid pH E Per 2 g	%
Adelosina longirostra	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Affinetrina gualtieriana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bolivina pseudoplicata	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cornuspira involvens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Daitrona sp.	5	5	9	8	15	16	11	7	0	0	5	23	10	21	5	11	13	33	7	27
Elphidium cf. *E. advenum*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium crispum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium macellum	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium margaritaceum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	9	0	0
Elphidium sp. 4	1	1	1	1	2	2	6	4	1	4	3	14	4	9	1	2	0	0	1	4
Elphidium sp. 5	3	3	17	14	4	4	35	23	8	29	7	32	6	13	13	30	8	21	5	19
Elphidium sp. 6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 5 of Cimerman and Langer	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 7 of Cimerman and Langer	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Haynesina depressula	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Lobatula lobatula	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	3	0	0
Massilina gualtieriana	0	0	0	0	1	1	1	1	0	0	0	0	1	2	0	0	0	0	0	0
Miliolinella subrotunda	2	2	2	2	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0
Miliolinella sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Peneroplis pertusus	2	2	3	3	1	1	0	0	2	7	0	0	0	0	0	0	0	0	0	0
Peneroplis planatus	2	2	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pileolina patelliformis	64	63	72	61	50	53	81	51	14	50	7	32	21	47	20	45	13	33	11	42
Pileolina patelliformis (plastogamous pair)	3	3	5	4	0	0	9	6	0	0	0	0	1	2	0	0	1	3	0	0
Pseudotriloculina sp.1 of Milker and Schmeidl	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0
Quinqueloculina annectens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina auberiana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina bosciana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina stelligera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina sp. 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rosalina globularis	15	15	8	7	12	13	10	6	3	11	0	0	2	4	1	2	2	5	2	8
Rosalina sp. 1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Spiroloculina ornata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Triloculinella dilatata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vertebralina striata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate calcareous sp. 1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 2	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 9	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 4	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 5	0	0	0	0	3	3	0	0	0	0	0	0	1	2	0	0	0	0	1	4
Indeterminate porcelaneous sp. 8	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 13	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 26	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 3.12 continued:

Species	Low pH A Per 2 g	%	Low pH B Per 2 g	%	Low pH C Per 2 g	%	Low pH D Per 2 g	%	Low pH E Per 2 g	%	Very Low pH A Per 2 g	%	Very Low pH B Per 2 g	%	Very Low pH C Per 2 g	%	Very Low pH D Per 2 g	%	Very Low pH E Per 2 g	%
Adelosina longirostra	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Affinetrina gualtieriana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bolivina pseudoplicata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cornuspira involvens	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	0	0	0
Daitrona sp.	76	75	61	69	133	77	92	70	108	66	37	90	67	87	75	87	47	80	66	82
Elphidium cf. *E. advenum*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium crispum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium macellum	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium margaritaceum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 3	0	0	0	0	0	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0
Elphidium sp. 4	5	5	10	11	4	2	10	7	11	7	1	2	1	1	4	5	1	2	6	7
Elphidium sp. 5	16	16	16	18	25	15	20	15	36	22	1	2	4	5	5	6	10	17	7	9
Elphidium sp. 6	0	0	0	0	3	2	3	2	0	0	1	2	2	3	0	0	0	0	0	0
Elphidium sp. 5 of Cimerman and Langer	0	0	0	0	0	0	3	2	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 7 of Cimerman and Langer	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Haynesina depressula	0	0	0	0	1	1	1	1	2	1	0	0	0	0	0	0	0	0	0	0
Lobatula lobatula	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Massilina gualtieriana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Miliolinella subrotunda	0	0	0	0	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0
Miliolinella sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Peneroplis pertusus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Peneroplis planatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pileolina patelliformis	4	4	1	1	3	2	1	1	3	2	0	0	2	3	0	0	1	2	2	2
Pileolina patelliformis (plastogamous pair)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
Pseudotriloculina sp.1 of Milker and Schmeidl	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina annectens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina auberiana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina bosciana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina stelligera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina sp. 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rosalina globularis	0	0	0	0	2	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
Rosalina sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Spiroloculina ornata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Triloculinella dilatata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vertebralina striata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate calcareous sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 21	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure 3.15: Mean (± 1 SD, n = 5) number of species of epiphytic foraminifera per 2 grams of air dried *Padina pavonica* thallus off Vulcano CO_2 seeps in May 2012. Results of Mann-Whitney U rank sum tests are depicted by letters with sites not connected by the same letter being significantly different (p < 0.05).

Shannon-Wiener diversity ranged from 2.29 at the reference sites to 0.45 in samples collected from the most acidified site, with corresponding Fisher Alpha and Pielou's evenness indices of 10.07 to 0.88 and 0.67 to 0.28, respectively. Kruskal-Wallis one-way analysis of variance on ranks revealed a statistically significant difference in the number of species between sites (p = <0.001, H = 22.663, degrees of freedom = 5). The mean number of individuals per replicate ranged from 22 to 458 with the lowest number of individuals occurring at the intermediate pH site (Figure 3.16). Kruskal-Wallis one-way analysis of variance on ranks revealed a statistically significant difference in the number of individuals between sites (p = <0.001, H = 22.156, degrees of freedom = 5).
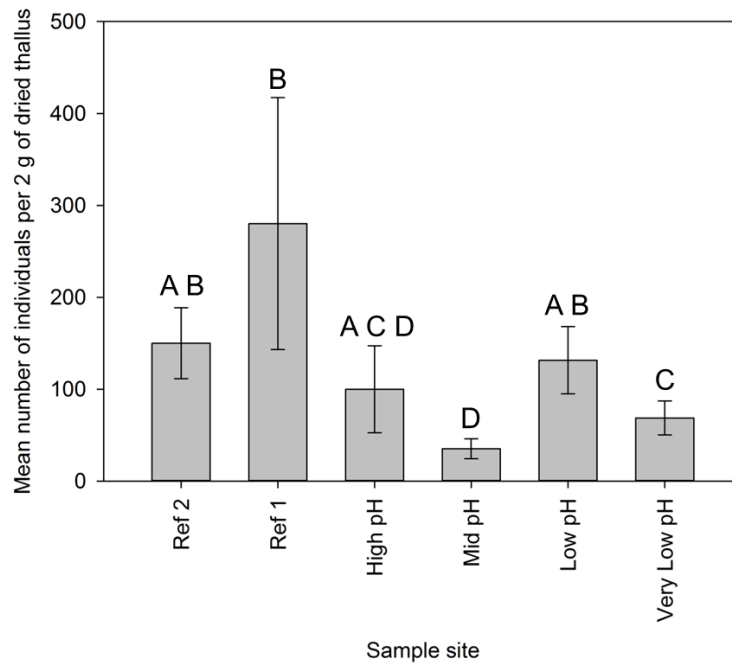
Figure 3.16: Mean (± 1 SD, n = 5) number of individuals of epiphytic foraminifera per 2 grams of air dried *Padina pavonica* thallus off Vulcano CO_2 seeps in May 2012. Results of Mann-Whitney U rank sum tests are depicted by letters with sites not connected by the same letter being significantly different ($p < 0.05$).

The most abundant species was *Pileolina patelliformis* with a total of 1541 individuals (40% of total). The relative abundance of *P. patelliformis* decreased across the gradient from approximately 56.5% at one of the reference sites to 1.5% closest to the CO_2 seeps (Figure 3.17). The next most abundant species was *Daitrona* sp. with 875 individuals (23% of total). The relative abundance of *Daitrona* sp. increased across the gradient from 0.3% at reference sites to 85.5% at high CO_2. The assemblages were dominated by calcareous forms at reference sites (pH ~8.19) and by agglutinated forms nearer to the seeps (pH ~7.71) (Figure 3.18). The dominant taxa in the low pH conditions were *Daitrona* sp., which has an agglutinated test and *Elphidium* spp.
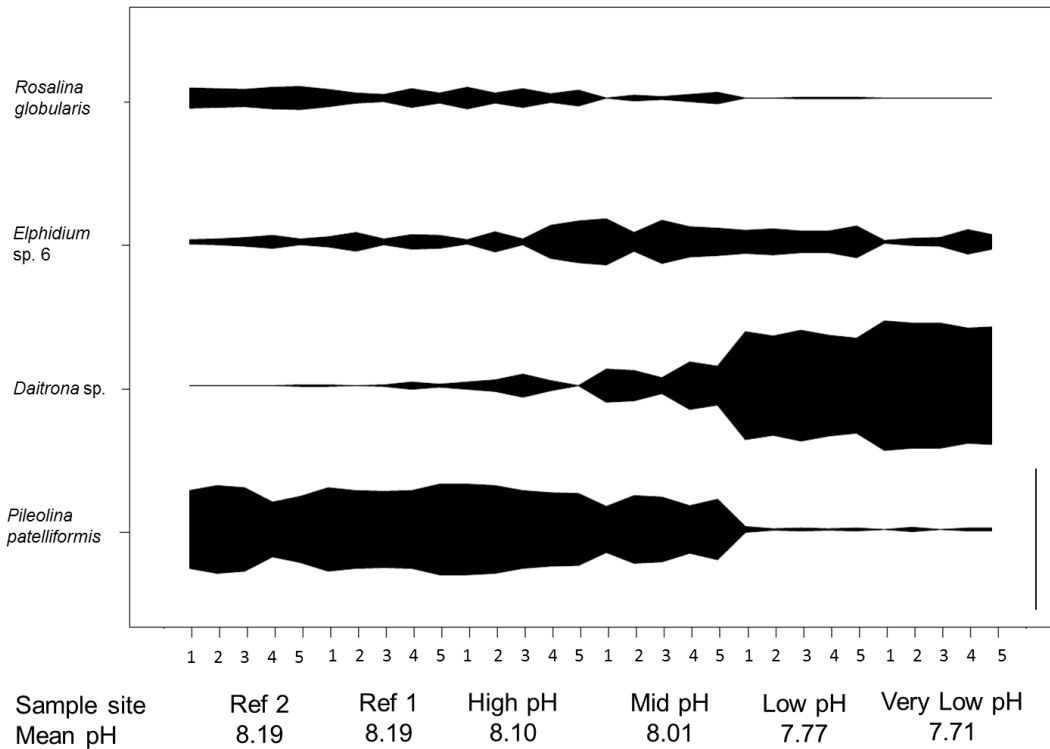
Figure 3.17: Relative abundance (%) of the four most abundant types of epiphytic foraminifera found on 2 g of dried *Padina pavonica* samples at Vulcano in May 2012. Numbers one to five are replicate numbers. The vertical scale bar on the bottom right hand side of the plot represents a relative abundance of 100 %.
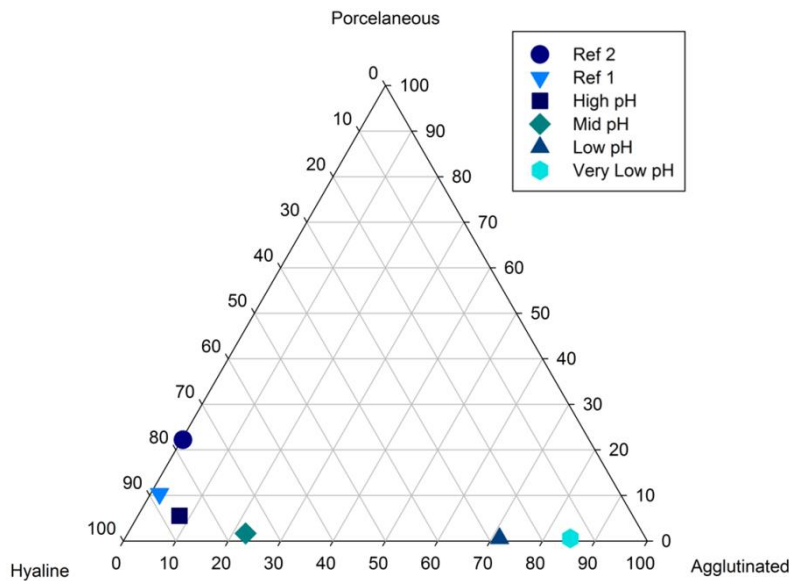
Figure 3.18: Proportions of porcelaneous, agglutinated and hyaline foraminifera taxa found on *Padina pavonica* thalli collected at Vulcano CO_2 seeps in May 2012. Bottom axis: percentage of agglutinated taxa, right axis: percentage of porcelaneous taxa, left axis: percentage of hyaline taxa.

Foraminiferal assemblages were similar at sample sites with similar CO_2 levels (Figure 3.19). One-way ANOSIM test also showed significant site differences in the assemblage (Global R statistic = 0.837, p = 0.001). Pairwise tests show which sites were responsible for the differences. The only sites that did not have a statistically significant difference in assemblage at the 0.05 significance level were: Ref 1 and High pH (Table 3.13). The assemblages of foraminifera on *Padina* seaweed surfaces were similar between a reference site and the high pH site. SIMPER revealed that the four taxa that contributed most to dissimilarity between sample sites were *P. patelliformis*, plastogamous pairs of *P. patelliformis*, *Daitrona* sp., and *R. globularis*.
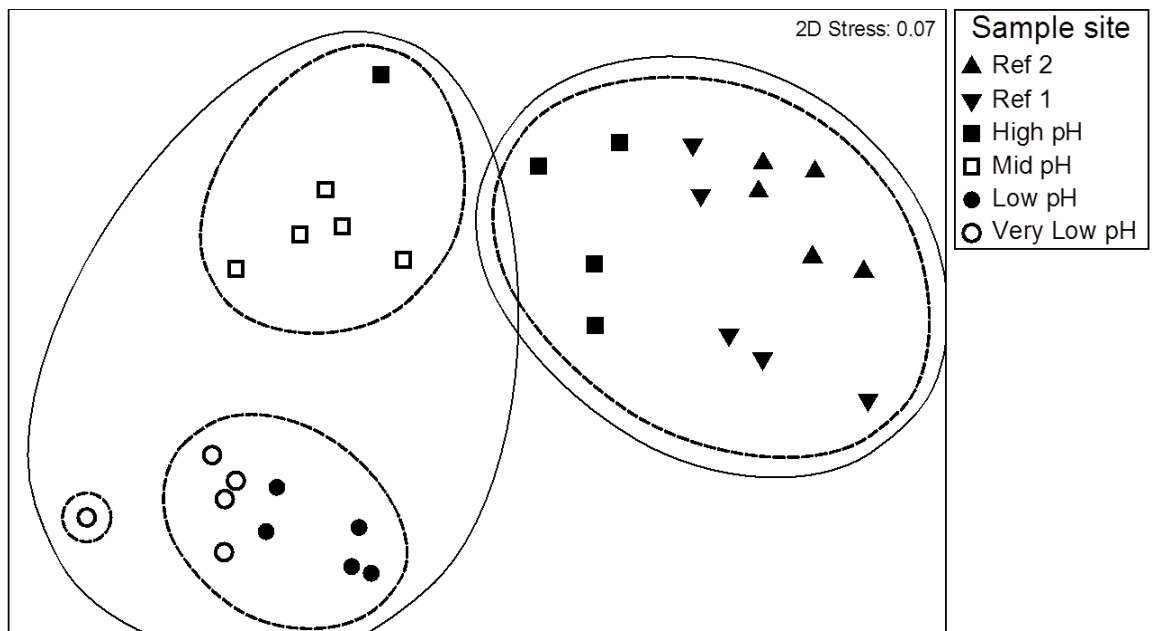
Figure 3.19: nMDS plot representing similarities between epiphytic foraminiferal assemblages found on air dried *Padina pavonica* thalli collected in May 2012 along a CO_2 gradient off Vulcano. The lines group samples at the 40 (solid) and 60 (dashed) percent similarity levels.

Table 3.13: Results of pairwise tests between sample sites for epiphytic foraminiferal assemblages found on air dried *Padina pavonica* thalli collected in May 2012 along a CO_2 gradient off Vulcano. The numbers are *p* values. Significant pairwise tests (< 0.05) are marked with asterisks.

	Ref 2	Ref 1	High pH	Mid pH	Low pH	Very Low pH
Ref 2		0.048*	0.008*	0.008*	0.008*	0.008*
Ref 1			0.063	0.008*	0.008*	0.008*
High pH				0.024*	0.008*	0.008*
Mid pH					0.008*	0.008*
Low pH						0.008*
Very Low pH						

3.3.3.6 Formalin preserved Padina pavonica *epiphytes May 2012*

The samples collected in May 2012 that had been preserved in formalin were divided into three assemblage types; 1) stained, 2) unstained (dead) and 3) attached. There were only 14 individuals that were stained dark magenta in at least half of their chambers. No further analysis, therefore, was done on the stained assemblage.

In the unstained assemblage, foraminifera were found in 17 of the 18 samples examined with 2165 individuals counted during this study (Table 3.14). There was a reduction in the number of species of epiphytic foraminifera along a calcium carbonate saturation gradient from reference sites ($\Omega \sim 5.29$) to high CO_2 conditions ($\Omega \sim 2.47$) nearer to the seeps (Figure 3.20).

Table 3.14: A list of the species of benthic foraminifera found within the dead (unstained) assemblage from *Padina pavonica* thalli samples preserved in formalin from Vulcano, collected in May 2012. Numbers per 2 grams of dry thallus are reported along with the percentage contribution.

Species	Ref 2 A Per 2 g	%	Ref 2 B Per 2 g	%	Ref 2 C Per 2 g	%	Ref 1 A Per 2 g	%	Ref 1 B Per 2 g	%	Ref 1 C Per 2g	%	High pH A Per 2 g	%	High pH B Per 2 g	%	High pH C Per 2 g	%
Bolivina pseudoplicata	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Bolivina striatula	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
Bolivina sp. 6	1	0	0	0	1	1	0	0	1	1	1	0	0	0	0	0	0	0
Cornuspira involvens	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Elphidium cf. *E. advenum*	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Elphidium macellum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium margaritaceum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
Elphidium sp. 4	2	1	1	1	3	2	3	1	1	0	1	0	1	2	3	4	2	7
Elphidium sp. 5	15	11	6	6	27	16	32	11	9	8	9	8	15	23	16	18	5	18
Elphidium sp. 6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 5 of Cimerman and Langer	1	1	1	1	0	0	2	1	0	0	0	0	2	3	0	0	0	0
Haynesina depressula	3	2	3	3	2	1	11	4	2	2	1	0	1	2	1	1	1	2
Lobatula lobatula	1	0	0	0	3	2	0	0	0	0	0	0	0	0	0	0	0	0
Massilina gualtieriana	0	0	0	0	1	1	0	0	1	0	0	0	3	5	1	1	1	2
Miliolinella subrotunda	2	1	0	0	3	2	1	0	0	0	0	0	0	0	1	1	0	0
Patellina corrugata	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Peneroplis pertusus	1	1	4	4	6	4	1	0	3	3	3	2	1	1	0	0	1	4
Peneroplis planatus	3	2	4	4	3	2	0	0	1	0	1	0	0	0	0	0	0	0
Pileolina patelliformis	81	59	68	68	55	33	182	66	83	73	88	80	37	54	60	67	16	60
Pileolina patelliformis (plastogamous pair)	8	6	4	4	11	7	21	8	5	5	3	2	2	3	2	2	0	0
Planorbulina mediterranensis	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Quinqueloculina annectens	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Quinqueloculina bosciana	0	0	1	1	0	0	0	0	1	0	0	0	0	0	1	1	0	0
Quinqueloculina stelligera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina sp. 1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina sp. 2	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina sp. 3	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Rosalina globularis	9	7	7	7	26	15	16	6	4	4	6	6	1	1	3	3	2	7
Rosalina sp. 1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	1	0	0
Triloculinella dilatata	2	1	0	0	1	1	1	0	0	0	0	0	0	0	1	1	0	0
Vertebralina striata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Wellmanellinella striata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
Indeterminate aggultinated sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
Indeterminate hyaline sp. 1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 5	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 10	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Indeterminate porcelaneous sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 4	3	2	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
Indeterminate porcelaneous sp. 5	3	2	1	1	7	4	3	1	1	0	1	0	1	1	0	0	0	0
Indeterminate porcelaneous sp. 8	2	1	0	0	9	6	1	0	1	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 11	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 16	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 20	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Indeterminate porcelaneous sp. 21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 30	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0

Table 3.14 continued:

Species	Mid pH A Per 2 g	%	Mid pH B Per 2 g	%	Mid pH C Per 2 g	%	Low pH A Per 2 g	%	Low pH B Per 2 g	%	Low pH C Per 2 g	%	Very Low pH A Per 2 g	%	Very Low pH B Per 2 g	%	Very Low pH C Per 2 g	%
Bolivina pseudoplicata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bolivina striatula	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bolivina sp. 6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cornuspira involvens	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium cf. *E. advenum*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium macellum	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium margaritaceum	2	10	0	0	0	0	1	100	1	100	4	100	1	100	7	100	0	0
Elphidium sp. 4	4	27	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 5	6	37	17	53	6	46	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 6	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 5 of Cimerman and Langer	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Haynesina depressula	0	0	4	12	1	4	0	0	0	0	0	0	0	0	0	0	0	0
Lobatula lobatula	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Massilina gualtieriana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Miliolinella subrotunda	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Patellina corrugata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Peneroplis pertusus	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Peneroplis planatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pileolina patelliformis	3	17	8	24	7	50	0	0	0	0	0	0	0	0	0	0	0	0
Pileolina patelliformis (plastogamous pair)	0	0	2	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Planorbulina mediterranensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina annectens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina bosciana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina stelligera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina sp. 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina sp. 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rosalina globularis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rosalina sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Triloculinella dilatata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vertebralina striata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Wellmanellinella striata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate agglutinated sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 5	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure 3.20: Mean (± 1 SD, n = 3) number of species of epiphytic foraminifera in the unstained assemblage from *Padina pavonica* thalli preserved in formalin collected from CO_2 seeps off Vulcano in May 2012. Results of Tukey's pairwise multiple comparison test are depicted by letters with sites not connected by the same letter being significantly different ($p < 0.05$).

The mean number of species ranged from 21 at Ref 2 to one at sample site Very Low pH (Figure 3.20). A one-way ANOVA revealed a statistically significant difference in the number of species between sites ($p = <0.001$, $F = 13.294$, degrees of freedom = 5). The mean number of unstained individuals per 2 grams of dried thallus ranged from two to 167 with the lowest mean number of individuals occurring at sample site Low pH (Figure 3.21). Kruskal-Wallis one-way analysis of variance on ranks revealed a statistically significant difference in the number of individuals per 2 grams of dried thallus between sites ($p = 0.008$, $H = 15.503$, degrees of freedom = 5). Shannon-Wiener diversity ranged from 1.76 at the reference sites to zero in samples collected from the most acidified site, with a corresponding Fisher Alpha index of 5.36 and 0.22. The most abundant species was *P. patelliformis* with a total of 1258 individuals (58.1% of unstained assemblage). No agglutinated individuals were found in the unstained assemblage.
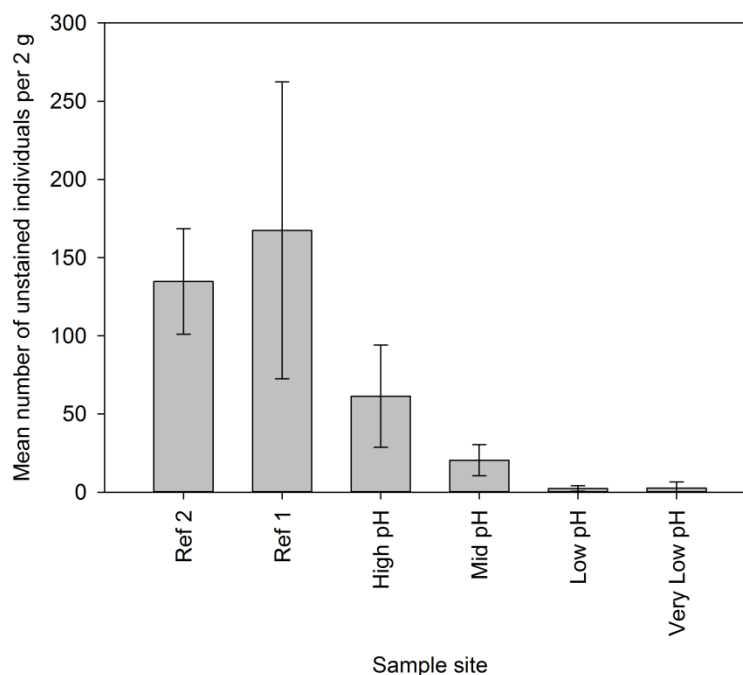
Figure 3.21: Mean (± 1 SD, n = 3) number of individuals of epiphytic foraminifera in the unstained assemblage per 2 grams of dried *Padina pavonica* thallus preserved in formalin collected from CO_2 seeps off Vulcano in May 2012.

No foraminifera were found in one sample (Very Low pH C) and this sample was removed from the nMDS analysis, as it was creating an outlier making the plot difficult to interpret. Foraminiferal assemblages were similar at sample sites with similar CO_2 levels (Figure 3.22). PERMANOVA revealed that there were significant site differences in the assemblage (pseudo-F = 9.2034, p (MC) = < 0.001). Those pairwise tests with p (MC) values above 0.05 (no significant difference) were Ref 2 and Ref 1, Ref 2 and High pH, Ref 1 and High pH, High pH and Mid pH and Low pH and Very Low pH (Table 3.15). SIMPER revealed that the four taxa that contributed most to dissimilarity between sample sites were *P. patelliformis*, *Elphidium* sp. 6, *R. globularis* and plastogamous pairs of *P. patelliformis*.
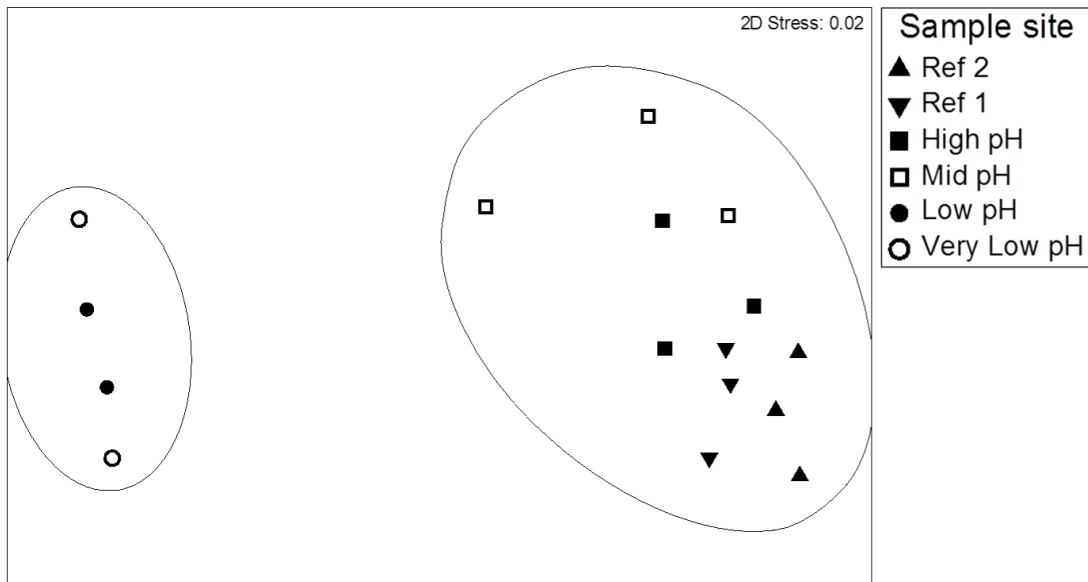
Figure 3.22: nMDS plot representing similarities between the unstained epiphytic foraminiferal assemblages collected from *Padina pavonica* thalli preserved in formalin, collected in May 2012 along a CO_2 gradient off Vulcano. The solid lines group samples at the 40 percent similarity level.

Table 3.15: Results of pairwise tests between sample sites for epiphytic foraminiferal assemblages from the unstained assemblage found on *Padina pavonica* thalli that had been preserved in formalin collected in May 2012 along a CO_2 gradient off Vulcano. The numbers are Monte Carlo *p* values. Significant pairwise tests (< 0.05) are marked with asterisks.

	Ref 2	Ref 1	High pH	Mid pH	Low pH	Very Low pH
Ref 2		0.331	0.077	0.015*	0.001*	0.016*
Ref 1			0.140	0.023*	0.001*	0.012*
High pH				0.123	0.001*	0.018*
Mid pH					0.003*	0.029*
Low pH						0.656
Very Low pH						

Foraminifera that remained on the thalli after washing (attached assemblage) were found in 16 out of the 18 samples collected in May 2012 that had been preserved in formalin with a total of 272 individuals (Table 3.16). Similarly to the unstained assemblage, there was a reduction in the number of species of epiphytic foraminifera along the pCO_2 gradient from reference sites to high CO_2 conditions nearer to the seeps (Figure 3.23).

Table 3.16: A list of the species of benthic foraminifera found within the attached assemblage from *Padina pavonica* thalli samples preserved in formalin from Vulcano, collected in May 2012. Numbers per 2 grams of dry thallus are reported along with the percentage contribution.

	Ref 2 A		Ref 2 B		Ref 2 C		Ref 1 A		Ref 1 B		Ref 1 C		High pH A		High pH B		High pH C	
Species	Per 2 g	%	Per 2 g	%	Per 2 g	%	Per 2 g	%	Per 2 g	%	Per 2 g	%	Per 2 g	%	Per 2 g	%	Per 2 g	%
Affinetrina gualtieriana	0	0	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bolivina sp. 6	1	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lobatula lobatula	0	0	0	0	2	0	0	0	0	0	1	13	0	0	0	0	0	0
Pileolina patelliformis	3	19	5	24	4	16	5	26	2	25	3	33	3	32	4	47	5	69
Pileolina patelliformis (plastogamous pair)	0	0	1	6	2	8	1	3	1	13	0	0	1	14	1	6	0	0
Daitrona sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium cf. *E. advenum*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	6	0	0
Elphidium macellum	0	0	0	0	0	0	1	3	0	0	0	0	0	5	0	0	0	0
Elphidium margaritaceum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 3	1	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 4	0	0	0	0	0	0	0	0	0	0	0	0	2	23	0	0	0	0
Elphidium sp. 5	1	4	1	6	1	6	5	29	1	13	0	0	0	0	2	18	2	23
Elphidium sp. 5 of Cimerman and Langer	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Haynesina depressula	0	0	0	0	1	6	0	0	0	0	0	0	1	9	0	0	0	0
Indeterminate sp. 1	1	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 4	0	0	1	3	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 5	1	4	0	0	1	4	1	3	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 8	1	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Massilina gualtieriana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Triloculinella dilatata	0	0	0	0	0	0	0	0	1	6	0	0	0	0	1	6	0	0
Miliolinella subrotunda	0	0	0	0	1	6	1	3	0	0	0	0	0	0	0	0	0	0
Peneroplis pertusus	3	19	3	15	1	6	1	3	2	25	1	7	0	5	0	0	1	8
Peneroplis planatus	0	0	1	3	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Planorbulina mediterranensis	0	0	1	3	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Pseudotriloculina rotunda	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Rosalina globularis	6	37	8	36	8	34	5	29	2	19	4	47	1	14	2	18	0	0
Quinqueloculina bosciana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina stelligera	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0

Table 3.16 continued:

Species	Mid pH A Per 2g	%	Mid pH B Per 2g	%	Mid pH C Per 2g	%	Low pH A Per 2g	%	Low pH B Per 2g	%	Low pH C Per 2g	%	Very Low pH A Per 2g	%	Very Low pH B Per 2g	%	Very Low pH C Per 2g	%
Affinetrina gualtieriana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bolivina sp. 6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lobatula lobatula	0	0	1	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pileolina patelliformis	1	33	3	20	1	17	0	0	0	0	0	0	0	0	0	0	0	0
Pileolina patelliformis (plastogamous pair)	0	0	2	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Daitrona sp.	0	0	0	0	0	0	0	0	1	25	3	57	0	0	0	0	0	0
Elphidium cf. *E. advenum*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium macellum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium margaritaceum	0	0	0	0	0	0	0	0	1	25	0	0	1	100	0	0	0	0
Elphidium sp. 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 4	0	0	0	0	0	0	0	0	1	25	1	29	0	0	1	100	0	0
Elphidium sp. 5	1	33	4	32	1	17	0	0	0	0	1	14	0	0	0	0	0	0
Elphidium sp. 5 of Cimerman and Langer	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Haynesina depressula	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Massilina gualtieriana	0	0	1	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Triloculinella dilatata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Miliolinella subrotunda	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Peneroplis pertusus	0	0	1	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Peneroplis planatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Planorbulina mediterranensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudotriloculina rotunda	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rosalina globularis	1	33	2	16	2	50	0	0	1	25	0	0	0	0	0	0	0	0
Quinqueloculina bosciana	0	0	0	0	1	17	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina stelligera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
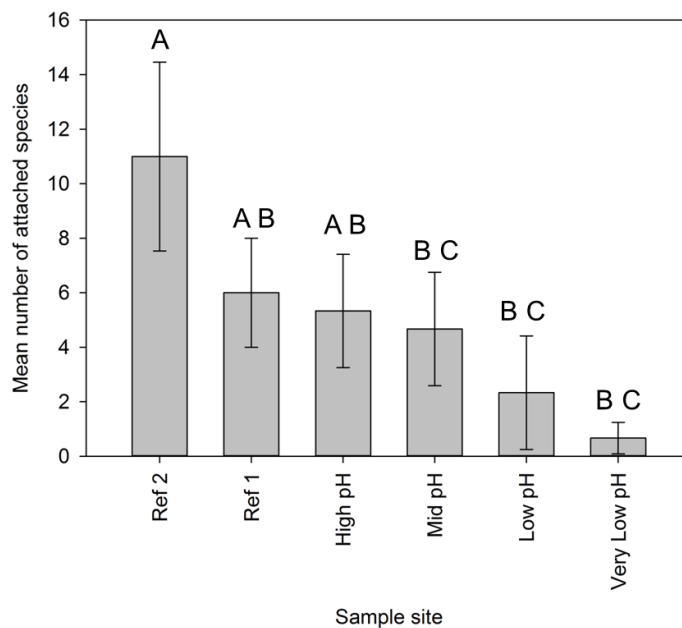
Figure 3.23: Mean (± 1 SD, n = 3) number of species of epiphytic foraminifera in the attached assemblage from *Padina pavonica* thalli preserved in formalin collected from CO_2 seeps off Vulcano in May 2012. Results of Tukey's pairwise multiple comparison test are depicted by letters with sites not connected by the same letter being significantly different ($p < 0.05$).

The mean number of attached species ranged from 11 at Ref 2 to one at sample site Very Low pH (Figure 3.23). A one-way ANOVA revealed a statistically significant difference in the number of species between sites ($p = 0.002$, $F = 7.745$, degrees of freedom = 5). The mean number of attached individuals per 2 grams of dried thallus ranged from one to 20 with the lowest number of individuals occurring at sample site

Very Low pH (Figure 3.24). A one-way ANOVA revealed a statistically significant difference in the number of individuals per 2 grams between sites (p = <0.001, F = 8.995, degrees of freedom = 5). Shannon-Wiener diversity ranged from 1.92 at the reference sites to zero in samples collected from the most acidified site, with a corresponding Fisher Alpha index of 5.36 and 0.80. The most abundant species was *R. globularis* with a total of 73 individuals (26.8% of the attached assemblage). The next most abundant species was *P. patelliformis* with a total of 69 individuals (25.3% of the attached assemblage). A total of five agglutinated individuals were found, belonging to the genus *Daitrona*.
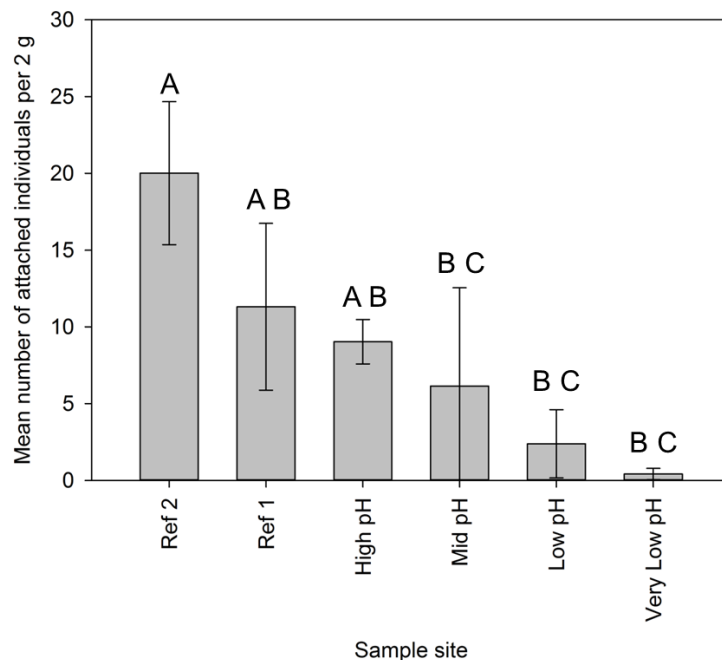
Figure 3.24: Mean (± 1 SD, n = 3) number of individuals of epiphytic foraminifera in the attached assemblage per 2 grams of dried *Padina pavonica* thallus preserved in formalin collected from CO_2 seeps off Vulcano in May 2012. Results of Tukey's pairwise multiple comparison test are depicted by letters with sites not connected by the same letter being significantly different (p < 0.05).

The two samples in which no foraminifera were found (Low pH A and Very Low pH C) were removed from the nMDS analysis as they were creating outliers making the plot difficult to interpret. Foraminiferal assemblages were similar at sample sites with similar CO_2 levels. The sample sites cluster into two distinct groups on an nMDS plot, those from the reference sites, highest pH areas and mid pH areas (Ref 2, Ref 1, High pH

and Mid pH) and those from the lowest pH areas (Low pH and Very Low pH) (Figure 3.25). PERMANOVA revealed that there were significant site differences in the assemblage (pseudo-F = 3.1882, p (MC) = 0.001). Those pairwise tests with p (MC) values below 0.05 (significant difference) were Ref 2 and Low pH, Ref 1 and Low pH and High pH and Low pH (Table 3.17). SIMPER revealed that the four taxa that contributed most to dissimilarity between sample sites were *R. globularis*, *Elphidium* sp. 6, plastogamous pairs of *P. patelliformis* and *P. pertusus.*
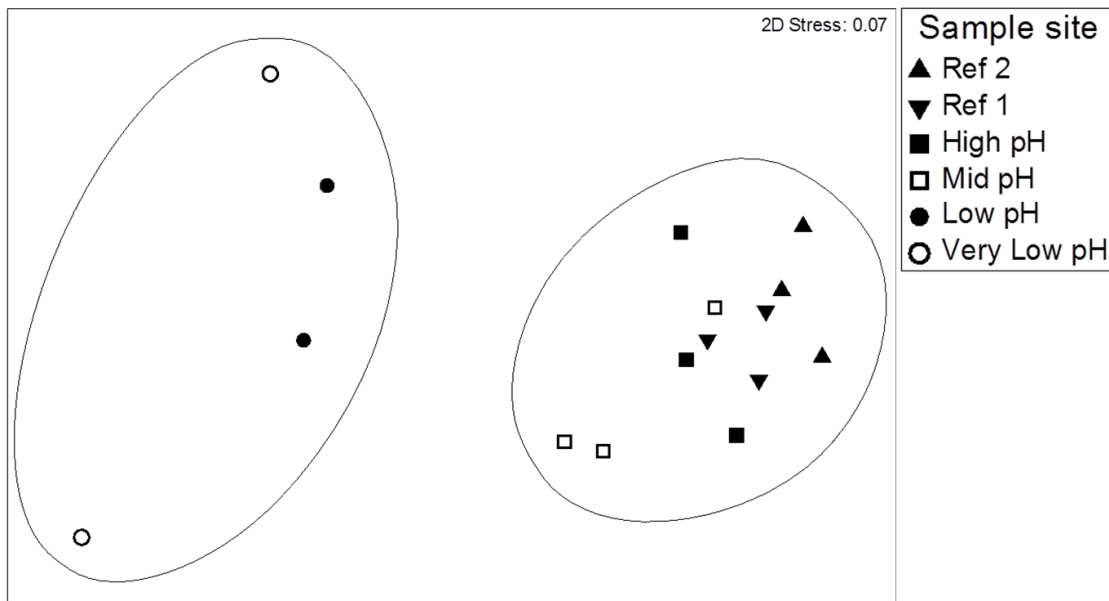
Figure 3.25: nMDS plot representing similarities between the attached assemblages from *Padina pavonica* thalli preserved in formalin collected in May 2012 along a CO_2 gradient off Vulcano. The solid lines group samples at the 40 percent similarity level.

Table 3.17: Results of pairwise tests between sample sites for attached foraminiferal assemblages from *Padina pavonica* thalli preserved in formalin collected in May 2012 along a CO_2 gradient off Vulcano. The numbers are Monte Carlo p values. Significant pairwise tests (< 0.05) are marked with asterisks.

	Ref 2	Ref 1	High pH	Mid pH	Low pH	Very Low pH
Ref 2		0.325	0.109	0.067	0.019*	0.073
Ref 1			0.645	0.248	0.024*	0.073
High pH				0.227	0.042*	0.099
Mid pH					0.052	0.090
Low pH						0.584
Very Low pH						

3.3.3.7 SEM

In total, 104 individuals were examined using a scanning electron microscope (SEM). Fifteen of these were examined at a higher magnification (x 4000), of which, only one showed signs of dissolution. The individual with signs of dissolution had surface pitting and belonged to the species *Triloculinella dilatata*, collected from the reference site, Ref 2, in May 2012. *Triloculinella dilatata* have high-magnesium calcite tests (Bentov and Erez, 2006), which may mean that they are even more vulnerable to the effects of ocean acidification, but the fact that this particular specimen was collected from a reference site, suggests that it was not undergoing dissolution due to low pH. There were no deformities (such as abnormally shaped chambers) in any of the individuals examined under the SEM (Figure 3.26 and Figure ii).
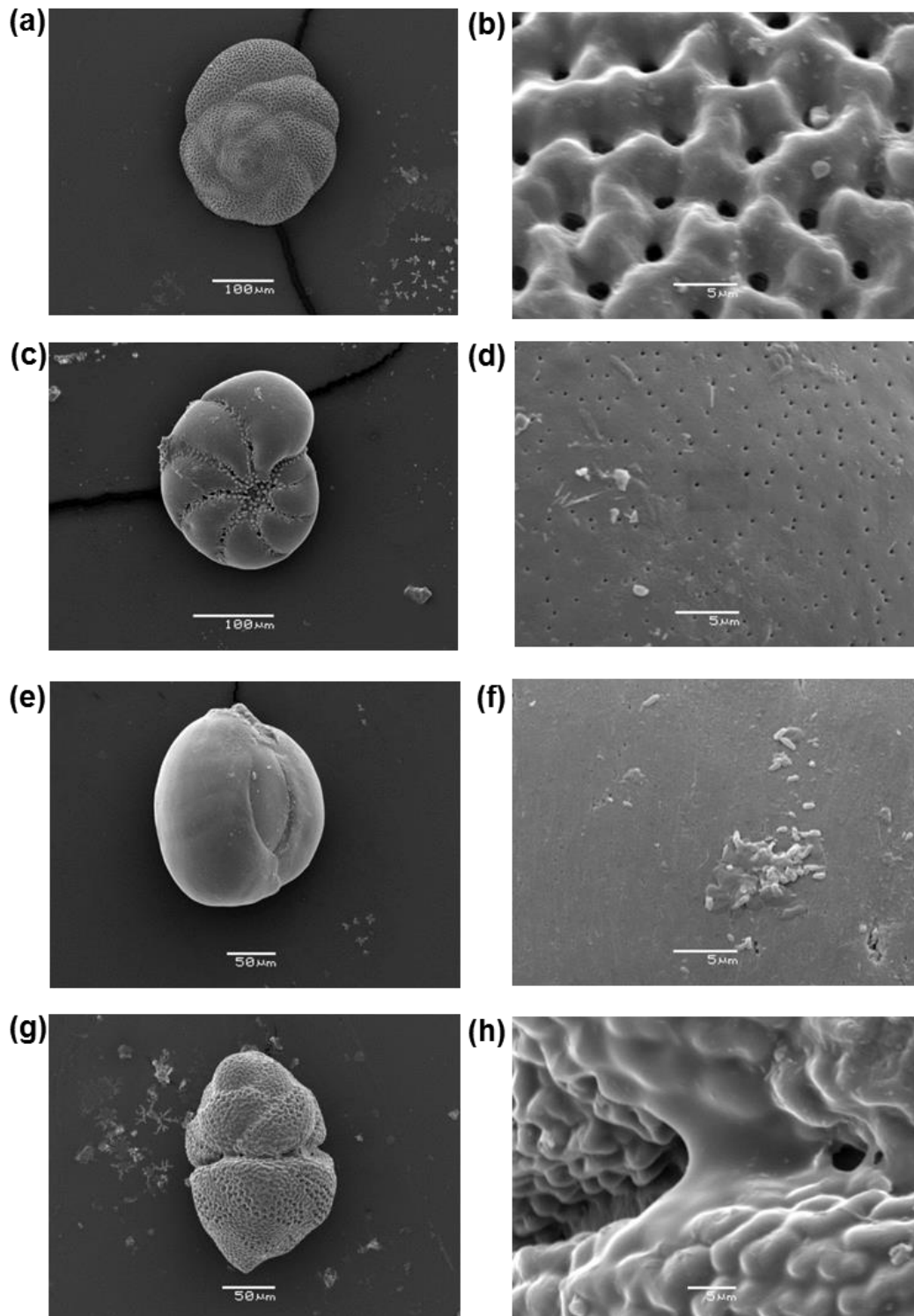
Figure 3.26: Surface preservation of selected epiphytic foraminifera found on air dried *Padina pavonica* thalli collected off Vulcano in May 2012. (a) *Pileolina patelliformis* from site Ref 2 (pH ~8.19), (b) wall detail of final chamber; (c) *Haynesina depressula* from site Ref 1 (pH ~8.19), (d) wall detail of final chamber; (e) *Miliolinella subrotunda* from site Ref 2 (pH ~8.19), (f) wall detail of final chamber; (g) A plastogamous pair of *P. patelliformis* from site Ref 2 (pH ~8.19), (h) detail of the join between the two specimens.

3.3.4 Discussion

The reasons for the absence of benthic foraminifera in the sediment samples from Vulcano are not clear, but it may be a result of the high energy environment, causing benthic foraminifera to be winnowed away (Cockey et al., 1996). The sediment size ranged from coarse sand to medium sand. This may provide an explanation for the absence of foraminifera. The coarse grain size and non-cohesive nature of the sediment provides an indication that the process of winnowing may be occurring in this shallow water location. The high energy environment will sort and remove finer sediment grains, leaving behind coarser grains. This would explain the absence of dead, as well as living, foraminifera. It is also possible that the coarse grain size of the sediment resulted in low food availability (Oxford et al., 2004). In addition, the sediment type at Vulcano may be inhospitable for living benthic foraminifera as it contained shards of glassy particles that were formed a few centuries ago due to the reworking of volcaniclastic material as Vulcanello merged with the main island of Vulcano (Romagnoli et al., 2012). The sediment has also had material added to it from pyroclastic flows from the last eruption of Vulcano from 1888 – 1890 (Dellino et al., 2011). As all samples along the gradient were declared barren in respect to benthic foraminifera, in this particular instance, variation in mean sediment size was not considered important in determining species composition.

As photosynthetic activity of marine plants and algae increases seawater pH (Semesi et al., 2009a, Manzello et al., 2012, Hendriks et al., 2014), it was predicted that seaweed and seagrasses would protect small calcareous organisms from acidified conditions near to CO_2 seeps by raising calcium carbonate saturation levels in the diffusion boundary layer (DBL). Johnson et al. (2012), who examined *P. pavonica* along the same CO_2 gradient at Vulcano, found that $CaCO_3$ content of the macroalgae reduced with CO_2 enrichment, but that photosynthetic rates increased. In the current study there was a reduction in calcified foraminifera in high CO_2 conditions (*ca.* 1675 µatm). Saderne and Wahl (2013) found that fucoid algal epiphytes were resistant to

pCO_2 levels *ca.* 1200 µatm, but at around 3150 µatm the tube worms (*Spirorbis spirorbis*) had reduced growth and settlement rates, although calcified (*Electra pilosa*) and non-calcified (*Alcyonidium hirsutum*) bryozoans were not impacted. Saderne and Wahl (2013) also argue that photosynthesis of the fucoid algae modulates calcification of the smallest epiphytes inhabiting the algal DBL.

The degree of buffering will depend on hydrodynamics and structural characters of seagrasses or macroalgal stand (Cornwall et al., 2014, Hendriks et al., 2014). When photosynthetic biomass is high, these habitats can experience pH values above that of ambient seawater, enhancing calcification rates of associated organisms (Semesi et al., 2009a) and *P. pavonica* is not the only species of macroalgae in the habitat. The standing stock of *P. pavonica* was clearly insufficient at Vulcano to allow a similar foraminiferal community to develop at the low pH sites compared to the reference sites. *Padina pavonica* may buffer the pH in the DBL during the daytime, but algal respiration at night causes large daily fluctuations in pH (Cornwall et al., 2013) and it may be periodic exposure to corrosive water that excluded some of the calcified foraminifera. The strong, but variable currents that are known to occur at Vulcano (Lucila et al., 1996, Boatta et al., 2013, Vizzini et al., 2013, Milazzo et al., 2014) may mean that there is insufficient time for high pH to develop within macroalgae stands for long periods (Cornwall et al., 2014).

At Vulcano in May 2012, there was a shift in the community assemblage of epiphytic foraminifera on *P. pavonica* thalli as pH decreased along a natural CO_2 gradient from one dominated by *P. patelliformis* and other calcareous taxa to one dominated by *Daitrona* sp., which is agglutinated. This shift occurred where mean pH reduced from ~ 8.01 to ~ 7.77 with a change in the mean Ω_{Calc} from 4.06 to 2.89. Sudden shifts in community assemblages have been identified before at other shallow water CO_2 seeps. An ecological shift has been found at a mean pH of 7.8 – 7.9 at Ischia (Hall-Spencer et al., 2008) and there were no calcareous foraminifera below a mean pH of ~

7.6 at Ischia, where agglutinated foraminifera dominated the foraminiferal assemblage (Dias et al., 2010). In this study, only two miliolid (porcelaneous) foraminiferal tests were found at the lowest pH site in May 2012 where there was a large increase in the proportion of agglutinated foraminifera. *Daitrona* sp., which was the only agglutinated taxon present in the samples, attach sediment particles to a proteinaceous or non-calcite matrix rather than a calcite cement (Sen Gupta, 1999b).

Elphidium spp. were also common on *P. pavonica* in high CO_2 conditions. The presence of *Elphidium* spp. in the low calcite saturation area shows that they were able to calcify and maintain their calcium carbonate tests, although a small percentage (1%) were deformed. No deformities were found in samples from Ischia (Dias et al., 2010) and the Gulf of California (Pettit et al., 2013), but deformities have been noted in culture experiments (e.g. Khanna et al. 2013). The *Elphidium* taxon is stress tolerant (Alve, 1995a, Frontalini and Coccioni, 2008) and has low magnesium calcite tests (below 4 mol% $MgCO_3$) (Bentov and Erez, 2006), which may explain their ability to survive in high CO_2, low calcite saturation conditions. They are also known for their ability to move on a variety of surfaces (Kitazato, 1988) and their response to light allows them to remain epiphytal (Manley and Shaw, 1997, Murray, 2006, Sadri et al., 2011). The excellent preservation of the few foraminifera that were examined under the SEM suggests that they were not experiencing dissolution of their tests due to the low pH conditions.

Padina pavonica deposits calcium carbonate in the form of aragonite needles extracellularly on the thallus surface and this calcification has been found to reduce near to CO_2 seeps off Vulcano (Johnson et al., 2012). A reduction in calcification is expected to reduce the thallus surface area. A reduction in thallus surface area may be a reason for fewer foraminifera in the low pH areas. In addition, it may be that foraminifera find it harder to attach to thalli that are not calcified, thereby leading to a reduction in individuals. This would mean that the reduction in the number of

foraminifera is a secondary effect of the low pH. If decreased calcification of *P. pavonica* was making it more difficult for the foraminifera to attach, then it would be expected that no epiphytic foraminifera would be found on thalli that are no longer calcified. The fact that there were still individuals attached to the thalli in low pH conditions would suggest that lack of calcification of the thalli is not making it more difficult for the epiphytic foraminifera to attach.

There tended to be a large amount of variation in the number of species of epiphytic foraminifera found on *P. pavonica* thalli between the two reference sites. This suggests that there is high degree of variability in the distribution of epiphytic foraminifera across small spatial scales (Semeniuk, 2000), despite environmental conditions remaining the same. Decreases in the number of species of foraminifera found across the CO_2 gradient could, therefore, be a result of variability in distributions rather than pH. Consistent decreases across the CO_2 gradient, however, indicate that changes in carbonate chemistry parameters are responsible for the shifts seen.

In contrast to the findings of this present chapter, Langer (1993) found a low diversity of epiphytic assemblages on *P. pavonica* thalli collected around Vulcano in August 1986 and February and August 1987. A total of 37 individuals belonging to eight species were found from examination of ten complete *P. pavonica* plants. Only species with short life spans (two to five months) were observed. The present study has found species with long-life spans (up to 1 year) such as *Planorbulina mediterranensis* as epiphytes on *P. pavonica* thalli.

Although a direct comparison between samples is not possible due to differing number of replicates, there were more foraminifera found on *P. pavonica* thalli that were collected in May compared to September. The samples from May showed a clear reduction in foraminiferal abundance across the CO_2 gradient, whereas samples from September did not. It could be that there were too few individuals in September for any

clear patterns to emerge. The higher number of individuals in May could be due to the spring bloom, which usually occurs in the Mediterranean Sea between the middle of March and the end of May (Vidussi et al., 2000, Andersen et al., 2001, Thyssen et al., 2014). Epiphytic foraminiferal abundance will be controlled by the temporal availability of the substrate (Langer, 1993, Debenay and Payri, 2010).

Another way in which some foraminifera may be protected from the effects of ocean acidification is through hosting algal symbionts. Many species of tropical foraminifera host algal symbionts. Through examining changes in pH in the DBL around symbiont-bearing foraminifera, it was found that photosynthesis of the symbionts only partly protected foraminifera against ocean acidification (Glas et al., 2012).

There were some caveats to the methods used. The low numbers of individuals that picked up the rose Bengal stain could be because there were few living individuals or, more likely, it could be an artefact of the staining methods used. There is much debate over the use of the rose Bengal staining technique (Walton, 1952, Murray, 1991a, Bernhard, 2000, Murray and Bowser, 2000, Bernhard et al., 2006) and the length of time for which samples have been left in the rose Bengal stain has varied greatly amongst researchers. A staining time of three hours was used in this study following the method used by Sadri et al. (2011) who also examined epiphytic foraminifera. As a means of standardising the rose Bengal staining technique, Schönfeld et al. (2012) recommended using a staining time of at least 14 days. The staining time of three hours, as used in this study, is much less than this and could mean that the foraminifera did not have sufficient time to pick up the stain (Schönfeld et al., 2012). This would lead to an underestimation of the number of living individuals. For this particular investigation, where the effects of ocean acidification were being examined, it was considered more appropriate to underestimate, rather than overestimate, the number of living individuals.

The samples that had been preserved in formalin were divided into three assemblage types; 1) stained, 2) unstained (dead) and 3) attached. Attached refers to the foraminifera that remained on the thalli after washing and were, therefore, not processed by staining with rose Bengal. The individuals that were not processed by staining with rose Bengal were picked directly from examining the thalli under a stereo-binocular microscope, therefore, there is no way of knowing if they were living or dead at the time of collection. This was also the case for the samples that had been air dried as the foraminifera were picked directly from the thalli. Arguably, these foraminifera that were still attached to the thalli after washing, or drying, were living at the time of collection. Any epiphytic foraminifera that were attached to the thalli and leaves must have been living recently in order to attach themselves and any that had been washed onto the thalli as dead individuals are likely to have been washed off during collection of the samples. Langer (1993) counted individuals that were still attached to the thalli as living. Davaud and Septfontaine (1995) found that most epiphytic foraminifera are easily removed as suspended load, therefore, even some living individuals may have been lost during sample collection. Poag (1982), however, found that encrusting types can be so firmly cemented that they commonly remain attached, even in fossil assemblages. *Padina pavonica* is thought to have a perennial life cycle, but dies back in the winter and, therefore, any attached foraminifera cannot have been on the thalli for longer than one year. The CO_2 gradient off Ischia and Vulcano is known to remain consistent across years (e.g. Hall-Spencer et al., 2008, Kerrison et al., 2011, Johnson et al., 2012, Boatta et al., 2013, Johnson et al., 2013), therefore, it can be proposed that the attached foraminifera would have lived in similar pH conditions to those measured in this investigation. This means that the information provided by the assemblage that remained attached to the thalli is still very useful.

3.4 Conclusions

There were too few samples collected from Ischia to draw any firm conclusions and additional sampling would be required to determine whether seagrass meadows may offer refugia to calcareous foraminifera with future ocean acidification. It was surprising to find that there were no benthic foraminifera in sediment samples collected along a shallow water CO_2 gradient off Vulcano. It is likely that the sharp particles were inhospitable. This meant that it was not possible to determine whether results from Ischia and Papua New Guinea were repeatable for sediment dwelling foraminifera in high pCO_2 conditions off Vulcano. There was a dramatic reduction in the number of species of epiphytic foraminifera on the brown seaweed *P. pavonica* along a shallow water CO_2 gradient off Vulcano. In samples collected in May, there was an assemblage shift from domination by calcareous taxa at reference sites (pCO_2 ~ 470 µatm) to domination by agglutinated taxa near to the seeps (pCO_2 ~ 1860 µatm). The hypothesis that algal surfaces would provide refugia for assemblages of calcified foraminifera along a gradient of overlying seawater acidification was not supported. Profound impacts on calcified foraminifera have also been found in sediments along carbonate saturation gradients (Dias et al., 2010, Fabricius et al., 2011, Uthicke et al., 2013) which has serious implications for the survival of calcareous foraminifera with future ocean acidification. Higher biomass stands of seagrass meadows, kelp forests or seaweed farms may be capable of mitigating the effects of ocean acidification at local scales. It is expected that ocean acidification will result in changes in foraminiferal assemblage composition and agglutinated forms may become more prevalent.

Chapter 4: The settlement of benthic foraminifera on artificial collectors along a natural CO₂ gradient

Abstract

The number of organisms that successfully recruit to an area will determine its community structure and population dynamics. To date, no research has been undertaken on how the settlement of foraminifera will be affected by ocean acidification; shallow water CO_2 seeps provide a useful means by which this can be examined. The aims of this chapter were to examine settlement of benthic foraminifera onto artificial collectors placed along a shallow water CO_2 gradient off the Island of Vulcano in Italy. There was a reduction in the number of benthic foraminifera found on artificial collectors as pH reduced from mean pH 8.19 to 7.71. No benthic foraminifera were found in areas with the lowest pH. This suggests that dead individuals undergo post-mortem dissolution under the highest CO_2 conditions and the associated reduction in calcite saturation state, which reached a minimum of Ω 1.82 near to the seeps. These results have global significance in the face of shoaling aragonite and calcite saturation horizons, suggesting that a decrease in calcium carbonate saturation state will lead to a reduction in the preservation of foraminifera on the sea floor and a reduction in the number of foraminifera buried within the sediment.

4.1 Introduction

Shallow water CO_2 seeps are extremely useful for examining the chronic effects of ocean acidification on marine communities (Hall-Spencer et al., 2008) and they are especially useful for examining ecosystem processes, such as settlement and recruitment, that cannot easily be studied under laboratory conditions (Cigliano et al., 2010, Kroeker et al., 2013b). Free-Ocean-Carbon-Dioxide-Enrichment (FOCE) experiments can also be used as an alternative approach to examine ecosystem processes (Arnold et al., 2012). FOCE experiments are analogous to terrestrial free-air-carbon-enrichment experiments. They allow the manipulation of pH under otherwise natural conditions. One of the major limitations of FOCE experiments, however, is that they do not allow migration of fauna into or out of the experimental area. The number of

organisms that settle in an area will determine population size and dynamics as well as community structure (Kurihara, 2008), making it important to understand the impact of ocean acidification on settlement.

Foraminifera play a significant role in the Earth's CO_2/CO_3^{2-} budget (Lee and Anderson, 1991, Langer et al., 1997) and their response to ocean acidification is likely to have consequences for inorganic carbon cycling. A major foraminiferal die-off because of their inability to settle may act as a negative feedback on atmospheric CO_2 levels and lead to a reduction in globally precipitated calcium carbonate (Dissard et al., 2010a).

The purpose of this study was to examine assemblages of benthic foraminifera that settled on artificial collectors placed across a natural CO_2 gradient. It is thought that the number and types of species that arrive on an artificial substrate will change according to the pH conditions of the area (Kurihara, 2008, Doropoulos et al., 2012). The settlement of many different organisms has been examined in relation to ocean acidification. These include biofilms (Lidbury et al., 2012, Johnson et al., 2013), macroalgae (Kroeker et al., 2013b), microfauna (Cigliano et al., 2010, Ricevuto et al., 2012), gastropods (Milazzo et al., 2014) and corals (Albright et al., 2008, Kurihara, 2008, Albright et al., 2010, Albright and Langdon, 2011, Doropoulos et al., 2012), as explained below.

Johnson et al. (2013) found that periphyton communities settling on perspex slides altered significantly as CO_2 concentrations increased across a shallow water CO_2 gradient off the Island of Vulcano, Italy. There was a significant increase in diatom abundance in CO_2 enriched areas. This was thought to be because elevations in CO_2 stimulated the primary productivity of the diatoms. In a similar experiment across the same CO_2 gradient, Lidbury et al. (2012) found that biofilm production was highest in seawater with high pCO_2. Kroeker et al. (2013b) examined recovery patterns of marine species following physical disturbance along a shallow water CO_2 gradient off the

Island of Ischia, Italy. They found that recovery was more predictable under acidified conditions because the recovery resulted in simple, algal dominated assemblages. The effects of ocean acidification on the settlement of benthic invertebrates and microfauna, including benthic foraminifera, was examined by Cigliano et al. (2010). They placed artificial collectors (scouring pads formed by an enrolled nylon net) along a natural CO_2 gradient off the Island of Ischia, Italy and examined the community composition of the organisms that settled on the collectors. The artificial collectors were attached to buoyed moorings 1 m from the seabed in approximately 3 m water depth. They found that increased levels of CO_2 had a significant effect on the settlement of many benthic invertebrates and microfauna. As pH decreased from 8.09 – 8.15 at reference sites, to 7.08 – 7.79 near to the seeps, the number of species and individuals of benthic foraminifera found on the artificial collectors decreased. In the artificial collectors from the lowest pH site, only five individual foraminifera were found that belonged to the species *Elphidium aculeatum* and *Elphidium depressulum*. Ricevuto et al. (2012) found similar patterns in the settlement of benthic invertebrates to Cigliano et al. (2010), when they placed artificial collectors along the same CO_2 gradient off Ischia, although they did not examine foraminifera. Examination of the impact of ocean acidification on the reef-building gastropod *Dendropoma petraeum* revealed that recruitment success was adversely affected because seawater with low calcium carbonate saturation prevented juveniles from cementing themselves to the parent reef (Milazzo et al., 2014).

Albright et al. (2008) examined the effect of aragonite saturation state on the recruitment of a common Caribbean reef coral *Porites astreoides*. They found that saturation state did not have a significant effect on percent settlement of *P. astreoides*, but that growth rate was positively correlated with saturation state. In another study of *P. astreoides*, Albright and Langdon (2011) found that settlement was reduced by 55 – 60% at pCO$_2$ of 800 µatm relative to controls (380 µatm). Examination of the impact of ocean acidification on the recruitment of the threatened Caribbean coral *Acropora palmata* found that fertilisation, settlement and growth were all negatively impacted by

increasing pCO$_2$ (Albright et al., 2010). They estimate that the cumulative impact of ocean acidification on fertilization and settlement success will result in a 73% reduction in the number of larval settlers on the reef under pCO$_2$ conditions predicted by the end of this century. Settlement of the coral *Acropora millepora* was reduced under CO$_2$ concentrations of 800 and 1300 µatm (Doropoulos et al., 2012). All of these studies suggest that although there are significant species specific differences, future ocean acidification is likely to affect settlement and recruitment of benthic organisms and, therefore, population size and community structure (Kurihara, 2008).

The presence of benthic foraminifera on artificial collectors will depend upon dispersal mechanisms. Benthic foraminifera are thought to be able to disperse and colonise new areas through passive or active methods (Alve, 1999). The only active dispersal mechanism for benthic foraminifera is through self-locomotion within, or on the sediment and this is only considered to be efficient over short distances (Alve, 1999). The two most important passive dispersal methods are thought to be the suspension and transport of various growth stages (Alve, 1999) and the release and transport of propagules or embryonic juveniles (Alve and Goldstein, 2003). The hydraulic regime of any one area plays an important role in determining the dispersion and colonisation of benthic foraminifera (Alve, 1999). A high energy environment tends to result in a short transit time, meaning that there is no time for pioneer or opportunistic assemblages to develop (Alve, 1999) and the major components of the nearest ambient seafloor assemblage are likely to colonise a new habit within days. In low energy environments, transit time is long for most species. Colonisation will typically follow a classic successional pattern with a high abundance of a pioneer assemblage initially, followed by the development of assemblages with an increasing number of specialised species. In low energy environments, colonisation can take several years (Alve, 1999).

The colonisation of sediment by benthic foraminifera has been examined previously, but not in relation to ocean acidification. In an examination of formerly anoxic

environments (where anoxic conditions had prevailed for more than five years at water depths between 2 and 50 m) in Norway, Alve (1995b) found that agglutinated foraminifera dominated living assemblages bordering anoxic environments and it took more than one year after re-aeration before the areas became suitable for colonisation by benthic foraminifera. Benthic foraminifera are able to colonise areas with high copper concentration (>2000 ppm) at a water depth of 63 m within a 32 week experiment (Alve and Olsgard, 1999). At a depth of 65 m off shore from Atlantic City, New Jersey, recolonisation of boxes by living benthic foraminifera had occurred after ten weeks (Ellison and Peck, 1983) and at a water depth of 125 m, colonisation of sediment filled boxes occurred within six weeks of deployment (Buzas et al., 1989). In the Panama Basin at a water depth of 3912 m, Kaminski et al. (1988) found that the agglutinated foraminifera *Reophax* was the most successful coloniser. The abundance of living individuals in recolonisation trays was one-tenth to one-third that of background abundance after nine months (Kaminski et al., 1988). In shallower water, colonisation appears to be more rapid. Buzas (1993) found that the colonisation rate of foraminifera was rapid with living densities stabilising within three weeks at 1 m water depth in the Indian River, Florida. Colonisation occurred in the same rank order as the ambient fauna.

Benthic foraminifera can be dispersed through the release and transport of propagules or embryonic juveniles (Alve and Goldstein, 2003). The main transport mechanisms of dead benthic foraminifera are thought to be bed load and suspended load, although transport on floating plants, ice, mass flow and turbidity currents are also possible (Murray et al., 1982, Murray, 1991a, Alve, 1995b, Alve, 1999). Transport as bed load is thought to be common in shallow water areas in sediments of sand grade or coarser. Here, tests are transported along the sea bed. Transport as suspended load occurs where dead benthic foraminifera thrown into suspension in the intertidal zone or by the re-working of subtidal sediments during storms (Murray, 1991a). Benthic foraminifera have previously been found in samples from plankton tows (Murray et al., 1982, John,

1987) suggesting that benthic foraminifera can be suspended in the water column. John (1987) reported the regular occurrence of the benthic foraminifera *Reophax scottii* in plankton samples taken at 10 m water depth in the North Sea.

The aims of this chapter are to examine the impact of ocean acidification on the settlement of benthic foraminifera and to see if the results of Cigliano et al. (2010) are reproducible at another shallow water CO_2 seep. The hypothesis that there will be a change in the assemblage of benthic foraminifera found on artificial collectors placed along a gradient of overlying seawater acidification was tested.

4.2 Materials and methods

4.2.1 Study area

The Vulcano CO_2 seep study sites were the same as those described in Chapter 3 (Figure 4.2). A gradient in pCO_2 occurs along a shallow water (0 – 5 m water depth) rocky shore (Kerfahi et al., 2014). This site is suitable for ocean acidification research since there are gradients in CO_2 that lack confounding gradients in salinity, alkalinity or in chemicals such as H_2S (Boatta et al., 2013). The sediment along this CO_2 gradient was formed a few centuries ago due to the reworking of volcaniclastic material as Vulcanello merged with the main island of Vulcano (Romagnoli et al., 2012) and additional sediment has recently been added following the last eruption of Vulcano from 1888-1890 (Dellino et al., 2011). The sediment contained shards of glassy volcanic grains.

4.2.2 Sample collection

Artificial collection pads, identical to those used by Cigliano et al. (2010), were placed along a CO_2 gradient at Vulcano in May 2010 and September 2011. The artificial collectors were approximately 8 cm in diameter and made from thick nylon netting which was rolled into an oblate spheroid (Figure 4.1). The artificial collection pads used

provide an analogue for other substrates on which benthic foraminifera can settle, such as macroalgae or algal turf. The artificial collection pads were known to be barren before placement, they can be easily manipulated and they provide a standard substrate, alleviating some of the problems associated with standardising sample collections, such as trying to collect samples of exactly the same size (Gobin and Warwick, 2006).
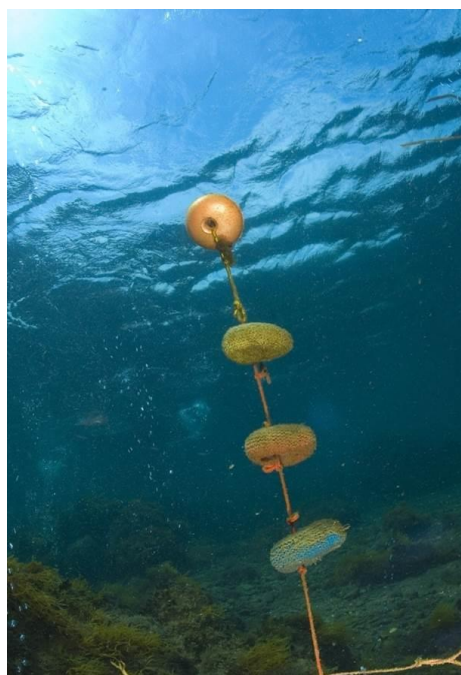
Figure 4.1: An example of the artificial collection pads which were used to examine the settlement of benthic foraminifera. Photograph taken by J. Hall-Spencer.

Artificial collectors were placed at four different locations along a CO_2 gradient in May 2010 (Ref 2, Mid pH, Low pH and Very Low pH) by colleagues from the University of Palermo, Italy and left for one month at six different locations in September 2011 (Ref 2, Ref 1, High pH, Mid pH, Low pH and Very Low pH) and left for 11 days (Figure 4.2). The collectors were placed approximately 0.5 m above the seabed and attached to bricks with nylon fishing line in approximately 2 m water depth. Four replicate collectors were placed at each location in May 2010 and three replicate collectors were placed in September 2011. Upon removal, the artificial collectors were placed in plastic bags underwater and gently taken ashore. They were stored in sealed plastic containers containing 4% buffered formalin until analysis.
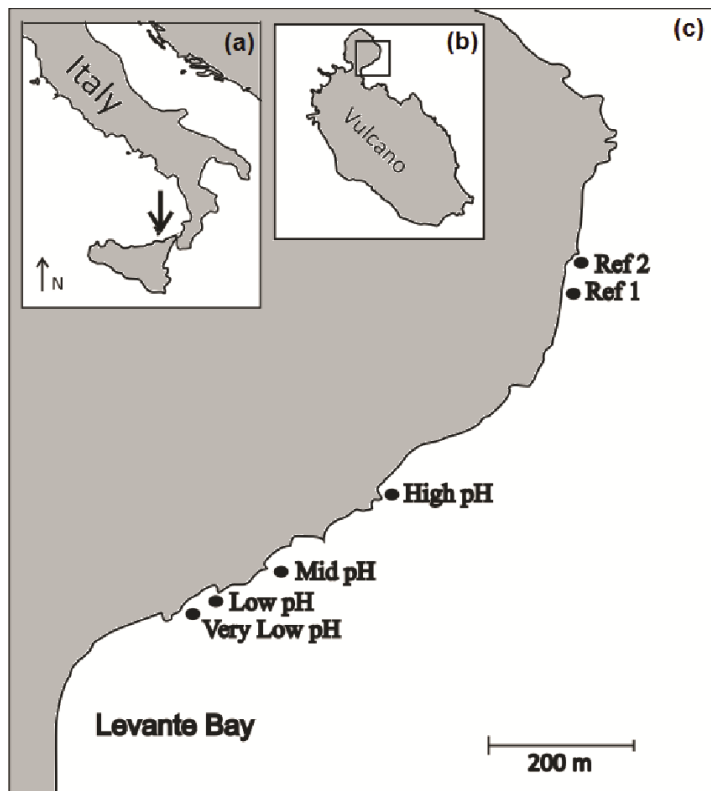
Figure 4.2: Study area at Vulcano. (a) Italy with arrow marking Vulcano, part of the Aeolian Island chain, northeast Sicily, (b) Vulcano, (c) Location of sample sites. Very Low pH was at 38°25′9″ N, 14°57′38″E, Ref 2 was at 38°25′20″N, 14°58′3″E.

Once in the laboratory, the collectors were washed over a 63 μm sieve. After rinsing for several minutes, the collectors were cut and unravelled over the sieve and washed again (Cigliano et al., 2010). The material retained on the sieve was stained with rose Bengal (1 g/L) for three hours (Sadri et al., 2011), re-washed to remove excess stain and placed in 70% Industrial Methylated Spirit (IMS) (Cigliano et al., 2010, Schönfeld et al., 2012). The samples were placed in 70% IMS to be consistent with colleagues from the University of Palermo, who had started to process the artificial collectors that were placed along the gradient in May 2010. They, in turn, were following the methods outlined in Cigliano et al. (2010). The unravelled netting of the artificial collectors was examined under a stereo-binocular microscope and any foraminifera remaining on the netting were removed and placed onto micropalaeontological slides.

The samples that were placed along the gradient in May 2010 were collected by colleagues from the University of Palermo, Italy. These samples were washed over a

144

63 µm sieve, but the retained material was not stained with rose Bengal initially. Once rinsed, the samples were placed into 70% IMS and seawater. Benthic foraminifera were picked out and placed in glass vials containing 70% IMS and seawater. The benthic foraminifera were then sent to Plymouth University for further analysis. Once received at Plymouth University the samples were stained with rose Bengal (1 g/L) for three hours (Sadri et al., 2011) and washed over a 63 µm sieve to remove excess stain. The samples were placed in 70% IMS.

All samples were initially wet picked by placing the material into a Petri dish and examining under a stereo-binocular microscope. Any foraminifera were removed with a paint brush and placed into a separate Petri dish. To prevent the micropalaeontological slides from becoming too wet, the foraminifera (in the Petri dish) were first placed in a drying oven at 30ºC for 24 hours. Once dry, the foraminifera were put onto micropalaeontological slides. Individuals were determined to have been live at the time of collection if they were stained dark magenta in at least half of their chambers (Bernhard et al., 2006). This excluded counting individuals with just a faint red dot in one chamber. Foraminifera were identified to species level, where possible, using Cimerman and Langer (1991) and Milker and Schmiedl (2012) and counted.

4.2.3 Transplant experiment

To see if there was a change in the assemblage of foraminifera found on artificial collectors when transplanted between sites, a transplant experiment was conducted for approximately one month between May and June 2013. Six artificial collectors were placed at the mid pH site and another six were placed at Reference Site 2. The collectors were placed approximately 0.5 m above the seabed and attached to bricks with nylon fishing line in approximately 2 m water depth. At both of the sample sites, the collectors were placed in a line equidistant from the coastline (Figure 4.3).
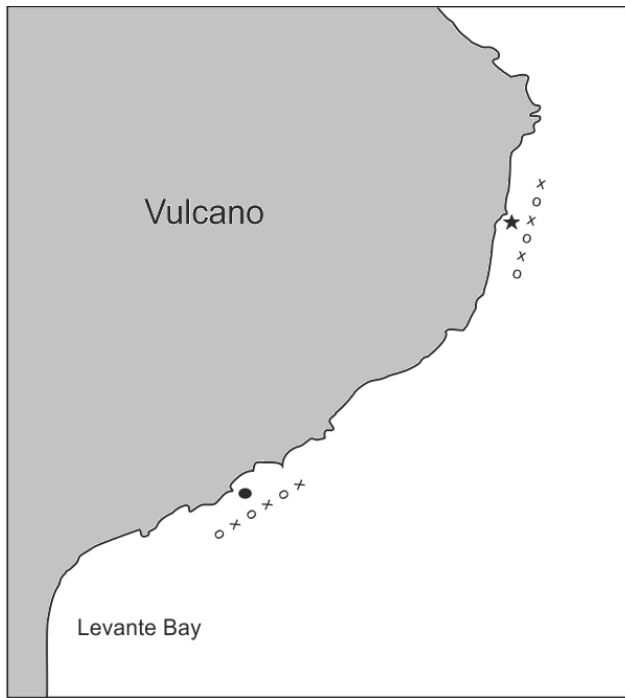
Figure 4.3: Approximate positioning of artificial collectors during a transplant experiment conducted offshore Vulcano May – June 2013. Solid circle marks sample site Mid pH. Star marks sample site Ref 2. X marks the approximate positioning of artificial collectors that were controls. o marks the approximate positioning artificial collectors that were transplanted between sites. Not to scale.

After 11 days, three collectors from Mid pH were transferred to Ref 2 and three collectors from Ref 2 were transferred to Mid pH. The remaining collectors were left in place to provide controls. Alternate collectors along the line were moved to try and limit any potential edge effects. When transplanting the collectors, they were placed in the same position along the sample line from which they had been removed and a plastic bag was placed over each collector to try and reduce the amount of material dislodged during movement. Nine days after the collectors had been transplanted, all collectors were retrieved from the water. The collectors had been deployed for a total of 20 days. Upon removal, the fishing line was cut to allow the collectors to be removed. The artificial collectors were placed in plastic bags underwater, taken ashore and placed in labelled plastic containers containing 4% buffered formalin. Once in the laboratory, the collectors were processed in the same way described in Section 4.2.2.

4.2.4 SEM

Foraminifera were examined under a JEOL JSM 5600 LV scanning electron microscope (SEM). Individuals were mounted on aluminium SEM stubs and sputter coated in an Emitech K550 gold sputter coater. To assess the effects of dissolution, test walls were examined at a higher magnification. Dissolution was noted if foraminiferal tests showed etching, pitting, fragmentation or enlarged pores. The final chamber was examined where possible as this is the thinnest and, therefore, shows damage quicker (Hansen, 1999).

4.2.5 Statistical analysis

For the May 2010 and September 2011 datasets, untransformed foraminiferal community assemblage data were used to calculate Shannon-Wiener diversity, Fisher Alpha and Pielou's evenness indices using PRIMER v.6. The data were then square root transformed to down-weight the contribution of dominant taxa. An ANOSIM test was conducted to test for similarities between sites and to determine if samples within the same site were more similar to each other than samples from different sites using PRIMER v.6. Further statistical analysis was not undertaken due to the limited number of samples.

4.3 Results

4.3.1 Carbonate chemistry

The carbonate chemistry values used in this study are the same as those reported in Chapter 3. Samples were collected between May 2011 and May 2013. Mean pH ranged from 8.19 at the reference sites to 7.71 at the Very Low pH site, which is closest to the seeps. The pH decreased across the gradient from reference sites to sample site Very Low pH (Table 3.5) and was lower and more variable near to the seeps. Seawater saturation of calcite ranged from a mean value of Ω 5.29 at the reference sites to Ω 2.47 closest to the seeps.

4.3.2 May 2010

Upon retrieval of the artificial collectors, two of the Mid pH replicates were missing. Unfortunately, because of differences in processing techniques between the University of Palermo and Plymouth University, not all of the samples could be stained with rose Bengal (Table 4.1).

Table 4.1: Sample site names, the number of replicates that were still in place upon collection and the number of replicates that were processed by staining with rose Bengal for the artificial collectors that were placed along a shallow water CO_2 gradient off Vulcano in May 2010.

Sample Site	Number of replicates collected	Number of replicates processed by staining with rose Bengal
Ref 2	4	4
Mid pH	2	0
Low pH	4	2
Very Low pH	4	2

In the samples that were stained with rose Bengal there were low numbers of living individuals meaning that statistical analysis was not conducted. There were a total of 464 stained individuals belonging to 24 species (Table 4.2).

Table 4.2: A list of the species of benthic foraminifera found within the stained (living) assemblage from artificial collectors placed along the CO_2 gradient at Vulcano in May 2010. Numbers found on the artificial collector are reported along with the percentage contribution.

Species	Ref 2 1	%	Ref 1 2	%	Ref 2 3	%	Ref 2 4	%	Low pH 3	%	Low pH 4	%	Very Low pH 3	%	Very Low pH 4	%
Ammonia sp. 3	0	0	0	0	1	0	4	2	0	0	0	0	0	0	0	0
Bolivina pseudoplicata	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
Elphidium sp. 5	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 5 of Cimerman and Langer	0	0	0	0	6	2	0	0	0	0	0	0	0	0	0	0
Haynesina depressula	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
Lobatula lobatula	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0
Massilina gualtieriana	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Miliolinella subrotunda	0	0	1	2	8	3	2	1	0	0	0	0	0	0	0	0
Peneroplis pertusus	0	0	0	0	23	9	9	6	0	0	0	0	0	0	0	0
Peneroplis planatus	0	0	0	0	24	9	9	6	0	0	0	0	0	0	0	0
Pileolina patelliformis	0	0	0	0	12	5	8	5	0	0	0	0	0	0	0	0
Planorbulina mediterranensis	0	0	0	0	3	1	6	4	0	0	0	0	0	0	0	0
Quinqueloculina annectens	0	0	22	54	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina auberiana	0	0	0	0	3	1	1	1	0	0	0	0	0	0	0	0
Quinqueloculina bosciana	0	0	1	2	2	1	2	1	0	0	0	0	0	0	0	0
Quinqueloculina stelligera	0	0	4	10	0	0	0	0	0	0	0	0	0	0	0	0
Rosalina globularis	0	0	4	10	138	53	96	59	0	0	0	0	0	0	0	0
Triloculinella dilatata	1	50	6	15	16	6	10	6	0	0	0	0	0	0	0	0
Vertebralina striata	0	0	0	0	1	0	3	2	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 5	1	50	0	0	0	0	1	1	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 8	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 11	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 31	0	0	2	5	12	5	9	6	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 35	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0

All of the stained individuals were found in artificial collectors that had been placed at the reference site. No stained individuals were found in the Low or Very Low pH site (Figure 4.4). At the reference site, there was a wide variation in the percentage of individuals that were living, ranging from 10% to 50%. The most abundant species was *Rosalina globularis* with a total of 238 individuals amounting to 51% of the living assemblage.
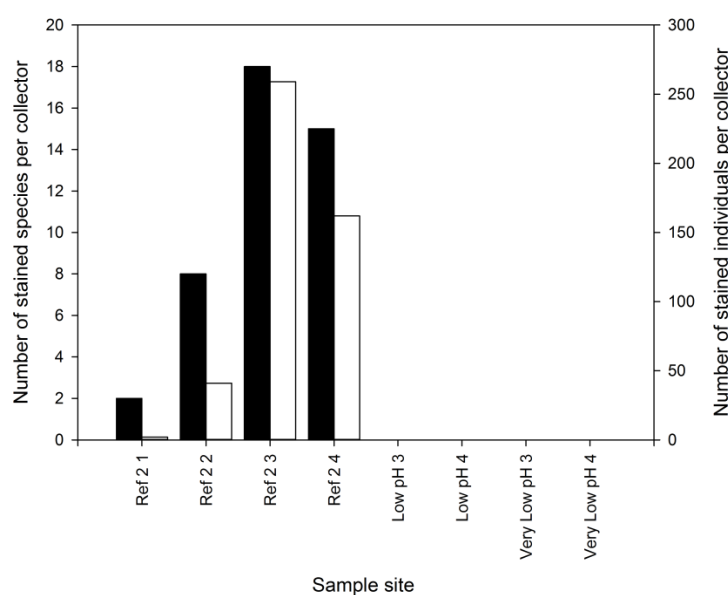
Figure 4.4: Number of living (stained) species and individuals of foraminifera found on artificial collectors placed offshore Vulcano in May 2010. Black bars: number of species, white bars: number of individuals.

When the total assemblage was examined, to include samples that had not been processed by staining with rose Bengal, at least one individual was found in nine out of the 14 samples examined with a total of 5198 individuals counted and 60 species (Table 4.3).

Table 4.3: A list of the species of benthic foraminifera found within the total assemblage from artificial collectors placed along the CO_2 gradient at Vulcano in May 2010. Numbers found on the artificial collector are reported along with the percentage contribution.

Species	Ref 2 1	%	Ref 1 2	%	Ref 2 3	%	Ref 2 4	%	Mid pH 1	%	Mid pH 2	%
Adelosina longirostra	19	2	10	1	0	0	0	0	0	0	0	0
Affinetrina gualtieriana	0	0	2	0	0	0	0	0	0	0	0	0
Ammonia sp. 2	1	0	0	0	0	0	0	0	0	0	0	0
Ammonia sp. 3	1	0	29	1	12	1	71	5	7	6	0	0
Bolivina difformis	2	0	8	0	2	0	14	1	0	0	0	0
Bolivina pseudoplicata	1	0	32	2	7	1	13	1	0	0	0	0
Bolivina striatula	0	0	3	0	0	0	1	0	0	0	0	0
Brizalina sp. 2 of Cimerman and Langer	1	0	0	0	0	0	0	0	0	0	0	0
Bulimina sp. 3	0	0	1	0	0	0	0	0	0	0	0	0
Cornuspira involvens	0	0	1	0	0	0	0	0	0	0	0	0
Elphidium aculeatum	0	0	0	0	0	0	16	1	0	0	0	0
Elphidium macellum	1	0	2	0	1	0	0	0	0	0	0	0
Elphidium sp. 4	1	0	2	0	2	0	5	0	3	3	0	0
Elphidium sp. 5	10	1	16	1	7	1	24	2	44	37	0	0
Elphidium sp. 6	1	0	1	0	1	0	1	0	0	0	0	0
Elphidium sp. 5 of Cimerman and Langer	20	2	6	0	54	7	0	0	0	0	0	0
Fursenkoina acuta	0	0	0	0	0	0	1	0	0	0	0	0
Haynesina depressula	2	0	7	0	1	0	9	1	0	0	0	0
Lobatula lobatula	13	2	45	2	13	2	36	2	0	0	0	0
Massilina gualtieriana	1	0	0	0	1	0	2	0	1	1	0	0
Miliolinella subrotunda	98	11	100	5	12	1	45	3	7	6	0	0
Peneroplis pertusus	33	4	212	11	27	3	95	7	0	0	0	0
Peneroplis planatus	14	2	88	5	25	3	90	6	0	0	0	0
Pileolina patelliformis	15	2	104	5	72	9	110	8	10	8	0	0
Pileolina patelliformis with float chamber	0	0	1	0	1	0	3	0	0	0	0	0
Planorbulina mediterranensis	6	1	15	1	6	1	15	1	0	0	0	0
Pseudotriloculina rotunda	0	0	0	0	0	0	2	0	0	0	0	0
Quinqueloculina annectens	62	7	139	7	0	0	0	0	0	0	0	0
Quinqueloculina auberiana	81	9	91	5	37	5	50	3	1	1	0	0
Quinqueloculina bosciana	1	0	11	1	3	0	10	1	0	0	0	0
Quinqueloculina stelligera	13	2	30	2	0	0	4	0	0	0	0	0
Rectuvigerina elongatastriata	0	0	0	0	0	0	1	0	0	0	0	0
Rosalina globularis	326	38	709	37	386	47	594	41	26	22	0	0
Rosalina sp. 1	1	0	2	0	3	0	1	0	0	0	0	0
Sigmoilina costata	7	1	12	1	1	0	10	1	0	0	0	0
Spiroloculina ornata	3	0	4	0	0	0	2	0	0	0	0	0
Trifarina angulosa	0	0	0	0	0	0	0	0	0	0	0	0
Triloculina adriatica	0	0	0	0	0	0	1	0	0	0	0	0
Triloculina marioni	0	0	0	0	0	0	1	0	0	0	0	0
Triloculinella dilatata	112	13	161	8	67	8	50	3	1	1	0	0
Uvigerina sp. 1 of Cimerman and Langer	0	0	1	0	1	0	0	0	0	0	0	0
Vertebralina striata	3	0	6	0	6	1	6	0	0	0	0	0
Indeterminate hyaline sp. 7	1	0	1	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 11	0	0	0	0	0	0	1	0	0	0	0	0
Indeterminate porcelaneous sp. 4	1	0	1	0	0	0	7	0	0	0	0	0
Indeterminate porcelaneous sp. 5	1	0	3	0	0	0	20	1	1	1	0	0
Indeterminate porcelaneous sp. 8	5	1	7	0	4	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 10	0	0	0	0	0	0	1	0	0	0	0	0
Indeterminate porcelaneous sp. 11	0	0	1	0	1	0	1	0	0	0	0	0
Indeterminate porcelaneous sp. 16	0	0	0	0	0	0	1	0	0	0	0	0
Indeterminate porcelaneous sp. 18	0	0	0	0	0	0	0	0	1	1	0	0
Indeterminate porcelaneous sp. 27	0	0	1	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 31	1	0	49	3	56	7	118	8	14	12	0	0
Indeterminate porcelaneous sp. 32	1	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 33	1	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 34	0	0	1	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 35	1	0	26	1	12	1	15	1	3	3	0	0
Indeterminate porcelaneous sp. 36	1	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 37	3	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 38	1	0	0	0	0	0	0	0	0	0	0	0

Table 4.3 continued:

Species	Low pH 1		Low pH 2		Low pH 3		Low pH 4		Very Low pH 1		Very Low pH 2		Very Low pH 3		Very Low pH 4	
		%		%		%		%		%		%		%		%
Adelosina longirostra	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Affinetrina gualtieriana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ammonia sp. 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ammonia sp. 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bolivina difformis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bolivina pseudoplicata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bolivina striatula	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brizalina sp. 2 of Cimerman and Langer	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bulimina sp. 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cornuspira involvens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium aculeatum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium macellum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 5 of Cimerman and Langer	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fursenkoina acuta	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Haynesina depressula	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lobatula lobatula	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Massilina gualtieriana	1	50	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Miliolinella subrotunda	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Peneroplis pertusus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Peneroplis planatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pileolina patelliformis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pileolina patelliformis with float chamber	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Planorbulina mediterranensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudotriloculina rotunda	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina annectens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina auberiana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina bosciana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina stelligera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rectuvigerina elongatastriata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rosalina globularis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rosalina sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sigmoilina costata	1	50	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Spiroloculina ornata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Trifarina angulosa	0	0	0	0	0	0	0	0	0	0	1	100	0	0	0	0
Triloculina adriatica	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Triloculina marioni	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Triloculinella dilatata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Uvigerina sp. 1 of Cimerman and Langer	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vertebralina striata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate hyaline sp. 11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 35	0	0	0	0	0	0	0	0	1	100	0	0	0	0	0	0
Indeterminate porcelaneous sp. 36	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 37	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 38	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

There was a reduction in the number of species from the reference site (Ref 2) to the lowest pH area. The mean number of species decreased from 37 at Ref 2 to one in the lowest pH area (Figure 4.5). The mean number of individuals also decreased across the gradient from 1269 at Ref 2 to just one in the lowest pH area. The extremely low number of individuals in the low pH and very low pH site (many samples had no individuals) meant that statistical analysis was not conducted (Alve, 1995b). Three out

of the four individuals that were found in the mid and low pH samples were porcelaneous. No agglutinated foraminifera were found in any of the samples. The most abundant species was *R. globularis* with a total of 2041 individuals amounting to 39% of the total assemblage.
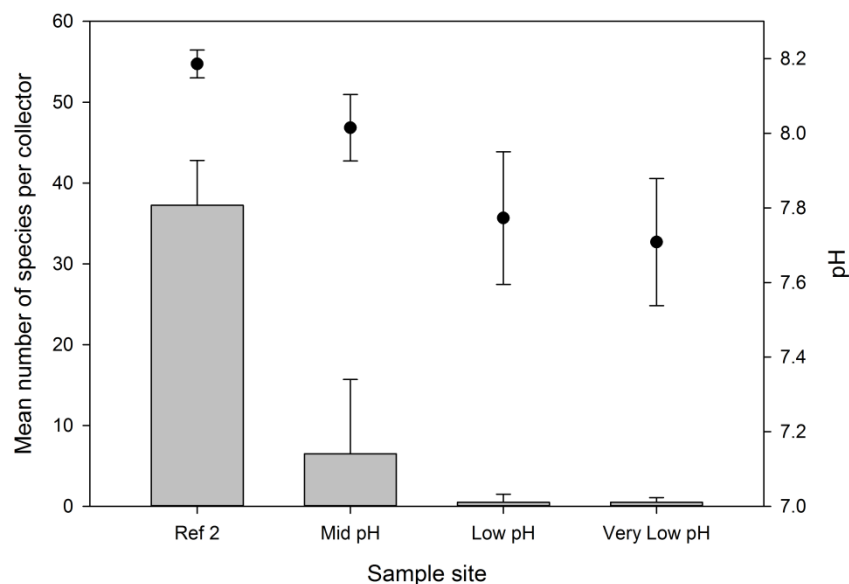

Figure 4.5: Mean (± 1 SD, n = 4 except for Mid pH where n = 2) number of species (grey bars) of foraminifera belonging to the total assemblage found on artificial collectors placed offshore Vulcano in May 2010 with mean (± 1 SD, n = 8 - 11) pH (filled circles) between May 2011 and May 2013.

4.3.3 September 2011

When retrieving the artificial collectors, all three replicates from sample site Mid pH were missing and one replicate from sample site Very Low pH was also missing (Table 4.4).

Table 4.4: Sample site names and the number of replicates that were still in place upon collection of artificial collectors placed along a shallow water CO_2 gradient off Vulcano in September 2011.

Sample Site	Number of replicates collected
Ref 2	3
Ref 1	3
High pH	3
Mid pH	0
Low pH	3
Very Low pH	2

There were a total of 383 stained individuals belonging to 20 species (Table 4.5). No stained foraminifera were found in artificial collectors that had been placed in the Low or Very Low pH site (Figure 4.6). At the reference site, there was a wide variation in the percentage of individuals that were living, ranging from 7% to 86%. The most abundant species was *Vertebralina striata* with a total of 182 individuals amounting to 48% of the stained assemblage. A one-way ANOSIM test revealed that there were no statistically significant differences in the species assemblage between Ref 2, Ref 1 and High pH (Global R statistic = 0.16, p = 0.225).

Table 4.5: A list of the species of benthic foraminifera found within the stained (living) assemblage from artificial collectors placed along the CO_2 gradient at Vulcano in September 2011. Numbers found on the artificial collector are reported along with the percentage contribution.

	Species	Ref 2 A	%	Ref 2 B	%	Ref 2 C	%	Ref 1 A	%	Ref 1 B	%	Ref 1 C	%	High pH A	%	High pH B	%	High pH C	%	Low pH A	%	Low pH B	%	Low pH C	%	Very Low pH A	%	Very Low pH B	%
Affinetrina gualtieriana	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ammonia sp. 3	0	0	1	1	0	0	0	0	4	8	8	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bolivina pseudoplicata	0	0	0	0	0	0	0	0	1	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bulimina sp. 3	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium macellum	0	0	2	2	0	0	0	0	4	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elphidium sp. 5 of Cimerman and Langer	0	0	0	0	2	3	0	0	0	0	1	1	0	0	0	0	3	12	0	0	0	0	0	0	0	0	0	0
Fursenkoina acuta	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lobatula lobatula	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Miliolinella subrotunda	0	0	0	0	0	0	0	0	1	2	10	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Peneroplis pertusus	0	0	15	16	14	19	0	0	0	0	0	0	0	0	1	50	1	4	0	0	0	0	0	0	0	0	0	0
Peneroplis planatus	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pileolina patelliformis	0	0	1	1	3	4	1	6	11	21	19	19	0	0	0	0	6	24	0	0	0	0	0	0	0	0	0	0
Planorbulina mediterranensis	0	0	2	2	1	1	2	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina auberiana	0	0	0	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quinqueloculina bosciana	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rosalina globularis	1	8	14	15	17	23	1	6	3	6	23	23	0	0	0	0	3	12	0	0	0	0	0	0	0	0	0	0
Triloculinella dilatata	0	0	9	10	1	1	0	0	1	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vertebralina striata	12	92	47	51	34	45	14	78	25	48	34	33	2	100	1	50	12	48	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 4	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indeterminate porcelaneous sp. 31	0	0	0	0	0	0	0	0	1	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
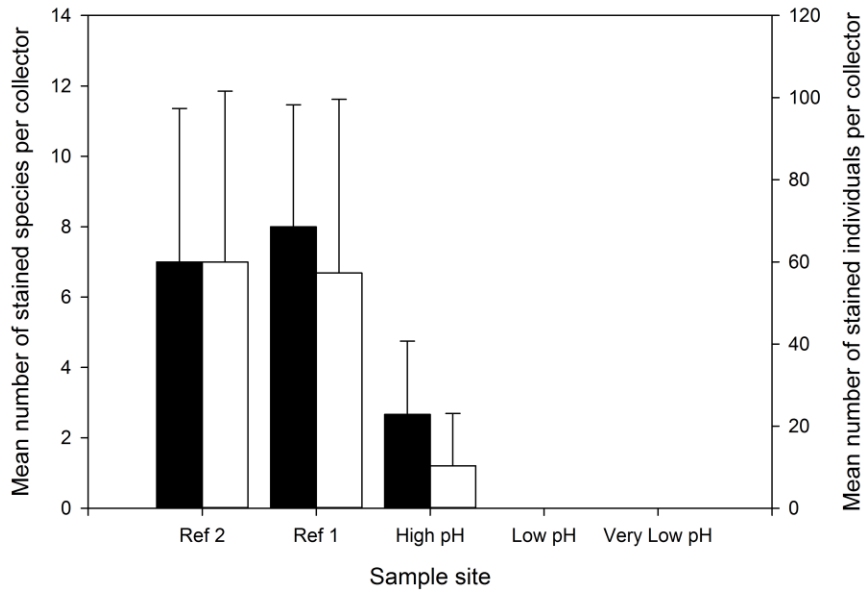
Figure 4.6: Mean (± 1 SD, n = 3 except for Very Low pH where n = 2) number of living (stained) species and individuals of foraminifera found on artificial collectors placed offshore Vulcano in September 2011. Black bars: number of species, white bars: number of individuals.

4.3.4 Transplant experiment

At the end of the transplant experiment many of the collectors were missing (Table 4.6). One replicate was missing from the reference site control, the mid pH control and the samples that had been transferred from the reference site to the mid pH site. This meant that the only set of samples with three replicates were the collectors that had been transferred from Mid pH to Ref 2. As a result, no statistical analysis was conducted on the data, but the results provide an indication as to what may be expected with future ocean acidification and provide the basis for follow-up research.

Table 4.6: Sample names and corresponding treatment for the recruitment pad transplant experiment conducted between May and June 2013. Missing means that at the end of the experiment, these collectors were no longer in place.

Sample Name	Treatment	
Ref 2 NT 1	Reference site control	
Ref 2 NT 2	Reference site control	
Ref 2 NT 3	Reference site control	Missing
Ref 2 TR 1	Transplant from Mid pH to Reference site 2	
Ref 2 TR 2	Transplant from Mid pH to Reference site 2	
Ref 2 TR 3	Transplant from Mid pH to Reference site 2	
Mid pH NT 1	Mid pH control	
Mid pH NT 2	Mid pH control	
Mid pH NT 3	Mid pH control	Missing
Mid pH TR 1	Transplant from Reference site 2 to Mid pH	
Mid pH TR 2	Transplant from Reference site 2 to Mid pH	
Mid pH TR 3	Transplant from Reference site 2 to Mid pH	Missing

There were extremely low numbers of stained individuals with a total of 19 individuals belonging to four species (*Bolivina pseudoplicata*, *Peneroplis pertusus*, *Peneroplis planatus* and *Daitrona* sp.) (Table 4.7). All of the peneroplids found were thought to be adults due to their large size. There were a total of five miliolid individuals in the stained (living) assemblage. All of these individuals were found in samples collected from the reference site (four were from the reference site controls and one was from a sample that had been transplanted from Mid pH to Ref 2). There were 13 agglutinated individuals. All of these were found in samples that had been transplanted from the reference site to the mid pH site. As a result of the low number of individuals, no further analysis was conducted on the stained (living) assemblage.

Table 4.7: A list of the species of benthic foraminifera found within the stained (living) assemblage from the artificial collector transplant experiment conducted along the CO_2 gradient at Vulcano May – June 2013. Numbers found on the artificial collector are reported along with the percentage contribution.

Species	Ref 2 NT 1	%	Ref 2 NT 2	%	Ref 2 TR 1	%	Ref 2 TR 2	%	Ref 2 TR 3	%	Low pH NT 1	%	Low pH NT 2	%	Low pH TR 1	%	Low pH TR 2	%
Bolivina pseudoplicata	0	0	0	0	0	0	0	0	1	14	0	0	0	0	0	0	0	0
Daitrona sp.	0	0	0	0	0	0	0	0	6	86	0	0	0	0	3	100	4	100
Peneroplis pertusus	2	50	0	0	1	100	0	0	0	0	0	0	0	0	0	0	0	0
Peneroplis planatus	2	50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

4.3.5 SEM

Sixty-five individuals were examined under the SEM (22 from May 2010 and 43 from September 2011). Two of the individuals were examined under the SEM because they appeared to have chambers when viewed under the light microscope, but their wall was soft when touched with a fine paint brush. The calcareous test of these individuals appears to have dissolved with just the inner organic lining remaining (Figure 4.7). A similar occurrence of natural dissolution, where the calcareous test dissolved, exposing the organic lining, has been reported previously by Murray and Alve (1999).

Figure 4.7: SEM image of an individual with the calcareous test dissolved and the inner organic lining remaining. Specimen found in an artificial collector from sample site Low pH placed offshore Vulcano in May 2010. Scale bar: 50 µm.

4.4 Discussion

There was a reduction in the number of species of foraminifera on artificial collectors as pCO$_2$ increased from reference sites to high pCO$_2$ areas near to the seeps. No foraminifera (either stained or unstained) were found in the artificial collectors from the lowest pH areas. The hypothesis was supported; there was a shift in the assemblage found on artificial collectors placed along a gradient of overlying seawater acidification. The absence of foraminifera in the lowest pH areas suggests that even dead individuals experienced post-mortem dissolution, were not washed into the artificial collectors in the first place, or washed off before collection.

Previous work examining the settlement of benthic foraminifera on artificial collectors across a CO_2 gradient off Ischia, Italy, found similar patterns (Cigliano et al., 2010). A total of 239 individuals were found, belonging to 11 taxa. *Elphidium aculeatum* was the dominant species. This species had been reported previously in sediment samples collected across the CO_2 gradient off Ischia (Dias et al., 2010), suggesting that it was a locally dominant taxon. There were significantly fewer individuals and taxa in the low pH conditions (pH ~ 7.4) compared to reference sites (pH ~ 8.1) (Cigliano et al., 2010). In the lowest pH areas, no individuals were found in sample sites from the north side of Castello d'Aragonese and only five were found from the south side. Cigliano et al. (2010) found fewer individuals than the current study, even though sampling took place during the spring. The reason for the lower number of individuals found by Cigliano et al. (2010), however, could be due to the fact that they used a 100 µm sieve instead of a 63 µm sieve. This means that many of the smaller individuals were probably washed away. Cigliano et al. (2010) did not use a staining technique, so there is no way to compare the number of living individuals found.

In this study the loss of individuals from the artificial collectors occurred where mean pH fell from ~ 8.01 to ~ 7.77 corresponding to a change in the mean Ω_{Calc} from 4.06 to 2.89. Sudden shifts in community assemblages have been identified at shallow water CO_2 seeps before. Hall-Spencer et al. (2008) found a major ecological tipping point at a mean pH of 7.8 – 7.9 at Ischia where most calcified organisms disappeared from the assemblage and Dias et al. (2010) found no calcareous foraminifera below a mean pH of ~ 7.6 at Ischia, where agglutinated foraminifera dominated the assemblage. Similarly, at CO_2 seeps off Papua New Guinea, no foraminifera were found at sites with a mean pH below ~ 7.9 and even sites with higher pH (mean pH ~8.00) contained foraminifera with pitted or eroded tests (Fabricius et al., 2011). Near to the CO_2 seeps with mean pH below ~7.9, the symbiont-bearing foraminifera *Marginopora vertebralis* was absent from seagrass blades, but was present in densities of over 1000 m^{-2} at nearby reference sites off Papua New Guinea (Uthicke and Fabricius, 2012). The

decline in non-calcifying taxa was less steep, but at pH ~7.9 they were also absent (Uthicke et al., 2013).

There were more living foraminifera in the artificial collectors that were placed along the gradient in May 2010 compared to September 2011. This could be due to the spring bloom, which usually occurs in the Mediterranean Sea between the middle of March and the end of May (Vidussi et al., 2000, Andersen et al., 2001, Thyssen et al., 2014) and colonisation is known to depend upon the time of year (Alve, 1995b). Despite the differences in the numbers of individuals between May and September, the overall pattern was still the same; there were few foraminifera in the lowest pH areas. Alve (1995b) also reported consistent distributional patterns despite studying re-colonisation at four different seasons. The artificial collectors were left in the water for different lengths of time in May and September, so any direct comparisons between the seasons are not possible.

There was large within-site variability. The reasons for this are not fully understood, but could be due to sampling bias. It is likely that the artificial collectors were moved by different amounts during collection, which may have resulted in the loss of different quantities of material from replicates. It could also be that whilst in place, certain replicates were subject to stronger currents than others, or, currents with variable orientation; the currents are known to be variable at Vulcano (Lucila et al., 1996, Boatta et al., 2013, Vizzini et al., 2013, Milazzo et al., 2014).

The extremely low number of living individuals in the transplant experiment suggests that the artificial collectors were not left in place long enough for foraminifera to settle on them. The low number of individuals could also be a result of the timing of the deployment. The experiment was conducted between May and June, which was probably too late to collect the high number of individuals associated with the spring bloom (Vidussi et al., 2000, Andersen et al., 2001, Thyssen et al., 2014).

The most abundant species in the living assemblage in May 2010 was *R. globularis* and in September 2011 it was *V. striata*. These, and many of the other species, have previously been found as epiphytes around Vulcano (Langer, 1993) and *V. striata* and *R. globularis* were the most abundant species found on *Padina pavonica* thalli in September 2011 (see Chapter 3). This suggests that the foraminifera may be washed up onto the artificial collectors from the nearby macroalgae, rather than from the sediment. This would also fit with the absence of foraminifera in the surrounding sediment, as shown in the previous chapter. Large adult foraminifera, such as *V. striata* and peneroplids (which were also found on the nearby macroalgae), are not normally transported as suspended load (Murray, 1991a), but epiphytic foraminifera can be easily removed as suspended load (Davaud and Septfontaine, 1995). If this were the case, then it could be changes in macroalgae along the gradient that are driving changes in the foraminiferal assemblages found in the artificial collectors.

No agglutinated foraminifera were found in any of the samples examined from May 2010 or September 2011. This finding is surprising, as previous investigations along shallow water CO_2 seeps have found an increase in the proportion of agglutinated foraminifera as pH reduces (Dias et al., 2010). Cigliano et al. (2010), however, found no agglutinated foraminifera in artificial collectors that had been placed along a CO_2 gradient for one month at Ischia. A total of 25 agglutinated foraminifera were found in the samples from the transplant experiment, 13 of which were stained. This is surprising given that no agglutinated foraminifera were found in samples from May 2010 or September 2011. There were too few agglutinated foraminifera to be able to determine if there was a significant pattern in the abundance of agglutinated foraminifera between the reference sites and mid pH site.

No planktonic foraminifera were found in any of the samples examined. This was also the case for artificial collectors placed off Ischia, Italy (Cigliano et al., 2010). The

artificial collectors were probably too near to the coast for sufficient numbers of planktonic foraminifera (Arnold and Parker, 1999). *Rosalina* spp., which were found in the samples, are known to have a planktonic stage for which they build a float chamber (Alve, 1999). None of the *Rosalina* found in this study, however, had a float chamber.

The low numbers of living individuals was not surprising given that the vast majority of transported benthic foraminifera are reported to be dead (Alve, 1995b). There was, however, a lot of variation in the percentage of living individuals. This raises questions as to the reliability of using rose Bengal to determine living individuals. There is much debate over the use of the rose Bengal staining technique (Walton, 1952, Murray, 1991a, Bernhard, 2000, Murray and Bowser, 2000, Bernhard et al., 2006) and the length of time for which samples have been left in the rose Bengal stain has varied greatly amongst researchers. A staining time of three hours was used in this study following the method used by Sadri et al. (2011). As a means of standardising the rose Bengal staining technique, Schönfeld et al. (2012) recommended using a staining time of at least 14 days. The staining time of three hours, as used in this study, is much less than this and could mean that the foraminifera did not have sufficient time to pick up the stain (Schönfeld et al., 2012). This would lead to an underestimation of the number of living individuals. For this particular investigation, where a biological process was being examined, it was considered more appropriate to underestimate, rather than overestimate, the number of living individuals.

The factors controlling the pattern of colonisation by benthic foraminifera are still poorly understood (Alve, 1995b) and distributions are very patchy in sediment (Murray, 1991b). The majority of foraminifera found in the artificial collectors were adults. Longer living species, such as *Planorbulina mediterranensis*, which can have life spans of up to one year (Langer, 1993), would not have had time to grow to adult size during the short time for which the artificial collectors were in place. They, therefore, must have been transported as adults. This this is surprising as larger living foraminifera are

known to be able to withstand very high current velocities, which suggests that they are not easily entrained (Alve, 1999).

Goldstein and Alve (2011) found that the exposure of collection sites was the most important factor in determining species richness. Differences in wave and current strength along the gradient could, therefore, be a reason for the differences in the number of species. Currents are mostly wind-driven at Vulcano and known to be strong, but variable (Lucila et al., 1996, Boatta et al., 2013, Vizzini et al., 2013, Milazzo et al., 2014) at all sample sites. The low number of stained individuals suggests that the foraminifera were being transported as dead tests. Once foraminifera die they can no longer attach using their pseudopodia and are easily entrained into the water column along with other sedimentary particles (Schönfeld et al., 2012). The absence of foraminifera from the low pH areas, however, suggests that even dead individuals that are transported onto the collectors experience post-mortem dissolution once in the high CO_2 conditions.

Vizzini et al. (2013) warn against relating biological changes at volcanic CO_2 seeps solely to pH. They found trace element enrichment along the CO_2 gradient at Vulcano and warn that this could be a driver of any biological changes seen across this gradient. The presence of heavy metals could be a potential cause of the absence of foraminifera in the lowest pH areas. Heavy metal pollution, however, causes test deformation in foraminifera (Olugbode et al., 2005) and no consistent test deformation was found in the calcareous foraminifera that were present in the low pH areas.

Many samples were lost during the experiments, this suggests that the artificial collectors were not attached securely enough. Attaching the fishing line to an underwater moored object would be beneficial, but at the time of conducting the experiment such objects were not available. In addition, the moored objects could then add a confounding factor and would make the placement of replicates difficult.

Although shallow water CO_2 seeps are extremely useful to ocean acidification studies, they are not ideal predictors (Hall-Spencer et al., 2008). This is in part because of the proximity of populations not affected by ocean acidification that may be able to migrate into the area. This is particularly the case when examining the settlement of benthic organisms. In the future there are unlikely to be areas unaffected by ocean acidification, from which the foraminifera can recruit. The pH variability at the seeps also means that organisms near to the seeps experience periods of high as well as low pH.

4.5 Conclusions

There was a reduction in the number of species and individuals of benthic foraminifera found on artificial collectors placed along a shallow water CO_2 gradient. The low number of stained (living) individuals suggests that it was mainly dead foraminifera, which are transported as suspended load, that were being washed up onto the artificial collectors from outside of the bay or from the surrounding macroalgae. The absence of foraminifera from the lowest pH areas indicates that the dead foraminifera might be undergoing post-mortem dissolution in the high CO_2 conditions.

Chapter 5: The impact of ocean acidification on major and trace element concentrations and boron isotopic composition of benthic foraminifera

Abstract

The boron isotopic composition of foraminiferal tests can be used as a palaeo-proxy for ocean pH. The relative proportions of the two main species of boron (boric acid and the borate ion) are known to change according to the pH of the seawater and this is reflected in the isotopic ratio of foraminiferal tests. This chapter examines the trace element and boron isotopic composition of benthic foraminifera collected across natural gradients in pCO$_2$ caused by shallow water CO$_2$ seeps off shore Papua New Guinea and the Island of Vulcano in Italy. There was a decrease in δ^{11}B as mean pH decreased from 7.99 – 7.82 for smaller (250 – 500 µm) *Amphistegina lessonii*, but not for larger (>500 µm) *Amphistegina lessonii* or *Calcarina spengleri*. The small sample size and pH variability at the seep sites makes it hard to draw any firm conclusions, but suggests that in the larger foraminifera, where there are more symbionts, photosynthetic activity dominates over ambient pH. This has implications for the choice of species when using boron isotopes to reconstruct ocean pH and shows the potential that shallow water gas seeps can have in validating the boron isotope paleo-pH proxy.

5.1 Introduction

Geochemical records of environmental change are frequently used to determine past environmental conditions. The trace element composition of foraminiferal tests and the boron isotopic composition of marine biogenic carbonates (δ^{11}B$_{carb}$) are examples of such geochemical records (Lea, 1999, Pearson and Palmer, 1999, 2000). Trace element composition can be used to infer a variety of past oceanographic conditions (Lea, 1999) and the boron isotopic composition of biogenic calcite has been used as a palaeo-proxy for ocean pH (e.g. Spivack et al., 1993, Sanyal et al., 1995; 1997, Pearson and Palmer, 2000, Palmer and Pearson, 2003).

The elemental composition of foraminiferal tests reflects seawater composition and the physical and biological conditions (i.e. vital effects) present during precipitation. Many

foraminiferal tests are composed of pure calcite, normally about 99% by weight $CaCO_3$ (Lea, 1999). Major and trace elements make up the remaining 1% and occur in calcitic foraminiferal tests at individual abundances of 0.25% or less (Lea, 1999). Major and trace elements can be divided into different groups according to their use as a proxy (Table 5.1).

Table 5.1: Major and trace elements in benthic foraminiferal calcite. The concentrations given are normal observed ranges. From Lea (1999).

Element	Ion in calcite	Concentration in benthic foraminifera (mol/mol Ca)	Proxy
Calcium	Ca^{2+}	1	
Carbon	CO_3^{2-}	1	
Magnesium	Mg^{2+}	$0.5 – 1.0 \times 10^{-3}$	Temperature
Sodium	Na^+		Salinity
Strontium	Sr^{2+}	$0.9 – 1.6 \times 10^{-3}$	Seawater chemistry, pressure
Boron	$B(OH)_4^-$	60×10^{-6}	pH
Lithium	Li^+		Seawater chemistry
Manganese	Mn^{2+}	$1 – 500 \times 10^{-6}$	Diagenesis
Barium	Ba^{2+}	$1.5 – 5 \times 10^{-6}$	Alkalinity
Neodymium	Nd^{3+}		Seawater chemistry
Cadmium	Cd^{2+}	$0.02 – 0.25 \times 10^{-6}$	Phosphate
Uranium	UO^{2+}		Carbonate ion

The first studies of trace elements in foraminifera tests used Sr, Mg and Na to establish Cenozoic ocean chemical histories (Bender et al., 1975, Graham et al., 1982). Further work on trace elements demonstrated that foraminiferal Cd could be used to determine past nutrient phosphate concentrations (Boyle, 1981, Boyle and Keigwin, 1982, Hester and Boyle, 1982).

More recent investigations have continued to use trace elements to determine past environmental conditions (Lea, 1999). For instance, Lea (1995) examined Ba/Ca and Cd/Ca of benthic foraminifera from a deep ocean core in the Cape Basin. Results indicated small variations in bottom water nutrient concentrations in Circumpolar Deep Water over the last 450 000 years. The reconstruction of past surface ocean phosphate concentrations from Cd/Ca ratios of planktonic foraminifera found similar phosphate

utilisation in the Subantarctic during the Last Glacial Maximum to that of today (Elderfield and Rickaby, 2000). Results suggested much smaller utilisation in the polar Southern Ocean which is thought to be a result of expansion of glacial sea ice.

Another example is the Mg/Ca ratio of planktonic foraminifera which was established as a reliable temperature proxy by Nürnberg et al. (1996) and used to estimate glacial-interglacial sea surface temperature (Mashiotta et al., 1999). Results suggested that sea surface temperature was about 2.5 °C cooler in the subantarctic Pacific and 4.0 °C cooler in the subantarctic Indian Ocean during glacials (Mashiotta et al., 1999). Elderfield and Ganssen (2000) examined Mg/Ca ratios of eight species of planktonic foraminifera and found that the variation of Mg/Ca ratios with temperature was similar for all eight species. Temperatures over the Last Glacial Maximum that were reconstructed from Mg/Ca ratios agreed with two other palaeotemperature proxies; faunal abundance and alkenone saturation. Lea et al. (2000) examined Mg/Ca of planktonic foraminifera from equatorial Pacific sediment cores and found that tropical Pacific sea surface temperatures were 2.8 ± 0.7 °C colder than present during the Last Glacial Maximum.

It is important to determine the ocean carbonate system in the past, as this is essential for understanding past changes in ocean acidification, pCO$_2$ and climate (Rae et al., 2011). In addition, if more information is known about ocean acidification events in the past, these can be used to inform what may happen in the future (Hönisch et al., 2012). Boron isotopes are of particular interest in ocean acidification studies as they are used as a palaeo-proxy for ocean pH. In seawater there are two main species of boron; the borate ion (B(OH)$_4^-$) and boric acid (B(OH)$_3$). The concentration of these two species changes according to the pH; borate ion concentration decreases as pH decreases. There are two stable isotopes of boron; ^{10}B and ^{11}B. The ratio of these is constant in seawater, but there is an isotopic fractionation between the two aqueous species of boron. This isotopic fractionation arises because there is a stronger bonding of boron in

boric acid than in the borate ion (when heavy isotopes are held in strong bonds, molecular vibrational energies are lowered most, lowering overall system energy). This leads to each of the species of boron having different $\delta^{11}B$ values (defined as the fractional difference between ^{11}B and ^{10}B). The offset between the two is 27 permil and constant over the whole pH range (Klochko et al., 2006). As the relative abundance of $B(OH)_4^-$ and $B(OH)_3$ is pH dependent, it means that $\delta^{11}B$ is also pH dependent (Sanyal et al., 1996). Therefore, if the boron isotopic composition in a substrate that incorporates only one of the boron species ($B(OH)_3$ or $B(OH)_4^-$) can be measured, through knowing the fractionation factor (α) (isotopic fractionation between $B(OH)_3$ and $B(OH)_4^-$) and the isotopic composition of boron in seawater, the pH can be calculated.

The use of the $\delta^{11}B$ proxy is based on the assumption that only the $B(OH)_4^-$ species is taken up by foraminiferal calcite and that no isotopic fractionation occurs during incorporation into shells (Hemming and Hanson, 1992, Yu et al., 2010). If $B(OH)_4^-$ were the only species to be incorporated, a direct correlation between B/Ca and seawater pH would be expected (Yu et al., 2010). The suggestion that $B(OH)_4^-$ adsorbs to growing carbonate crystals (Hemming et al., 1995, Hemming et al., 1998) and is the only species incorporated into the carbonate structure (Hemming and Hanson, 1992) arose from early analysis of marine carbonates in relation to boron isotopes. These studies found a narrow range in boron isotopic composition from a wide variety of samples (e.g. corals, bivalves, ooids) and that $B(OH)_4^-$ was incorporated into the carbonate skeleton by substitution of bicarbonate ions (Hemming and Hanson, 1992). Subsequent laboratory studies on synthetic calcite (Hemming et al., 1995) has given strong support for the hypothesis that $B(OH)_4^-$ is the only species being incorporated into the carbonate structure. A recent study, however, suggests that both $B(OH)_3$ and $B(OH)_4^-$ could be incorporated into biogenic carbonates (Klochko et al., 2009). If true, this would complicate the use of $\delta^{11}B$ as a seawater pH proxy (Yu et al., 2010).

Application of the $\delta^{11}B$ proxy also requires accurate knowledge of the isotopic fractionation (α) between $B(OH)_3$ and $B(OH)_4^-$. The size of this fractionation can be estimated from measured or theoretical estimations of vibrational frequencies and zero point energies. There has been much debate over α in the literature (Kakihana et al., 1977, Liu and Tossell, 2005, Pagani et al., 2005, Zeebe, 2005, Klochko et al., 2006), adding further complication to the use of the boron isotope proxy. The fractionation factor was first reported as 1.0194 by Kakihana et al. (1977), but calculations were based on an incorrectly allocated vibrational frequency (Rustad and Bylaska, 2007). More recent work has given values of ~ 1.025 – 1.035 (Oi et al., 1991, Oi, 2000, Liu and Tossell, 2005, Zeebe, 2005, Rustad et al., 2010) and a recent determination by Klochko et al. (2006) gave a value of 1.0272 ± 0.0006 at 25 °C. This value is now widely accepted.

The boron isotope palaeo-pH proxy has been used to: reconstruct seawater pH through glacial-interglacial cycles (Sanyal et al., 1995, Sanyal et al., 1997, Hönisch and Hemming, 2005), interpret changes in upwelling over glacial-interglacial cycles (Sanyal and Bijma, 1999, Palmer and Pearson, 2003), create pH depth profiles (Palmer et al., 1998), reconstruct pH over the last 21 and 60 million years (Spivack et al., 1993, Pearson and Palmer, 1999, 2000) and determine changes in pCO$_2$ and ice volume during the middle Miocene (Foster et al., 2012).

The boron isotopic ratio of foraminifera tests was used to estimate that the deep Atlantic and Pacific oceans had a pH 0.3 ± 0.1 units higher during the last glaciation (Sanyal et al., 1995). During the penultimate glaciation, pH was similarly 0.3 ± 0.1 units higher in the equatorial Pacific Ocean compared to the present day deep ocean (Sanyal et al., 1997). Hönisch and Hemming (2005) examined boron isotope data of planktonic foraminifera over two glacial cycles to calculate aqueous pCO$_2$ from the pH data and compared this with the known atmospheric pCO$_2$ from ice core data. The data

showed a strong correlation with the Vostok pCO$_2$ record (Hönisch and Hemming, 2005).

Palaeo-pH reconstructions based on boron isotopic composition of foraminifera revealed that two major upwelling zones behaved differently during the glacial periods compared to today. There was no significant change in surface ocean pH in the eastern Pacific, but the pH of the surface ocean off northwest Africa was 0.2 ± 0.07 units higher during the glacial period compared to the Holocene (Sanyal and Bijma, 1999). This suggests that the eastern equatorial Pacific upwelling system was a larger source of CO$_2$ to the atmosphere during the last glacial period, whereas the area of upwelling off northwest Africa was a significantly smaller source of CO$_2$ (Sanyal and Bijma, 1999). Palmer and Pearson (2003) also found that the western equatorial Pacific was a strong source of CO$_2$ to the atmosphere between 13 800 and 15 600 years ago.

Palmer et al. (1998) examined boron isotopes from planktonic foraminifera that calcified at different water depths in the tropical ocean. Results revealed that pH-depth profiles across the five time windows examined from the middle Miocene to the late Pleistocene were similar to the modern ocean (Palmer et al., 1998). From measuring the boron isotopic ratio of bulk foraminifera samples from a deep-sea sediment core, Spivack et al. (1993) found that 21 million years ago surface ocean pH was 7.4 ± 0.2, but increased to the present day value of approximately 8.2 ± 0.2 about 7.5 million years ago. This suggests that atmospheric CO$_2$ concentrations were much higher 21 million years ago than today, but an independent second proxy for another carbonate chemistry parameter is needed to calculate pCO$_2$. Construction of a pH profile for the middle Eocene tropical Pacific Ocean, from measuring the boron isotopic ratio in contemporaneous planktonic foraminifera, revealed that atmospheric pCO$_2$ was probably similar to modern concentrations (Pearson and Palmer, 1999). Pearson and Palmer (2000) examined boron isotopes of planktonic foraminiferal tests to estimate the surface ocean pH over the past 60 million years. From this they reconstructed

atmospheric CO_2 concentrations and found that from about 60 to 52 million years ago atmospheric CO_2 concentrations were more than 2000 ppm with an erratic decline 55 – 40 million years ago. Atmospheric CO_2 concentrations are thought to have remained below 500 ppm from approximately 24 million years ago (Pearson and Palmer, 2000). Foster et al. (2012) examined the boron isotopic composition of planktonic foraminifera from two deep-sea sites across the middle Miocene Climatic Optimum (MCO). They found that changes in the cyrosphere were well correlated to changes in the concentration of atmospheric CO_2. Absolute values of CO_2 during the MCO were between 350 and 400 ppm and levels either side of the MCO were similar to, or lower than, pre-industrial levels (200 – 260 ppm). They concluded that ice volume and climate variations during the middle Miocene probably involved a more dynamic Antarctic Ice Sheet, but there was also some component of land-based ice in the northern hemisphere.

Foraminifera have been used extensively in both the development and the application of the boron isotope pH-proxy. They are useful to such studies in that they occur worldwide, have an excellent fossil record, are available in relatively large numbers and the occurrence of planktonic and benthic forms allow differences between the surface and the deep ocean to be examined. In order to establish the pH dependence of the boron isotopic fractionation, experimental studies have been conducted where foraminifera have been cultured in the laboratory under different pH conditions (Sanyal et al., 1996, Sanyal et al., 2001). Sanyal et al. (1996) cultured the planktonic foraminifera *Orbulina universa* at four different pH values (7.0 ± 0.05, 8.15 ± 0.05, 8.60 ± 0.05 and 9.00 ± 0.10) in order to determine the pH dependence of boron isotope fractionation between seawater and planktonic foraminifera. The boron isotopic composition of the foraminifera showed a clear relationship with pH; heavier boron isotopic composition occurred at higher pH. Sanyal et al. (2001) carried out inorganic precipitation experiments and calibrated another planktonic foraminifera, *Globigerinoides sacculifer*, at pH values of 7.6 ± 0.05, 8.20 ± 0.05 and 8.60 ± 0.05.

Together these studies show that foraminifera follow the theoretical curve for borate, but that the boron isotopic values of *G. sacculifer* were offset from that of *O. universa* by ~3‰ and did not lie exactly on the theoretical curve (assuming that the fractionation of the two boron species was 19.4 permil). Differences in the isotopic fractionation between species were explained on the basis of vital effects, where respiration and photosynthesis appear to be the key physiological parameters controlling species-specific vital effects (Hönisch et al., 2003). Zeebe et al. (2003; 2008) used a numerical model showing that these biological processes are responsible for the observed offsets, but do not compromise the pH proxy.

Studies examining the isotopic composition of benthic foraminifera collected from natural environmental settings have also been conducted (Hönisch et al., 2008, Rae et al., 2011). Rae et al. (2011) found a predictable variation of $\delta^{11}B$ with pH across a wide range of benthic foraminiferal species when analysed under Multi-Collector Inductively Coupled Plasma Mass Spectrometry (MC-ICPMS). Epifaunal benthic foraminifera can have a more stable habitat resulting in fewer ecological complications than those associated with the examination of planktonic species (Rae et al., 2011). Infaunal species typically have lower $\delta^{11}B$ than epifaunal species and the offsets can be highly variable due to strong pH gradients in the sediment. Rae et al. (2011), therefore, argue against the use of infaunal foraminifera to reconstruct bottom-water pH. Deep-sea epifaunal species, however, can still experience pH conditions offset from bottom water pH due to decomposition of organic matter following the spring phytoplankton bloom.

In addition to the use of foraminiferal $\delta^{11}B$, B/Ca ratios of foraminiferal calcite can be used to reconstruct pH. Yu et al. (2010) examined $\delta^{11}B$ and B/Ca in the benthic foraminifera *Cibicidoides wuellerstorfi* from the Caribbean Sea over the last 160 000 years. Consequently, they argue that both proxies are accurate in estimating deep ocean pH. In an examination of benthic foraminifera from Late-Holocene samples from the Atlantic, Rae et al. (2011) found that the B/Ca of individual *Cibicidoides* species

showed a good correlation with changes in carbonate ion concentration, but the B/Ca of different species morphotypes varied considerably. As a result, they argue that δ^{11}B provides a more robust proxy.

In order to further develop the δ^{11}B proxy, it is important to examine modern biocalcifying organisms that have grown in locations where seawater pH is well quantified and calibrate δ^{11}B values against pH (Rae et al., 2011, Anagnostou et al., 2012). Natural CO_2 seeps provide an opportunity to examine the boron isotopic composition of calcareous organisms from a natural environmental setting. This will be a way of testing the reliability of the boron isotopic composition using samples from a natural setting. A study which collected samples across a shallow water CO_2 gradient off the Island of Ischia, Italy, was conducted on the coral *Cladocora caespitosa* (Trotter et al., 2011). Trotter et al. (2011) found a decrease in δ^{11}B$_{carb}$ with decreasing seawater pH, indicating that the boron isotope system is strongly dependent upon pH. Anagnostou et al. (2012) examined the boron isotopic composition of the deep-sea coral, *Desmophyllum dianthus*, collected from ambient pH ranges of 7.57 – 8.05. The δ^{11}B values recorded were greater than those measured for tropical corals and indicate physiological modification of pH in the calcifying space. All calibrations are empirical (i.e. a calibration study provides a relationship between δ^{11}B and pH under a defined set of boundary conditions), but palaeoceanographers typically use those calibrations in other settings (e.g. last glacial).

The aim of this chapter is to examine the major and trace element composition and the boron isotopic composition of benthic foraminifera, collected along natural CO_2 gradients. Differences in δ^{11}B offset between two species of foraminifera and between different size fractions of the same species will also be examined.

It is hypothesised that as seawater pH decreases, $\delta^{11}B_{carb}$ of benthic foraminiferal tests will also decrease. It is also hypothesised that there will be a $\delta^{11}B$ offset between species, as others have shown (e.g. Sanyal et al., 2001).

5.2 Study sites

5.2.1 Vulcano

The Vulcano CO_2 seep study sites were the same as those described in Chapter 3 (Figure 3.5). This site is suitable for ocean acidification research since there are gradients in pCO_2 that lack confounding gradients in salinity, alkalinity or in chemicals such as H_2S (Boatta et al., 2013).

5.2.2 Papua New Guinea

Shallow water gas seeps fringe the D'Entrecastraux Islands, Milne Bay Province, Papua New Guinea (Fabricius et al., 2011). This study site was first used in ocean acidification research by Fabricius et al. (2011) and has since been established as a suitable study site by further investigations (Johnson et al., 2012, Uthicke and Fabricius, 2012, Russell et al., 2013, Uthicke et al., 2013, Fabricius et al., 2014). Samples were collected at the Upa-Upasina Reef (north-western Normanby Island) (Figure 5.1). This was the largest seep site with thousands of bubble streams emerging along a reef slope. The seeps are located along an active tectonic fault line (Fabricius et al., 2014). Seeps were most intense near to the shore at <0.5 m water depth, but were found down to 5 m water depth. Reference sites were located 500 m south of the seep site, with a topography similar to the seep site (Fabricius et al., 2011). Tidal range in the region was >1 m (Russell et al., 2013).
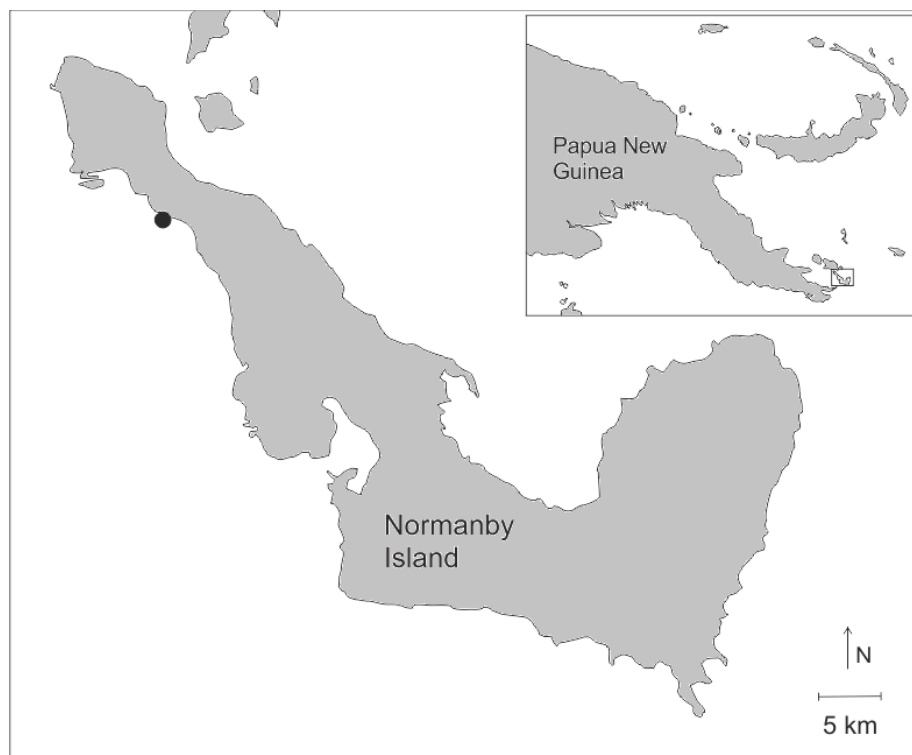
Figure 5.1: Study area off Papua New Guinea. Insert shows Papua New Guinea with box marking Normanby Island. Black circle marks the Upa-Upasina seep site.

5.3 Methods

5.3.1 Sample collection at Vulcano

The foraminiferal samples used for trace element and boron isotope analysis were those that were found as epiphytes on air dried *Padina pavonica* thalli that were collected offshore Vulcano in May 2012. The collection of these samples is described in Chapter 3, Section 3.3.2.3.

5.3.2 Sample collection at Papua New Guinea

Sediment samples were collected at the Upa-Upasina seep site off-shore Papua New Guinea by Dr Sven Uthicke from the Australian Institute of Marine Science in May 2012. Surface sediment samples from the top 1 cm of sediment were collected by SCUBA divers from 13 different sites along the CO_2 gradient. Samples were not stained with rose Bengal, instead they were left to air dry and sent to Plymouth

179

University. For four of the sample sites, two replicates were provided, meaning that a total of 17 samples were sent to Plymouth.

Once in the laboratory, sediment samples were dry sieved through a sieve stack consisting of four sieves with apertures of 500 µm, 250 µm, 125 µm and 63 µm. Each size fraction was weighed. Taking one size fraction at a time, the sample was placed onto a picking tray and examined under a stereo-binocular microscope. Foraminifera were dry picked using a fine paint brush and placed onto micropalaeontological slides.

5.3.3 Selection of species

One species from Vulcano was selected for examination of major and trace elements and boron isotopic composition. The choice of species was limited by the numbers present within the samples and the size of the individuals. Ideally, a large species that occurred in all samples across the CO_2 gradient would have been selected, but very few species were found in all samples. The calcareous species that was the most abundant in the low pH sample sites, therefore, had to be selected for analysis. The species chosen for analysis was an *Elphidium* species, which was found as an epiphyte on *P. pavonica* thalli.

The two most abundant species from the Papua New Guinea samples were chosen for analysis; these were *Amphistegina lessonii* and *Calcarina spengleri.* These are both epifaunal and have diatom symbionts (Uthicke et al., 2013). *Amphistegina lessonii* is often found fixed to filamentous algae or protected areas in small holes or boulders in reef rocks (Hohenegger, 2011). *Calcarina spengleri* is often found in areas with high water motion such as the reef crest (Hohenegger, 2011).

5.3.4 Selection of individuals

Each sample consisted of seven individuals which were lumped together to ensure that there was enough test material for analysis. Seven individuals of *Elphidium* sp. were

selected from sample sites Ref 1, High pH and Low pH from Vulcano (Table 5.2). Seven *C. spengleri* were selected for analysis from a reference site, a mid pH site and a low pH site from Papua New Guinea. Intact specimens that showed no signs of degradation or breakage, with a similar number of spines, were selected. All individuals selected were of a similar size, from the >500 μm size fraction. Seven medium sized *A. lessonii* were selected from the >500 μm size fraction from a reference site, a mid pH site and a low pH site from Papua New Guinea. Seven small *A. lessonii* were selected from the 250 – 500 μm size fraction from the same reference site and low pH site as the medium sized *A. lessonii* and an additional very low pH site.

To provide an indication of individual variation, three individual *A. lessonii* from the low pH site were also selected for examination. These specimens could be examined individually because they were larger (>500 μm) and, therefore, there was more test material to analyse. All *A. lessonii* selected were intact specimens with no signs of degradation or breakage following the recommendations of Hemming and Hönisch (2007). Photographs were taken of the selected individuals before further analysis.

All further analysis was carried out under the help and supervision of Dr James Rae using the facilities for boron isotope analysis run by Dr Gavin Foster, University of Southampton, UK.

Table 5.2: Samples that were analysed for major and trace elements and boron isotopes collected across shallow water CO_2 gradients offshore Vulcano and Papua New Guinea.

Sample number	Location	Sample site	Species	Size fraction	Number of individuals in sample	Analysis conducted
1	Vulcano	Ref 1	*Elphidium* sp.		7	Trace element
2	Vulcano	High pH	*Elphidium* sp.		7	Trace element
3	Vulcano	Low pH	*Elphidium* sp.		7	Trace element
4	Papua New Guinea	Ref	*Calcarina spengleri*	>500 µm	7	Trace element Boron isotopes
7	Papua New Guinea	Mid pH	*Calcarina spengleri*	>500 µm	7	Trace element Boron isotopes
9	Papua New Guinea	Low pH	*Calcarina spengleri*	>500 µm	7	Trace element Boron isotopes
5	Papua New Guinea	Ref	*Amphistegina lessonii*	>500 µm	7	Trace element Boron isotopes
8	Papua New Guinea	Mid pH	*Amphistegina lessonii*	>500 µm	7	Trace element Boron isotopes
10	Papua New Guinea	Low pH	*Amphistegina lessonii*	>500 µm	7	Trace element Boron isotopes
6	Papua New Guinea	Ref	*Amphistegina lessonii*	250 – 500 µm	7	Trace element Boron isotopes
11	Papua New Guinea	Low pH	*Amphistegina lessonii*	250 – 500 µm	7	Trace element Boron isotopes
15	Papua New Guinea	Very Low pH	*Amphistegina lessonii*	250 – 500 µm	7	Trace element Boron isotopes
12	Papua New Guinea	Low pH	*Amphistegina lessonii*	>500 µm	1	Trace element Boron isotopes
13	Papua New Guinea	Low pH	*Amphistegina lessonii*	>500 µm	1	Trace element Boron isotopes
14	Papua New Guinea	Low pH	*Amphistegina lessonii*	>500 µm	1	Trace element Boron isotopes

5.3.5 Cleaning of samples

All preparatory work and analysis was conducted by Dr James Rae in an over-pressured clean laboratory with boron-free HEPA filters (MegaCell ULPA Teflon®).

Boron-free HEPA filters were also used in the mass spectrometry laboratory. Nitrile gloves (Fisher Scientific) were worn throughout sample preparation to reduce contamination. The Milli-Q water system contained a boron-specific Q-Gard TBX1 cartridge. The following methods are those outlined in Rae (2011).

All plastic spin tubes and pipette tips used were acid cleaned prior to use. This involved cleaning in hydrochloric acid (HCL) overnight on a hot plate at 80 °C then rinsing three times with Milli-Q water. Immediately prior to use, tubes and pipette tips were rinsed with 10% suprapure nitric acid (HNO_3) then rinsed three times with Milli-Q.

Teflon vials (Savillex) were cleaned by wiping the outside with acetone then rinsed three times with Milli-Q. The inside of the vials were then wiped with acetone using plastic tweezers wrapped in a clean wipe. Vials were rinsed three times with Milli-Q and washed with 7 M reagent grade HNO_3 overnight on a hot plate at 120 °C. Vials were then rinsed three times with Milli-Q and placed together in a Teflon tub containing 7 M HNO_3 using plastic tweezers. The tub was left on a hot plate overnight at 120 °C. The tub was then rinsed three times with Milli-Q and the vials were removed using plastic tweezers and placed into another tub containing 0.5 M HNO_3. The tub containing the vials was left overnight on a hotplate at 120 °C. The vials were then rinsed with Milli-Q three times, capped and stored in an airtight box. Immediately prior to use, vials were filled with 10% suprapure HNO_3, placed on a hotplate at 120 °C for one hour and then rinsed three times with Milli-Q.

Foraminifera samples were placed under a stereo-binocular microscope and crushed between two acid cleaned glass slides. Between each sample these glass slides were rinsed thoroughly with Milli-Q water and wiped clean with a clean wipe. Care was taken to ensure that all chambers of the foraminifera were cracked open to expose all of the test to cleaning. The tests were not crushed excessively as this results in dust, which is

easily lost during cleaning. The crushed samples were then transferred to acid-cleaned, labelled plastic spin tubes (HDPE centrifuge tubes from Eppendorf).

Clay was removed from the samples by repeat utlrasonication and Milli-Q rinses. Approximately 50 µl of Milli-Q was added to each tube. Samples were then ultrasonicated for 30 seconds. Milli-Q was added to the tube to suspend the fragment-clay mixture. The fragments sank more rapidly than the clay, therefore, the clay could be pipetted out along with the Milli-Q. Approximately 50 µl of Milli-Q was left in the vial. This process was repeated three times.

In order to remove organic matter, oxidative cleaning was conducted with 1% Supra-pure hydrogen peroxide (H_2O_2), buffered with 0.1 M ammonium hydroxide (NH_4OH) (distilled in-house), 250 µl of this solution were added to the samples. The tubes were then placed on a hot plate at 70 ℃ for 30 minutes and were occasionally agitated to eliminate organic matter. After 30 minutes the hydrogen peroxide solution was **ri**nsed off three times with Milli-Q. To remove any contaminants that may have been absorbed onto the foraminifera fragments during cleaning, 0.0005 M HNO_3 was added to the samples for approximately 30 seconds. This solution was removed and the samples were rinsed twice with Milli-Q. Approximately 500 µl of Milli-Q was added to the samples before they were centrifuged for five minutes. This concentrated test fragments at the bottom of the tube and all remaining liquid was removed.

Test fragments were dissolved in 200 µl of 0.075 M HNO_3. The smallest possible volume of weak nitric acid was used to minimise potential contamination from reagents and prevent leaching of any remaining contaminant phases. Once fully dissolved the samples were centrifuged for five minutes. All of the sample, with the exception of ~ 10 µl at the bottom of the vial, was pipetted off into an acid washed Teflon vial.

5.3.6 Major and trace element analysis

A small amount (<5%) of the dissolved sample was analysed for major and trace elements (Ca, Mg, Na, Sr, B, Li, Mn, Ba, Nd, Cd, U and Al) using a Thermo Finnigan Element 2 Inductively Coupled Plasma Mass Spectrometer (ICPMS). A small aliquot (5 µl) of each sample was taken and diluted in 0.5 M HNO_3 to determine Ca matrix concentration. This was done by analysing against a trace element standard at a range of Ca concentrations. This was to ensure that all samples and standards were analysed at the same concentration. Measurement of B/Ca allows estimation of the sample size available for boron isotope analysis. Samples were corrected for instrument mass bias by sample-standard bracketing against an in-house gravimetric trace element standard based on carbonate, which was cross calibrated with other trace element laboratories. Consistency standards were also run to check for accuracy and reproducibility. After trace element analysis, it became apparent that B/Ca in samples of *Elphidium* sp. were too low for the analysis of boron isotopes.

5.3.7 Column chemistry

In order to ensure that samples were ionised similarly in the Multi-Collector Inductively Coupled Plasma Mass Spectrometer (MC-ICPMS) (used for boron isotope analysis), the sample matrix was removed. This was achieved by separating the boron in the samples from their matrix of dissolved foraminifera by ion exchange chromatography, using resin contained within a 20 µl micro-column following the methods outlined in (Foster, 2008). The resin used was Amberlite IRA 742, which is a boron specific anionic exchange resin (Kiss, 1988). This resin has a high affinity for $B(OH)_4^-$ and all boron in a solution at high pH. At high pH, boron stuck to the resin in a column, whilst matrix elements were washed off. Once matrix elements were removed, boron was collected from the resin by lowering the pH in the column. This gave the desired sample solution of pure boron in acid. Tests of column yields gave total yields of >95% of boron and showed no detectable fractionation (Foster, 2008).

5.3.8 Boron isotope analysis

Boron isotope analysis was conducted on a Thermo Finnigan Neptune MC-ICPMS. Instrumental mass bias was corrected for by sample-standard bracketing using a 50 ppb solution of NIST 951. Mass bias was corrected using the average of the bracketing standards analysed immediately before and after the sample. Background contamination was accounted for by measurement of bracketing blank solutions. These blank solutions consisted of the same 0.5 M HNO_3 used during sample preparation and analysis. Potential contamination from reagents used during dissolution (0.075 M HNO_3) was accounted for using a Total Procedural Blank (TPB) correction. TPB solutions were made up of an equal volume of 0.075 M HNO_3 and buffer to that used in samples. One TPB was run with every set of columns. All samples and consistency standards were measured twice in each sequence.

5.3.9 Data analysis

The boron isotopic ratio was expressed in delta notation relative to NIST 951 boric acid standard ($^{11}B/^{10}B$ = 4.04367) (Rae et al., 2011) and was calculated according to Equation 5.1:

$$\delta^{11}B(\permil) = \left[\left(\frac{^{11}B/^{10}B_{sample}}{^{11}B/^{10}B_{NIST951}}\right) - 1\right] \text{ x } 1000$$

[5.1]

To examine dissimilarities between samples a correlation-based Principle Components Analysis (PCA) was conducted on the trace element data using PRIMER v.6. The data was first normalised to ensure that there were dimensionless, comparable scales (Clarke and Warwick, 2001).

5.4 Results

5.4.1 Carbonate chemistry data

The carbonate chemistry values used in this study for the CO_2 gradient offshore Vulcano are the same as those reported in Chapter 3. Samples were collected

between May 2011 and May 2013. Although foraminifera samples used in this study were collected in May 2012, having carbonate chemistry data from before and after this time period provides a better indication of the variability at this site. The pH at Vulcano is known to be variable (Boatta et al., 2013). Mean pH ranged from 8.19 (minimum value = 8.12, maximum value = 8.25) at the reference site, to 7.77 (minimum value = 7.61, maximum value = 8.06) at the Low pH site, which was closer to the seeps. The pH is lower and more variable in the low pH site compared to the reference site (Figure 3.8). Seawater calcite saturation state at the sample sites used in this study ranged from a mean value of Ω 5.29 (minimum value = 4.54, maximum value = 6.01) at the reference site, to Ω 2.89 (minimum value = 1.77, maximum value = 4.54) at the low pH site.

Carbonate chemistry values used in this study for offshore Papua New Guinea were those reported by Uthicke et al. (2013). Although surface sediment samples were collected from 13 different sites along the CO_2 gradient, only foraminifera from three different sites could be used for major and trace element and boron isotope analysis. This was because of the low abundance of foraminifera found within the samples and the limited number of samples that could be analysed in this study. Carbonate chemistry data for the four sample sites used in this study are reported here (Table 5.3). Mean pH ranged from 7.99 (upper 95% interval = 8.02, lower 95% interval = 7.97) at the reference site, to 7.82 (upper 95% interval = 7.91, lower 95% interval = 7.74) at the Very Low pH site. The pH was lower, but more variable in the Low pH and Very Low pH sites, which were closer to the gas seeps (Figure 5.2). This was also the case across the shallow water CO_2 gradient at Vulcano (Chapter 3) and Ischia (Hall-Spencer et al., 2008). Seawater was supersaturated with respect to calcite at the sample sites, ranging from Ω 5.17 (±0.13) to Ω 3.67 (±0.64).

Table 5.3: Mean seawater pH and associated carbonate chemistry parameters measured in April 2011 at the three Upa-Upasina Reef sample sites used in this study. Carbonate chemistry parameters were calculated from total alkalinity (TA) and dissolved inorganic carbon (DIC) measurements at each site (number of samples (n) given below site names). Upper and lower 95% confidence intervals for pH and standard deviation for all other parameters are shown in brackets. Data from Uthicke et al. (2013).

Site	pH (Total)	TA (µmol/kgSW)	pCO$_2$ (µatm)	Ω_{Calc}
Reference site	7.99	2286 (21)	449 (15)	5.17 (0.13)
(n=6)	(7.97 – 8.02)			
Mid pH	7.95	2292 (20)	507 (84)	4.84 (0.54)
(n=5)	(7.90 – 8.01)			
Low pH	7.87	2307 (38)	639 (266)	4.37 (0.99)
(n=5)	(7.75 – 8.06)			
Very Low pH	7.82	2295 (27)	792 (157)	3.67 (0.64)
(n=6)	(7.74 – 7.91)			
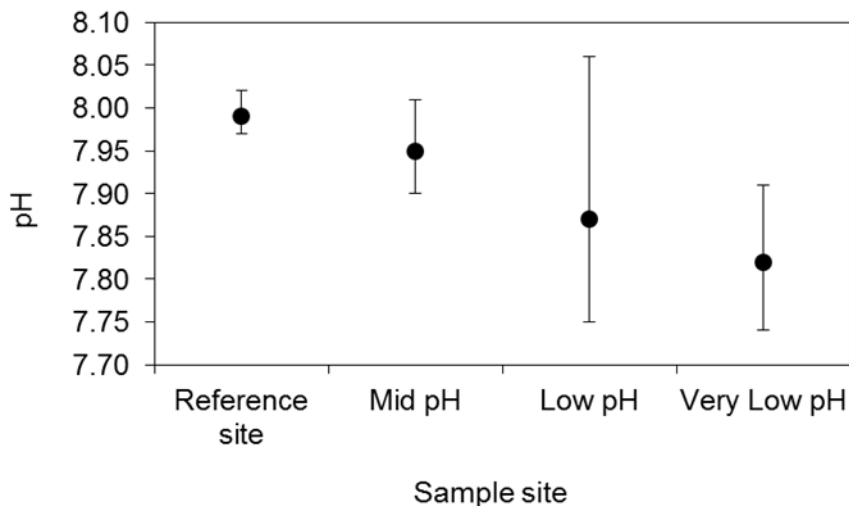
Figure 5.2: Mean (n = 5 – 6) pH measured across the CO$_2$ gradient at the Upa-Upasina Reef sample sites off Papua New Guinea in April 2011. Error bars represent the upper and lower 95% confidence intervals. Data from Uthicke et al. (2013).

5.4.2 Major and trace element concentrations

Major and trace element concentrations were variable between species, but also between different sized individuals of the same species (Figure 5.3). *Amphistegina lessonii* individuals of the same size, from the same sample site, tended to have the same trace element concentration. For most of the trace elements measured, *Elphidium* sp. had very different concentrations compared to *C. spengleri* or *A. lessonii*. B/Ca values ranged from 59.88 – 482.33 µmol/mol. There did not appear to be a trend in B/Ca with pH and there was a lot of variation between the different species and

188

between individuals from the same site. All *Elphidium* sp. measured had very low B/Ca values, regardless of the mean seawater pH of the sample site from which they were collected. When all samples were examined together, there was a general trend for a decrease in B/Ca as bicarbonate ion concentration increased, but this pattern was not consistent across species.
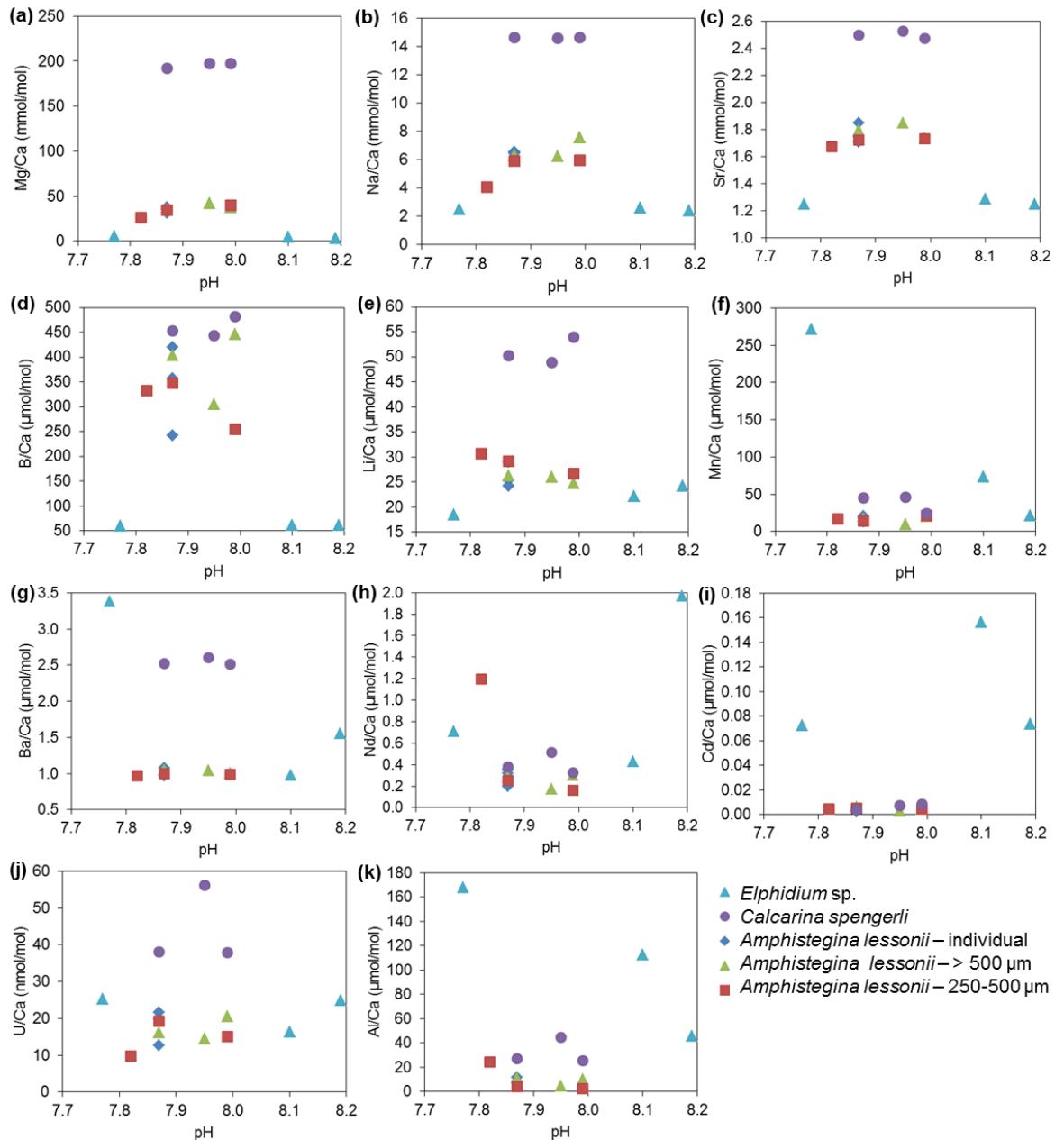

Figure 5.3: Major and trace element concentrations measured using ICPMS in tests of three different species of benthic foraminifera collected across shallow water CO_2 gradients. pH refers to the mean pH of the seawater at the sample site from which the foraminifera were collected. *Elphidium* sp. collected off Vulcano, Italy, *Calcarina spengleri* and *Amphistegina lessonii* collected from the Upa-Upasina Reef off Papua New Guinea. All data points were obtained from seven crushed individuals with the exception of *Amphistegina lessonii* – individual, which was obtained from a single specimen. (a) Mg/Ca, (b) Na/Ca, (c) Sr/Ca, (d) B/Ca, (e) Li/Ca, (f) Mn/Ca, (g) Ba/Ca, (h) Nd/Ca, (i) Cd/Ca, (j) U/Ca, (k) Al/Ca.

189

When PCA was conducted on all trace element data together, PC1 explained 54% of the total variation and PC2 explained a further 29% of the total variation. This means that the PCA should provide an accurate representation of similarity between samples. The samples did not group according to pH or sample site, but they did appear to group according to species (Figure 5.4). This suggests that the trace element concentrations measured from the same species of foraminifera were more similar to each other than concentrations measured from different species.
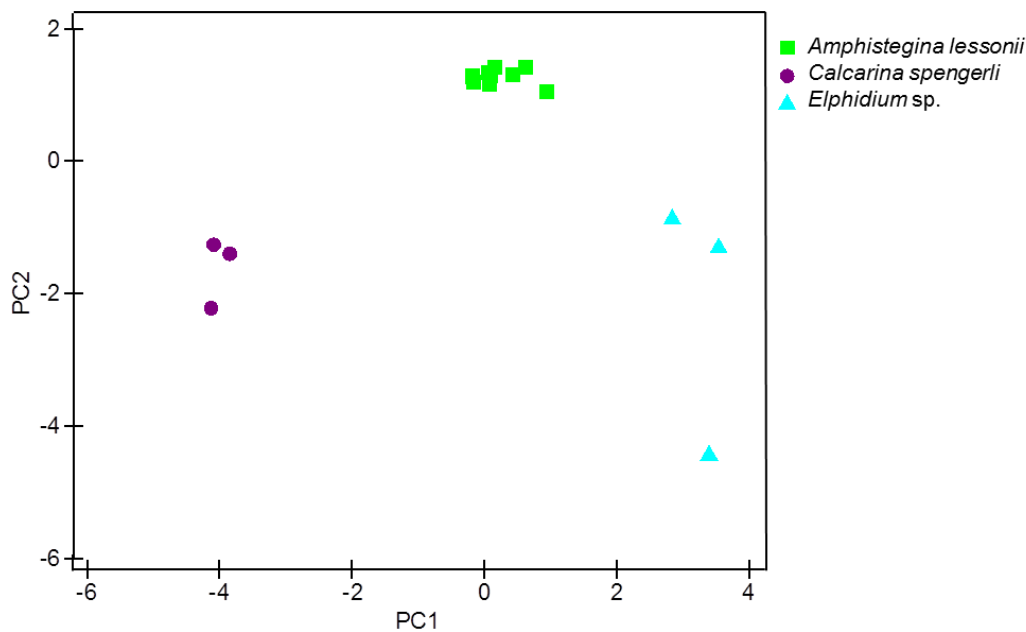
Figure 5.4: Major and trace element concentrations. 2-dimentional PCA ordination of trace element concentrations measured using ICPMS in tests of three different species of benthic foraminifera collected across shallow water CO_2 gradients. *Elphidium* sp. collected off Vulcano, Italy, *Calcarina spengleri* and *Amphistegina lessonii* collected from the Upa-Upasina Reef off Papua New Guinea. PC1 and PC2 together account for 83% of the total sample variability.

5.4.3 Boron isotopes

$δ^{11}B$ values ranged from 17.97 – 21.93‰. *Calcarina spengleri* had the lowest $δ^{11}B$ values and an individual *A. lessonii* had the highest (Figure 5.5). There was a decrease in $δ^{11}B$ as pH decreased for the *A. lessonii* taken from the 250 – 500 µm size fraction, but no apparent trend for the larger *A. lessonii* (>500 µm). There did not appear to be a trend in $δ^{11}B$ with pH for *C. spengleri*. There was large variation in $δ^{11}B$ for individual *A. lessonii* from the same sample site which were of a similar size.
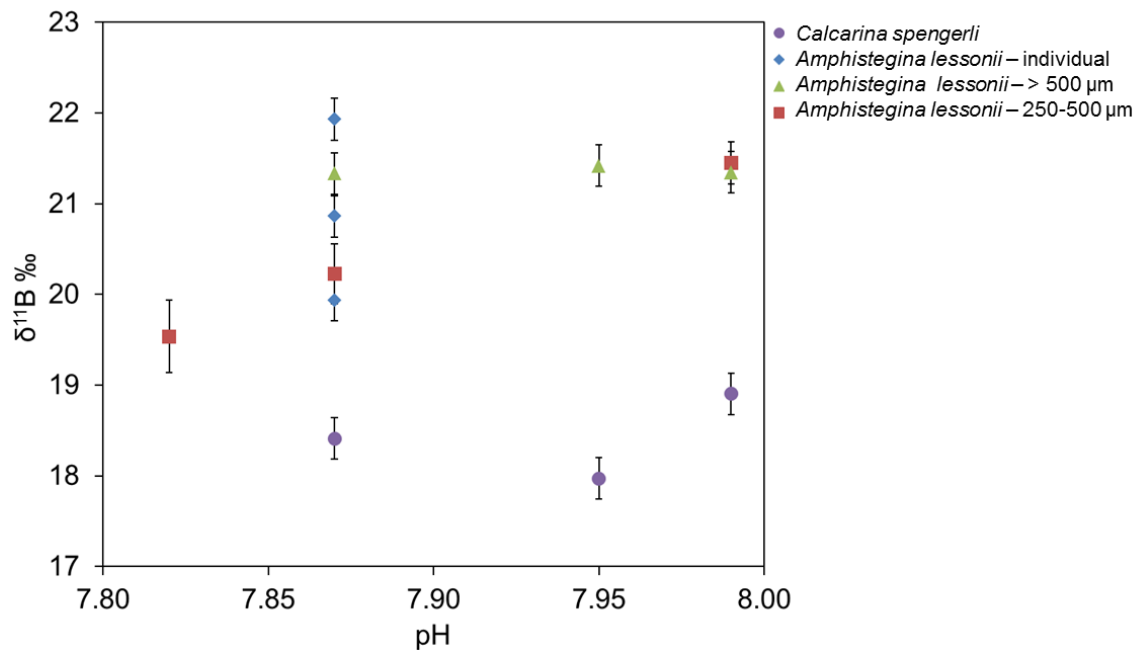
Figure 5.5: δ^{11}B (± 2 SD) of tests of *Calcarina spengleri* and *Amphistegina lessonii* collected across a shallow water CO_2 gradient at the Upa-Upasina Reef off Papua New Guinea measured using MC-ICPMS. pH refers to the mean pH (total scale) of the seawater at the sample site from which the foraminifera were collected. All data points were obtained from seven crushed individuals with the exception of *Amphistegina lessonii* – individual, which were obtained from a single specimen.

5.5 Discussion

The concentrations of major and trace elements were variable between species. This indicates the presence of vital effects (Yu and Elderfield, 2007, Rae et al., 2011), where biological processes override environmental signals. Concentrations were also variable between different sized individuals of the same species, indicating a possible ontogenetic effect. Allison and Austin (2008) found that Mg/Ca and Sr/Ca of the benthic foraminifera *Hyalinea balthica* were variable between individuals of the same size and also within individual chambers. Allison and Austin (2003) found that between test chambers there was significant variation in Mg and Sr concentration. Dissard et al. (2010a) found that changes in CO_3^{2-} concentration or total dissolved inorganic carbon did not affect the Mg distribution coefficient. On the contrary, Sr incorporation was enhanced under increasing CO_3^{2-} concentration. In another study, they found that salinity increased the incorporation of both Mg and Sr (Dissard et al., 2010b).

When examining B/Ca, Rae et al. (2011) found concentrations varied considerably between different morphotypes of the same species. In this study, the three *A. lessonii* individuals that were of a similar size and from the same sample site tended to have a similar trace element concentration.

For most of the major and trace elements measured, *C. spengleri* and *A. lessonii* had similar concentrations. *Elphidium* sp. had different concentrations presumably because they were collected from a different location from that of *C. spengleri* and *A. lessonii*. The difference in location means that *Elphidium* sp. calcified under different seawater conditions. In addition, the *Elphidium* sp. analysed in this study were all epiphytes on the brown seaweed *P. pavonica,* whereas *C. spengleri* and *A. lessonii* were found as epifauna in sediment samples. It could be that the microenvironment around the *P. pavonica* thalli, that can have a higher pH due to photosynthesis (Cornwall et al., 2013), altered the major and trace element incorporation into the test of *Elphidium* sp. In order to interpret the major and trace element concentrations, it is important to know how each of them is used as an environmental proxy.

5.5.1 Magnesium

Magnesium is used as a temperature proxy (Dueñas-Bohórquez et al., 2009), where higher concentrations of magnesium in foraminiferal tests are associated with warm climatic periods. Highest benthic foraminifera values are from the shallowest, warmest sites (Lea, 1999), but are controlled by whether the foraminifera produce low, intermediate or high Mg-calcite (Bentov and Erez, 2006). Mg/Ca values had a large range; 3.39 – 197.67 mmol/mol. These concentrations are higher than the typical benthic foraminiferal Mg/Ca range of 0.5 – 10 mmol/mol reported by Lea (1999). *Calcarina spengleri* had the highest concentrations of magnesium and *Elphidium* sp. had the lowest. *Calcarina spengleri* have high Mg-calcite tests, whereas *A. lessonii* and *Elphidium* spp. have low Mg-calcite tests (Bentov and Erez, 2006, Sinutok et al., 2014). *Amphistegina lessonii* are known to have higher Mg/Ca than smaller benthic

foraminifera, such as *Elphidium* (Raja et al., 2005). This explains the Mg/Ca offset between *C. spengleri*, *A. lessonii* and *Elphidium* sp. The use of this proxy is complicated by strong post-depositional effects on shell Mg in some species. There is preferential dissolution of magnesium enriched portions (Lea, 1999). This should not be a problem when examining magnesium concentrations in living, or recently living, foraminifera. The same species tended to have similar Mg/Ca regardless of pH. Allison et al. (2011) examined the Mg/Ca and Sr/Ca of *Elphidium williamsoni* tests which had been grown in culture over a range of pH conditions. They found that both Mg/Ca and Sr/Ca varied significantly within individual test chambers, but also between individuals cultured under the same conditions. Allison et al. (2010) did not observe a relationship between culture pH and test Mg/Ca. Mg/Ca of *Elphidium* measured in this study (3.88 – 5.55 mmol/mol) are within the range reported by Allison et al. (2011). Dueñas-Bohórquez et al. (2009) found that salinity had an overriding control on Mg incorporation in the test of the planktonic foraminifera *G. sacculifer*. This could be one reason for the different Mg/Ca values between the species examined. Seep sites offshore Vulcano, the location from which *Elphidium* sp. were collected, had higher salinities (~38) compared to seep sites offshore Papua New Guinea (~34). Toyofuku et al. (2000), however, found that salinity did not significantly influence Mg/Ca in two species of benthic foraminifera *Planoglabratella opercularis* and *Quinqueloculina yabei*, but Dissard et al. (2010b) found that an increase in salinity increased Mg incorporation in *Ammonia tepida*.

5.5.2 Strontium

Strontium concentrations in foraminiferal calcite may reflect seawater Sr/Ca or alternatively, it may reflect the influence of deep water carbonate ion saturation (Yu et al., 2014) as already suggested by Dissard et al. (2010a) based on a laboratory study with *A. tepida*. If foraminiferal calcite Sr/Ca reflects seawater Sr/Ca then it is possible to estimate global average river Sr fluxes (Lear et al., 2003). Yu et al. (2014) found a significant control of carbonate ion saturation on the incorporation of Sr in benthic

foraminiferal calcite, suggesting that seawater Sr/Ca has remained nearly constant during the late Pleistocene. Culture experiments have also found that Sr incorporation is enhanced under increasing carbonate ion concentration (Dissard et al., 2010a). Dueñas-Bohórquez et al. (2009) found that calcite saturation state was the main control on Sr incorporation in *G. sacculifer* tests (this implies that carbonate ion concentration was the main control, as calcium concentration was constant), whereas salinity did not have an influence on Sr/Ca. Sr/Ca values in this study ranged from 1.25 – 2.52 mmol/mol. This is slightly higher than the typical range for benthic foraminifera (Lea, 1999). *Calcarina spengleri* had the highest concentrations whereas *Elphidium* sp. had the lowest concentrations. The same species tended to have similar Sr/Ca values regardless of pH. Although the absolute values were different, patterns in Sr/Ca appeared to be very similar to those for Mg/Ca. Allison et al. (2011) did not find significant correlations between Mg/Ca and Sr/Ca between *E. williamsoni* tests cultured at the same pH.

5.5.3 Boron

Boron is conservative in seawater and the ratio of boron to calcium is nearly fixed in seawater (Lea, 1999). Any variations in B/Ca in foraminiferal tests, therefore, reflects the impact of physical and/or biological processes on calcification. The boron to calcium ratio is used as a palaeo-proxy for pH (Yu et al., 2007, Yu et al., 2010). It has the advantage over boron isotopes as it is easier to measure, but absolute values and trends are strongly species specific (Yu and Elderfield, 2007). It has been found to vary considerably in different morphotypes of the same species (Rae et al., 2011). B/Ca values are typically 130 – 230 µmol/mol for epifaunal foraminifera and 10 – 80 µmol/mol for infaunal foraminifera (Rae et al., 2011). This could possibly be because of higher bicarbonate ion concentrations in the sediment, meaning less boron in foraminiferal tests because there is a competition between boron and bicarbonate during uptake (Hemming and Hanson, 1992). The values measured in these samples are generally higher than the values reported by Rae et al. (2011) ranging from 59.88 –

482.33 µmol/mol. This could be a reflection of the large size of many of the foraminifera examined. There did not appear to be any trend in B/Ca with pH although there was a general trend for a decrease in B/Ca as bicarbonate ion concentration increased. This pattern, however, was not consistent across species.

5.5.4 Lithium

Lithium is a chemical proxy and records secular changes in ocean chemistry. Like boron, it is conservative in seawater and variability in shell content is mediated by changes in seawater composition. On long time scales, seawater lithium is thought to vary with the extent of sea floor spreading (Lea, 1999). Calcification rate, which may in turn be a function of carbonate ion concentration, may affect Li/Ca (Hall and Chan, 2004a, Lear and Rosenthal, 2006). Hall and Chan (2004a) found that incorporation of lithium onto foraminiferal tests did not appear to be influenced by changes in temperature, dissolution or pressure. In this study Li/Ca values ranged from 18.45 – 53.99 µmol/mol, which is within the typical range for planktonic foraminifera (Lea, 1999). *Calcarina spengleri* had the highest Li/Ca values and *Elphidium* sp. had the lowest. The reasons for this are not clear, but could be due to the differences in size of the foraminifera.

5.5.5 Manganese

Manganese is a digenetic proxy and reveals information about post-depositional history. It precipitates as a secondary coating on tests as magnesium carbonate, which is a secondary mineral that forms in reducing conditions typical of ocean floor sediments (Lea, 1999). The presence of manganese carbonate can also complicate the use of other trace elements as proxies because it can trap high levels of certain trace elements such as cadmium or barium (Lea, 1999). Mn/Ca values ranged from 9.81 – 271.41 µmol/mol. There was one *Elphidium* sample that had a value of 271.41 µmol/mol. All other samples had a value below 75 µmol/mol. This anomalous result

suggests potential contamination. There did not appear to be any trend in Mn/Ca with pH.

5.5.6 Barium

Barium is a nutrient proxy which is thought to be most similar to alkalinity and silicic acid (Hall and Chan, 2004b). Barium is depleted in surface waters though precipitation of barite (Lea, 1999). Ba/Ca values ranged from 0.97 – 3.38 μmol/mol. These values lie within the range reported for benthic foraminiferal tests (Lea, 1999). Ba/Ca of *Elphidium* sp. had a wide variation. For the two other species examined, Ba/Ca was similar regardless of pH. The Ba/Ca variation in *Elphidium* sp. tests, which were the only species examined from Vulcano, could reflect a difference in alkalinity between the sample sites. Although alkalinity was fairly constant across the shallow water CO_2 gradient at Vulcano (Chapter 3), it was more variable than values reported for the sample sites used in this study offshore Papua New Guinea (Uthicke et al., 2013).

5.5.7 Neodymium

Neodymium isotopes can be used as a chemical proxy for weathering and ocean circulation (Vance and Burton, 1999). This use of this proxy, however, is complicated because neodymium has a strong affinity for manganese and iron oxide phases that coat foraminiferal tests. Burton and Vance (2000), however, found that diagenetic Fe-Mn oxides could be removed from foraminifera through cleaning and that Nd isotopes reflected seawater composition. Nd/Ca values in this study ranged from 0.16 – 1.97 μmol/mol. *Elphidium* sp. tended to have higher Nd/Ca values. This could be because *Elphidium* sp. had higher concentrations of manganese, for which neodymium has a high affinity (Lea, 1999). There was a general trend for a decrease in Nd/Ca as pH increased.

5.5.8 Cadmium

Cadmium is a nutrient proxy, the behaviour of which closely mimics that of phosphate and it is thought to be the most direct nutrient analogue available (Lea, 1999, Yu et al., 2013). Cadmium occurs in low concentrations in the oceans and, therefore, the abundance in foraminiferal tests is very low (Lea, 1999). Potential complications with the use of this proxy are that manganese carbonate, formed by digenetic addition, can trap cadmium, increasing concentrations. Cd/Ca values ranged from 0.00 – 0.16 µmol/mol. This lies within the range typical for benthic foraminiferal tests reported by Lea (1999). Cd/Ca values for *Elphidium* sp. were much higher than for *C. spengleri* or *A. lessonii*. Again, this is likely to be because *Elphidium* sp. had higher concentrations of manganese carbonate, which is known to trap cadmium (Lea, 1999). There did not appear to be any trend in Cd/Ca with pH.

5.5.9 Uranium

Seawater concentrations of uranium can be a measure of the extent of suboxic sediments (Lea, 1999). To accurately determine seawater uranium content in the past, test calcite must incorporate uranium in proportion to the concentration in seawater (Russell et al., 1994). Russell et al. (1994) found that this was the case for *Amphistegina lobifera* grown in culture. Subsequent studies on planktonic foraminifera from cores, however, found that there was a temperature control on the incorporation of uranium into foraminifera tests (Russell et al., 1996). U/Ca values ranged from 9.82 – 56.12 nmol/mol. *Calcarina spengleri* had the highest U/Ca. This could be a reflection of the larger size of *C. spengleri*. Ni et al. (2007) found that there were increases in U/Ca of the planktonic foraminifera *G. sacculifer* and *Globigerinoides ruber* with increasing test size. They argue that this could be a result of changing growth rates. There did not appear to be any trends of U/Ca with pH. Russell et al. (2004) found that U/Ca of the planktonic foraminifera species, *O. universa*, grown in culture, decreased as seawater carbonate ion concentration increased. This was thought to be a result of the formation of stable complexes between uranyl ions and carbonate ions. A similar

conclusion was reached by Keul et al. (2013b) in a culture study of the benthic foraminifera *A. tepida*.

5.5.10 Aluminium

The presence of aluminium in foraminiferal tests is indicative of clay particles and indicates contamination (Barker et al., 2003, Elderfield et al., 2006, Rae et al., 2011). Al/Ca values ranged from 2.31 – 168.04 µmol/mol, but no correlation was seen between Al/Ca and B/Ca. Al/Ca values were highest for *Elphidium* sp. This is somewhat surprising as *Elphidium* sp. were found as epiphytes on *P. pavonica* thalli and were, therefore, unlikely to be contaminated with clay.

5.5.11 Boron isotopes

$\delta^{11}B$ values ranged from 17.97 – 21.93‰. This is similar to the range reported for benthic foraminifera from 23 late-Holocene samples (Rae et al., 2011). When *A. lobifera* were grown in culture in pH conditions ranging from 7.90 – 8.45 the range in $\delta^{11}B$ (17.86 – 26.27‰) was similar to the present study (Rollion-Bard and Erez, 2010).

There was a decrease in $\delta^{11}B$ as pH decreased for the *A. lessonii* taken from the 250 – 500 µm size fraction, but not for *A. lessonii* from the >500 µm size fraction or *C. spengleri*. The trend for the smaller *A. lessonii* supports the hypothesis and the use of the boron isotope proxy; there was a decrease in $\delta^{11}B$ as pH decreased. A lack of trend between $\delta^{11}B$ and pH for the larger *A. lessonii* and *C. spengleri* could suggest an ontogenetic effect. As foraminifera increase in size, the number of symbionts increases and, therefore, photosynthesis of the symbionts may start to influence the ambient pH (Hönisch and Hemming, 2004, Babila et al., 2014). Differences between species may be caused by different species of symbionts, different numbers of symbionts or different respiration rates of the foraminifera (which will work in the opposite direction to reduce pH) (Babila et al., 2014). Rollion-Bard and Erez (2010) suggested that a small amount of test calcium carbonate recorded the initial pH of seawater vacuolated by

foraminifera during calcification, which is linked the environmental seawater. They hypothesised that the rest of the test was precipitated at elevated pH values as the foraminifera elevate pH at the site of calcification. For *A. lobifera*, Rollion-Bard and Erez (2010) found this upper pH limit to be approximately pH 9. As foraminifera increase in size, the increased number of symbionts may mean that photosynthesis of the symbionts is sufficient to increase the pH that the foraminifera experience and, therefore, $\delta^{11}B$ does not reflect environmental seawater pH. Both shell size and shell weight are known to influence boron isotope values (Hönisch and Hemming, 2004). Hönisch and Hemming (2004) found that tests of the planktonic foraminifera *G. sacculifer* from the larger sieve size (515 – 865 μm compared to 250 – 380 μm) had higher (+2.1 - +2.3 ‰) $\delta^{11}B$ values. They suggest that this difference is caused by differences in symbiont photosynthetic activity. This accords with the findings from this investigation, the larger *A. lessonii* tending to have higher $\delta^{11}B$ values.

Calcarina spengleri had the lowest $\delta^{11}B$ values with no apparent trend between $\delta^{11}B$ and pH. Different offsets between species are thought to be caused by differences in the pH of the micro-environments in which the foraminifera calcified (Sanyal et al., 2001). The pH of the microenvironment can be altered by symbionts, therefore, different species of symbionts between *C. spengleri* and *A. lessonii* may be a cause of the differences. *Calcarina spengleri* and *A. lessonii* both have diatom symbionts (Uthicke et al., 2013). When examining symbionts in benthic foraminifera, Leutenegger (1984) grouped the diatoms in *C. spengleri* and *A. lessonii* into different types suggesting that they belong to different taxa, although they could not be taxonomically identified. The lower $\delta^{11}B$ values in *C. spengleri* suggests that the counteracting impact of respiration is stronger than photosynthesis in this species, reducing the pH that the foraminifera experience. Infaunal and epifaunal foraminifera tend to have different $\delta^{11}B$ values (Rae et al., 2011), but both *C. spengleri* and *A. lessonii* are epifaunal and consequently this cannot be a reason for the offset between these two species.

There was large variation in $\delta^{11}B$ for individual *A. lessonii* from the same site which were of a similar size. The reasons for this are not clear, but could reflect variability of the pH environment in which they calcified. The pH at the seep sites tends to be very variable (Kerrison et al., 2011, Boatta et al., 2013) making it very difficult to determine the exact pH environment in which the foraminifera calcified. Although all these *A. lessonii* were collected from the same sample site, it may be that they calcified in different pH conditions. For detailed, high precision chemical analysis, as for boron isotope analysis, where environmental factors need to be carefully controlled, it may be better to use laboratory experiments as a means of increasing our understanding of this proxy. It could also be possible that not all of the three individuals examined were living. The collection methods negated the possibility of staining the samples with rose Bengal to determine living individuals. The foraminifera selected for analysis were pristine with no signs of breakage and Uthicke et al. (2013) used the same criteria for selecting benthic foraminifera that were considered to be a good representation of the present-day community. If the individuals were not living, providing that they calcified in the same pH environment, this should not have resulted in differences in $\delta^{11}B$, as Rae et al. (2011) observed no influence of partial dissolution of benthic foraminiferal tests on $\delta^{11}B$.

The selection of intact specimens could have biased results. It could be that degraded specimens were grown at times when pH was, on average, lower and intact specimens were grown when pH was, on average, higher. An interesting future study would be to compare degraded and intact specimens.

It is important to know the boron isotopic composition of the seawater around the gas seeps to verify that this does not differ from other values measured for seawater. In the present study, it was not possible to collect these samples. The lack of this information, however, does not negate the use natural gradients in CO_2 to test the boron isotope proxy, but a larger sample size would be needed in future investigations. The boron

isotopic composition of corals collected across a shallow water CO_2 gradient offshore Ischia, Italy has previously been examined where the boron isotopic composition of seawater around the gas seeps was not measured (Trotter et al., 2011).

5.6 Conclusions

The concentration of major and trace elements were variable between species, as well as between different sized individuals of the same species. The smaller *Elphidium* sp., that were collected across a different shallow water CO_2 gradient, tended to have different major and trace element concentrations to *C. spengleri* or *A. lessonii*. A decrease in $\delta^{11}B$ with pH was found for smaller *A. lessonii*, but not for the larger *A. lessonii* or *C. spengleri*. This suggests that once the foraminifera reach a certain size, the number of symbionts is sufficient for photosynthesis to influence the ambient pH. There was a large variation in $\delta^{11}B$ for individual *A. lessonii* of the same size collected from the same pH conditions. This is thought to be a result of the pH variability around shallow water CO_2 seeps, or could be due to variability in $\delta^{11}B$ of seawater around the seeps and could mean that although the individuals were collected from the same sample site they did not calcify under the same pH conditions.

Chapter 6: General discussion, overall conclusions and future work

6.1 Summary of main findings

The majority of responses of shallow water calcified benthic foraminifera to increased pCO_2 were negative (Table 6.1), although in some cases there were too few individuals found to draw firm conclusions. Such a summary will mask subtle nuances within assemblage shifts, but allow for broad patterns to be identified.

Table 6.1: Summary of main findings presented in this thesis. Location refers to the shallow water CO_2 gradient from which samples were collected. Habitat type refers to the type of sample collected. Responses to pCO_2 for four assemblage parameters (number of species, number of individuals, the number of calcareous taxa and the number of agglutinated taxa) are represented by trend plots. N/A = not applicable, when there were too few individuals to draw firm conclusions from the data.

Location	Habitat type	Assemblage type	Number of species	Number of individuals	Calcareous taxa	Agglutinated taxa
Gulf of California, Mexico	Infaunal and epifaunal from sediment samples	Living	(no trend)	(no trend)	(no trend)	(no trend)
		Dead	(decreasing)	(no trend)	(decreasing)	(no trend)
Ischia, Italy	Infaunal and epifaunal from sediment samples	Living	N/A	N/A	N/A	N/A
		Dead	(decreasing)	(no trend)	(decreasing)	N/A
	Epiphytes on *Posidonia oceanica* leaves	Living	N/A	N/A	N/A	N/A
		Dead	(no trend)	(no trend)	(no trend)	N/A
Vulcano, Italy	Epiphytes on *Padina pavonica* thalli	Attached to thalli	(decreasing)	(decreasing)	(decreasing)	(increasing)
		Living	N/A	N/A	N/A	N/A
		Dead	(decreasing)	(decreasing)	(decreasing)	(increasing)
	Artificial collectors	Living	(decreasing)	(decreasing)	(decreasing)	N/A
		Dead	(decreasing)	(decreasing)	(decreasing)	N/A

6.1.1 Gulf of California, Mexico

The number of species and the number of individual foraminifera in the living (stained) assemblage, collected from CO_2 seeps in the Gulf of California, did not show a significant response to increased pCO_2. This could be because too few individuals were sampled for statistically rigorous patterns to emerge. Few living foraminifera were found in samples, even those with the highest pH (~7.88). The highest pH recorded was 7.88, this is below the value of 8.1, considered to be a global average for surface waters (Raven et al., 2005, Guinotte and Fabry, 2008), but it is expected that infaunal benthic foraminifera will experience lower pH conditions within the sediment pore water. Although the community of foraminifera present was expected for this region at these water depths (74 – 207 m) (Bandy, 1961, Murray, 1991a, Pettit et al., 2013), there were fewer individuals per unit area compared to previous investigations in this area (Bandy, 1961).

The number of species of the dead assemblage (those foraminifera that did not take up the rose Bengal stain) showed a significant decrease as pH decreased, but there was no significant change in the number of individuals. The very few agglutinated taxa that were present in samples did not show a response to pCO_2. There was evidence of post-mortem test dissolution, revealing that the foraminifera had been subjected to dissolution of their tests.

Foraminiferal assemblages examined in the Gulf of California (Pettit et al., 2013) appeared to be more resilient to increased pCO_2 than assemblages across shallow water CO_2 gradients off Ischia (Cigliano et al., 2010, Dias et al., 2010), Vulcano or Papua New Guinea (Fabricius et al., 2011, Uthicke and Fabricius, 2012, Uthicke et al., 2013). This was contrary to expectation; less resilience was expected due to the lower pH at sample sites in the Gulf of California. The increased resilience observed could be due to higher nutrient concentrations (Zeitzschel, 1969, Halfar et al., 2004, Halfar et al., 2006), compared to the oligotrophic conditions of the Mediterranean Sea, providing

more food and, therefore, energy for foraminiferal growth despite ocean acidification. Alternatively, a more stable saturation state in the Gulf of California could be a reason for the increased resilience. It is expected that a lack of strong temperature variations in the northern Gulf of California leads to less variation in Ω_{Calc}. Keul et al. (2013a) found that the saturation state was the carbonate chemistry parameter that controlled calcification rates, so this could explain the presence of calcareous foraminifera in low pH conditions (~7.55).

6.1.2 Ischia

At Ischia there were too few living (stained) individuals of benthic foraminifera found within the sediment or on *Posidonia oceanica* leaves to determine if there was any significant impact of ocean acidification on benthic foraminiferal assemblages. The low number of living individuals is thought to be a result of the time of year of sampling (November). This sample collection at Ischia was a pilot investigation and additional sampling would be required to determine whether seagrass beds may offer refugia to foraminifera in the face of global ocean acidification. In examination of seagrass invertebrate assemblages associated with *P. oceanica* meadows offshore Ischia, Garrard et al. (2014) found that many groups of invertebrate taxa were robust to the effects of ocean acidification. This suggests that the *P. oceanica* habitat may provide a refugia against ocean acidification.

Analysis of a small number of sediment samples indicated a decrease in the number of species of the unstained (dead) assemblage as pH reduced, whereas there was no change in the number of individuals. The decrease in the number of species supports previous findings across a CO_2 gradient off Ischia (Dias et al., 2010). At Ischia, responses of foraminiferal assemblages were similar between the north and south side of Castello d'Aragonese. Similar patterns in the distribution of calcified macrofauna and flora along the CO_2 gradient were previously reported by Hall-Spencer et al. (2008) and Porzio et al. (2011).

6.1.3 Vulcano

Foraminifera were unusually rare in the sediment at Vulcano; there were no benthic foraminifera found in sediment samples. The reasons for this were not clear, but could be a result of winnowing (Cockey et al., 1996), the coarse grain size of the sediment resulting in low food availability (Oxford et al., 2004), or the inhospitable nature of the sediment, which consisted of shards of glassy particles formed from the reworking of volcaniclastic material and pyroclastic flows during the last eruption of Vulcano (Dellino et al., 2011, Romagnoli et al., 2012).

In samples of *Padina pavonica* thalli and artificial collectors, where foraminifera were found, responses were similar, regardless of whether the assemblages being examined were those found as epiphytes or on artificial collectors. There was a dramatic reduction in the number of species and individuals as pCO_2 increased along a shallow water CO_2 gradient. The same responses were reported in previous investigations across a CO_2 gradient off Ischia (Cigliano et al., 2010, Dias et al., 2010) and Papua New Guinea (Fabricius et al., 2011, Uthicke et al., 2013), despite differences in assemblages examined (i.e. epiphytic, infaunal and epifaunal). There was also an assemblage shift in foraminifera found on the brown macroalga *P. pavonica* at Vulcano from domination by calcareous taxa at reference sites ($pCO_2 \sim 470$ µatm) to domination by agglutinated taxa near to the seeps ($pCO_2 \sim 1860$ µatm). This indicates that these algal surfaces will not provide refugia for calcareous foraminifera along a gradient of overlying seawater acidification, although dense stands of seagrass or kelp may do so because their photosynthesis may be sufficient to increase pH in the DBL above ambient (Cornwall et al., 2013).

The fact that the majority of the foraminifera found in the artificial collectors were adults would suggest that the foraminifera were not being transported as propagules or juveniles, which is thought to be the main mechanism of transport for attached or larger foraminifera (Alve, 1999, Alve and Goldstein, 2003). Larger living foraminifera are also

known to be able to withstand very high current velocities, which suggests that they are not easily entrained (Alve, 1999). The short time for which the artificial collectors were in place adds further support to the proposition that the foraminifera were not transported in the embryonic life stages. This is particularly the case for some of the longer living species such as *Planorbulina mediterranensis*, which can have life spans of up to one year (Langer, 1993). These longer lived species would not have had time to grow to adult size during the short time that the artificial collectors were in place. There were low numbers of stained individuals found on the artificial collectors, suggesting that they trapped mainly dead foraminifera, which were washed onto the artificial collectors as suspended load from sediment outside of the study area, or from surrounding macroalgae. Although no benthic foraminifera were found in sediment samples collected along the CO_2 gradient at Vulcano, it is possible that the foraminifera were being washed onto the collectors from sediment further out in the bay. Benthic foraminifera can be suspended in the water column and transported long distances (Murray et al., 1982, John, 1987). The absence of foraminifera from the lowest pH areas suggests that foraminifera were undergoing post-mortem dissolution in the high CO_2 conditions, were not washed onto the collectors in the first place, or were washed off before collection.

6.1.4 Major and trace elements and boron isotopic composition

The concentration of major and trace elements in benthic foraminifera collected across shallow water CO_2 gradients were variable between species, as well as between different sized individuals of the same species. Examination of the boron isotopic composition of two species of benthic foraminifera collected across a shallow water CO_2 gradient off Papua New Guinea led to contrasting results. There was a decrease in $\delta^{11}B$ with pH for smaller (250 – 500 µm) *Amphistegina lessonii*, but not for larger (>500 µm) *A. lessonii* or *Calcarina spengleri*. This is thought to be because of the presence of symbionts, which increase pH due to photosynthesis. In the larger foraminifera, where there are more symbionts, photosynthesis may dominate over

ambient pH (Babila et al., 2014). The pH variability around shallow water CO_2 seeps (Kerrison et al., 2011, Boatta et al., 2013) is thought to be a cause of the large variation in $\delta^{11}B$ for individual *A. lessonii* of the same size collected from the same pH conditions. The large variability in pH could mean that although the individuals were collected from the same sample site, they did not calcify under the same pH conditions. An extended period of calm weather could, for example, result in exceptionally high CO_2 levels, whereas extended stormy periods would lead to lower than average CO_2 levels due to mixing.

6.1.5 Implications for benthic foraminiferal assemblages

Although the response of foraminiferal assemblages to ocean acidification appear to be complex, overall, the findings presented in this thesis indicate that ocean acidification has a negative effect on shallow water, benthic foraminiferal assemblages. There was a decrease in the number of species and individuals and, in some cases, a shift in the assemblage from calcareous to agglutinated taxa as CO_2 levels increased. The findings presented in this thesis raise serious concerns for the survival of shallow water calcareous benthic foraminifera due to ocean acidification. This has implications for marine ecosystems (Uthicke et al., 2013, Doo et al., 2014). Foraminifera are extremely abundant in the sediment (Murray, 1991a) and larger benthic foraminifera, that contain algal symbionts, contribute to primary production through photosynthesis and organic carbon production (Doo et al., 2014). Benthic foraminifera are also important in nutrient cycling and probably assist microorganisms in the breakdown of fresh detrital material (Gooday et al., 1992). The wide range of trophic strategies that foraminifera adopt (Murray, 2006), makes it hard to predict impacts on trophic webs from a change foraminiferal population dynamics, but changes may impact diatoms, bacteria and the cycling of organic detritus (Gooday et al., 1992). In terms of predators, foraminifera are known to be preferentially selected for by scaphopod molluscs and some shrimps (Murray, 1979). Changes in the availability of foraminifera may, therefore, affect

populations of these organisms. As shrimps are commercially important, this has the potential to impact food security.

A decrease in calcareous foraminifera has implications for inorganic carbon cycling (Lee and Anderson, 1991, Langer et al., 1997, Dissard et al., 2010a, Doo et al., 2014) because a major foraminiferal die-off may act as a negative feedback on atmospheric CO_2 levels, as less calcification would result in less CO_2 released to the atmosphere (Riebesell et al., 2000, Zondervan et al., 2001, Ridgwell et al., 2007). Foraminifera play a role in the production of reef carbonates contributing to an estimated 4.8% of the global carbonate reef budget (Langer et al., 1997, Doo et al., 2014); their loss is likely to create problems for reef dynamics and some areas are reliant on foraminifera for the supply of beach sands (Uthicke et al., 2013, Doo et al., 2014). Benthic foraminifera were estimated to be the single most important contributor (ca. 30% of total sediments) to the sediment mass of Green Island, Australia (Yamano et al., 2000).

The results from this PhD also have global significance in the face of shoaling aragonite and calcite saturation horizons, it is likely that there will be a reduction in the preservation of calcium carbonate on the sea floor and a reduction in the number of foraminifera buried within the sediment as saturation state decreases. Shoaling saturation horizons also have implications for benthic ecology as calcified organisms, such as cold water corals, will be exposed to undersaturated waters, which is expected to affect their distribution (Guinotte et al., 2006, Tittensor et al., 2010, Jackson et al., 2014).

The findings from this investigation are supported by many laboratory studies on single species of benthic foraminifera (e.g. Kuroyanagi et al., 2009, Dissard et al., 2010a, Fujita et al., 2011, Haynert et al., 2011, Hikami et al., 2011, Sinutok et al., 2011, Glas et al., 2012, Keul et al., 2013a, Khanna et al., 2013, Reymond et al., 2013, Schmidt et al., 2014, Sinutok et al., 2014) and other investigations of assemblages from shallow water

CO_2 seeps (Cigliano et al., 2010, Dias et al., 2010, Fabricius et al., 2011, Uthicke and Fabricius, 2012, Uthicke et al., 2013).

Although populations of the small (< 3 cm high) brown macroalga, *P. pavonica*, do not seem to protect epiphytic foraminifera, high biomass stands of seagrass meadows, kelp forests or seaweed farms may be capable of mitigating the effects of ocean acidification at local scales because of high photosynthetic biomass (Semesi et al., 2009a), which will increase pH. High biomass stands may also reduce current flow sufficiently to allow time for high pH to develop for long periods (Cornwall et al., 2014). In addition, the presence of canopies within macroalgal stands can increase the thickness of the diffusion boundary layer substantially, leading to a larger area that is buffered against ocean acidification (Cornwall et al., 2013). Garrard et al. (2014) found that *P. oceanica* meadows around CO_2 seeps off Ischia were capable of providing refugia against the adverse effects of ocean acidification for invertebrates. Seaweeds and seagrasses can thrive in waters with naturally high pCO_2 (Martin et al., 2008, Porzio et al., 2011) and have the potential as a geoengineering solution by consuming dissolved CO_2 and raising local seawater pH (Gao and McKinley, 1994, Manzello et al., 2012, Chung et al., 2013, Cornwall et al., 2014, Hendriks et al., 2014). An increase in pH of up to 0.38 units is possible in the presence of seagrass meadows (Unsworth et al., 2012). The primary production of those seagrasses and macroalgae which are carbon limited increases as CO_2 levels rise (Connell and Russell, 2010, Manzello et al., 2012), which has led to a drive by the United Nations to conserve such habitats. A blue carbon project has been established in South Korea, where natural and made-made marine communities are being used to remove CO_2 in coastal regions. The higher the biomass of these plant communities, the more CO_2 is drawn down (Chung et al., 2013). A reduction in CO_2 emissions is, however, the only way to minimise long-term, large-scale risks posed by ocean acidification.

6.2 Overall conclusions

The overall conclusions from the work presented in this thesis are as follows:

1) There was a reduction in the number of species of foraminifera as pCO_2 increased across shallow water CO_2 gradients.

   This pattern tended to occur regardless of location and was evident across shallow water CO_2 gradients in the Gulf of California, offshore Ischia and offshore Vulcano. This reduction was also evident across different assemblage types; infaunal, epifaunal, epiphytic or those found on artificial collectors.

2) There was a loss of calcareous taxa from foraminiferal assemblages as pCO_2 increased across shallow water CO_2 gradients.

   This also occurred regardless of location and was evident across shallow water CO_2 gradients in the Gulf of California, offshore Ischia and offshore Vulcano. The reduction in calcareous taxa was also evident for different assemblage types (i.e. infaunal, epifaunal, epiphytic and those found on artificial collectors).

3) There was a shift in foraminiferal assemblages from calcareous to agglutinated taxa as pCO_2 increased across shallow water CO_2 gradients.

   Agglutinated foraminifera are expected to be more resilient to ocean acidification, and may even benefit from the loss of calcified competitors, as they do not produce calcium carbonate tests. Samples with ca. 100% agglutinated assemblages have been reported in the geological record (Jones, 1988, Charnock and Jones, 1990, Czarniecki, 1993), but it is not clear if these are a response to the environment or a result of dissolution of calcareous taxa during taphonomy or diagenesis (Dias et al., 2010). The materials that agglutinated foraminifera use to construct their test, however, may still be cemented together by calcite (Sen Gupta, 1999b), which they may have difficulty in producing if calcite saturation state reduces. This may mean that

they are not more resilient to ocean acidification. A shift in foraminiferal assemblages from calcareous to agglutinated forms has been reported previously from shallow water CO_2 gradients (Dias et al., 2010).

4) Small stands of macroalgae did not protect benthic foraminifera from the effects of ocean acidification.

The brown macroalga *P. pavonica* did not provide refugia for benthic foraminifera along a gradient of overlying seawater acidification. There was a reduction in the number of species and individuals of epiphytic foraminifera as pH reduced towards CO_2 seeps from mean pH ~8.19 to ~7.71. High biomass stands of seagrass meadows, kelp forests or seaweed farms, however, may be capable of mitigating the effects of ocean acidification at local scales because of high photosynthetic biomass (Semesi et al., 2009a), which is expected to increase pH. High biomass stands may also reduce current flow sufficiently to allow time for high pH to develop for long periods (Cornwall et al., 2014).

5) There was post-mortem dissolution of calcareous benthic foraminifera in the low pH conditions.

Samples examined from the Gulf of California (pH 7.55 – 7.88) had post-mortem test dissolution, suggesting that once the foraminifera were dead they were subject to dissolution of their tests. The absence of foraminifera (both stained and unstained) from artificial collectors in low pH conditions (mean pH ~ 7.71) off Vulcano, suggests that foraminifera were undergoing post-mortem dissolution in the high CO_2 conditions and/or they were not washed onto the collectors in the first place.

6) The settlement of benthic foraminifera was adversely affected by ocean acidification with a reduction in the number found on artificial collectors in the lowest pH conditions (mean pH ~ 7.71, minimum Ω = 1.82).

Foraminifera appeared to settle on artificial collectors as adults, rather than propagules or juveniles. Foraminiferal assemblages found on artificial collectors were similar to epiphytic assemblages, so it is possible that mainly dead foraminifera were transported onto the artificial collectors from surrounding macroalgae. In many cases, no benthic foraminifera were found on artificial collectors in the lowest pH conditions.

7) There was evidence for a decrease in $\delta^{11}B$ with a decrease in pH for smaller (250 – 500 μm) *A. lessonii*, but not for larger (>500 μm) *A. lessonii* or *C. spengleri* collected across a shallow water gradient in CO_2.

This is thought to be because of the presence of symbionts, which increase pH due to photosynthesis and suggests that in the larger foraminifera, photosynthesis dominates over ambient pH (Babila et al., 2014). There was a large variation in $\delta^{11}B$ for individual *A. lessonii* of the same size collected from the same pH conditions. This is thought to be a result of wide pH variability found around shallow water CO_2 seeps. Although individuals were collected from the same sample site, they may not have calcified under the same pH conditions. These preliminary findings open up the research possibility for using shallow water gas seeps to validate the boron isotope palaeo-pH proxy.

6.3 Methodological limitations

There are some limitations to the methods used in this thesis. In some cases there were no replicates. This is a large limitation, especially when sampling from the natural environment where there is often a large amount of variability and patchiness in the distribution of foraminifera (Buzas, 1970, Bernstein et al., 1978, Griveaud et al., 2010). The samples from the Gulf of California were collected during a multidisciplinary research cruise. Ideally, to avoid pseudo replication (Schönfeld et al., 2012), replicate samples would have been obtained from separate deployments of the Smith McIntyre grab. This, however, was not possible. No replicate cores were taken from the same

deployment. This was in part because the samples were used by other members of the research cruise in other analyses. At Ischia only one sample of each type was collected at each sample site. No replicates were collected because the experiment had been designed as a pilot investigation with additional sampling planned for April 2012, but for logistical reasons this additional sampling was not undertaken. Many of the artificial collectors placed across the shallow water CO_2 gradient off Vulcano were lost during the experiments. This resulted in a lack of replicates in some cases. Attaching the fishing line to an underwater moored object would be beneficial, but at the time of conducting the experiment such objects were not available. In addition, the moored objects could then add a confounding factor and would make the placement of replicates difficult.

The use of rose Bengal to determine living (or recently dead) individuals is another methodological limitation. There is much debate over the use of the rose Bengal staining technique (Walton, 1952, Murray, 1991a, Bernhard, 2000, Murray and Bowser, 2000, Bernhard et al., 2006) and the length of time for which samples have been left in the rose Bengal stain has varied greatly amongst researchers. A staining time of three hours was used in this thesis following the method used by Sadri et al. (2011) who also examined epiphytic foraminifera. As a means of standardising the rose Bengal staining technique, Schönfeld et al. (2012) recommended using a staining time of at least 14 days. The staining time of three hours, as used in this study, is much less than this and could mean that the foraminifera did not have sufficient time to pick up the stain (Schönfeld et al., 2012). This would lead to an underestimation of the number of living individuals. For these investigations, where the effects of ocean acidification were being examined, it was considered more appropriate to underestimate, rather than overestimate, the number of living individuals.

6.4 Limitations of research

Problems associated with the investigations conducted as part of this thesis have been discussed in each chapter. There are more general problems associated with the use of shallow water CO_2 seeps in ocean acidification research that require discussion. Although shallow water gas seeps can be an extremely useful means of examining the impact of chronic ocean acidification on marine organisms and communities, there are several limitations associated with their use. There is usually high variability in pH across the shallow water CO_2 gradients (Kerrison et al., 2011), so sample sites have to be selected that avoid, or account for, minima that exceed values predicted due to ocean acidification. Organisms at stations along a seep generated CO_2 gradient may be responding to the variability in pH (or pCO_2 or calcium carbonate saturation) rather than the low pH conditions directly. In shallow water marine environments, however, pH is often variable (Raven et al., 2005, Hofmann et al., 2011). Hofmann et al. (2011) found that pH variability could be 0.024 – 1.430 pH units with diel, semi-diurnal and stochastic patterns of variation due to changes in mixing, tidal excursions, biological activity, residence time or riverine inputs. Many organisms actually experience pH regimes that are not predicted for average surface waters until 2100 (Hofmann et al., 2011). If organisms are resilient to pH variability it is arguable that, where there are periods of relatively low pH, these organisms may be resilient to ocean acidification. Variability in CO_2 levels was rarely considered in early ocean acidification research (Johnson et al., 2012) and the response of organisms to constant low pH was investigated. Seep systems can reveal ecological responses to long-term increases in CO_2 levels that retain natural pH variability (Kerrison et al., 2011, Johnson et al., 2012).

Another issue with CO_2 seep research is that there can be immigration of organisms from ambient conditions to the seep sites. In the future, high pH conditions will not be available from which benthic organisms can recruit. Across shallow water CO_2 gradients, the only control over the carbonate system used so far, has been the choice and location of sample or experimental sites and other environmental parameters such

as temperature, salinity or nutrient concentration cannot be controlled (Barry et al., 2010b). The choice of sample sites is extremely important and sites need to be chosen that have identical temperature, salinity and alkalinity with pCO_2 as the key environmental variable.

The use of shallow water CO_2 seeps can also be logistically challenging. The seeps are often in remote locations and supplying fieldwork equipment or storing and shipping samples can be problematic. Lack of replicate shallow water CO_2 gradients that lacked confounding gradients in temperature, alkalinity and toxic compounds was a problem recently (Havenhand et al., 2010), but now increasing numbers of sites are being used to test observations initially made at Ischia (e.g. Vulcano (Johnson et al., 2012, Boatta et al., 2013, Johnson et al., 2013, Milazzo et al., 2014), Methana (Baggini et al., 2014, Bray et al., 2014), Papua New Guinea (Fabricius et al., 2011, Johnson et al., 2012)). The finding of similar ecological patterns across CO_2 gradients at multiple seep sites builds confidence in predictions. Considering these problems, it is no doubt best to use multiple approaches to help predict the likely effects of rapid ocean acidification (Rodolfo-Metalpa et al., 2011). Using a combination of approaches allows multiple environmental stressors to be examined, such as ocean acidification and increased temperature or eutrophication (Rodolfo-Metalpa et al., 2010; 2011, Reymond et al., 2013, Schmidt et al., 2014, Sinutok et al., 2014).

6.5 Future direction of research

There are many ways in which research into the impacts of ocean acidification on shallow water benthic foraminifera can be further developed. It would be interesting to examine benthic foraminiferal assemblages across additional natural gradients in CO_2. If the same patterns are found at additional shallow water seep sites, then this adds more support to previous findings from the Mediterranean Sea (Dias et al., 2010), the tropical Pacific (Fabricius et al., 2011, Uthicke et al., 2013) and those presented in this thesis. Notably there are no observations to date for the Arctic, Antarctic, Atlantic or

Indian Oceans. Sampling of foraminiferal assemblages across shallow water CO_2 gradients at different times of the year would allow for seasonal comparisons. It may be that foraminifera are more sensitive to the effects of ocean acidification at certain times of the year (e.g. warm periods may lead to increased metabolic rates, or dark periods may lead to low primary productivity and energy depletion). Epiphytic foraminifera could be sampled from additional species of macroalgae. It is possible that certain species of macroalgae, or high biomass habitats, such as seagrass beds and kelp forests, are better able to protect foraminifera against the adverse effects of ocean acidification.

Additional studies could involve conducting down-core studies at seep sites. Many of the shallow water gas seeps have been present for hundreds of years; therefore, it is expected that benthic foraminifera found with sediment cores collected across CO_2 gradients would show the effects of ocean acidification over time. It would be interesting to see how foraminifera preserved within the sediments have been affected by high CO_2 from the seeps in the past. This approach, however, might be more valuable at deep-sea CO_2 seep sites where sediment is not re-worked during storms.

Detailed investigations into deformation of foraminiferal tests across natural CO_2 gradients would be of interest. Deformation of foraminiferal tests with ocean acidification have previously been reported from laboratory investigations (Khanna et al., 2013), but none have been reported from examination of natural assemblages across shallow water CO_2 gradients (Dias et al., 2010, Pettit et al., 2013). Such deformations could be artefacts due to being kept in culture and is something that warrants further investigation.

An important follow-up study would be to examine the trace element concentrations and boron isotopic compositions of more species of foraminifera collected across natural CO_2 gradients. This could be combined with laboratory investigations, where

environmental factors and carbonate chemistry parameters can be carefully controlled, to see if results are comparable. A larger sample size would be required for future investigations and measurement of the boron isotopic composition of the seawater around the gas seeps, to verify that this does not differ from other values measured for seawater. It would also be important to measure the pH variability at the seeps, both daily and annually.

The mechanism for the production of calcite cement by agglutinated foraminifera is worthy of further investigation. The process may be similar to that for calcareous foraminifera, where a high internal pH is required to create a calcitic chamber (de Nooijer et al., 2008; 2009a; 2009b; 2014). If this were the case, then ocean acidification may have a deleterious impact on agglutinated foraminifera that produce a calcite cement, due to the increased energy demands associated with increasing pH to the levels required to produce the calcite cement. It is, however, possible that much higher bicarbonate concentrations may facilitate cementation of agglutinated foraminifera.

A major new direction of research would be investigation of the impact of shoaling aragonite and calcite saturation horizons on benthic calcified life, including foraminifera. Large areas of the seabed are becoming corrosive to calcium carbonate; the effects of which are not fully understood. It is vital to see how the ecology of sediment changes across the lysocline and the implications that this has for biodiversity, fisheries and food security.

Future investigations should focus on the adaptive potential of benthic foraminifera, to establish if there is a possibility that benthic foraminifera could evolve to overcome the impacts of ocean acidification in the future. Deep-sea benthic foraminifera have suffered mass extinctions during periods of high pCO$_2$ in the past, such as during the Palaeocene-Eocene Thermal Maximum PETM (e.g. Zachos et al., 2005, Hönisch et al., 2012). Some species of benthic foraminifera have survived periods of ocean

acidification in the past, but this does not mean that modern foraminifera will be insensitive to the effects of anthropogenic ocean acidification because of differences in the current rate of change (Penman et al., 2014).

The results presented in this thesis contribute valuable new information to the debate over the impact of ocean acidification. The examination of benthic foraminiferal assemblages from two previously unstudied shallow water CO_2 seep sites (Gulf of California and Vulcano) has been reported. This PhD thesis is also the first study to look at the trace element concentrations and boron isotopic composition of benthic foraminifera collected across natural gradients in pCO_2 caused by the presence of shallow water gas seeps. The reduction in species richness and diversity and the loss of calcareous species from assemblages as pCO_2 levels increase raises serious concerns for the survival of calcareous benthic foraminifera as the oceans continue to acidify. The loss of calcareous foraminifera has serious implications for marine ecosystems globally, through a reduction in organic carbon production, nutrient cycling and a potential change in populations of prey for foraminifera (bacteria, diatoms) and predators of foraminifera (molluscs, shrimps). A loss of foraminifera also has the potential to affect inorganic carbon cycling and may provide a negative feedback on atmospheric CO_2 levels. Societal impacts might include a reduction in tourism and loss of shoreline protection as foraminifera are important in maintaining coral cays. A reduction in CO_2 emissions is the only way to minimise long-term, large scale risks posed by ocean acidification.

APPENDICES

APPENDIX I: Faunal reference list and taxonomic notes

As this is not a detailed taxonomic study, a reference list of the taxa encountered is given below, with short taxonomic comments where appropriate. The taxa are listed alphabetically. The species names are given together with the original reference where this differs. Generic determinations are based on Loeblich and Tappan (1987). Information from World Register of Marine Species, Murray (1979), Cimerman and Langer (1991), Murray (1991a) and Milker and Schmiedl (2012). Those marked by an asterisk are illustrated in figures. The figure number is listed in brackets.

***Adelosina longirostra* (d'Orbigny)** = *Adelosina laevigata* d'Orbigny, 1846.
Classified as *Quinqueloculina laevigata* in Cimerman and Langer (1991).

***Affinetrina gualtieriana* (d'Orbigny)** = *Triloculina gualtieriana* d'Orbigny, 1839.

***Affinetrina* sp. 1.**

***Ammoglobigerina globigeriniformis* (Parker and Jones)** = *Lituloa nautiloidea* Lamarck var. *globigeriniformis* Parker and Jones, 1865.

***Ammonia beccarii* (Linné)** = *Nautilus beccarii* Linné, 1758.
Specimens from the Gulf of California classified as *A. beccarii* in Pettit et al. (2013), but after further consideration the specimens are not *A. beccarii* (Hayward et al., 2004) and have instead been designated as *Ammonia* sp. 1.

****Ammonia* sp. 1.** (Figure i a).

***Ammonia* sp. 2.**

***Ammonia* sp. 3.**

***Amphistegina lessonii* d'Orbigny, 1826.**

***Amphistegina lobifera* Larsen, 1976.**

***Amphistegina* sp. 1.**

***Bolivina acuminata* Natland 1946.**

***Bolivina acutula* (Bandy)** = *Bolivina advena* Cushman var. *acutula* Bandy, 1953.

***Bolivina difformis* (Williamson)** = *Textularia variabilis* var. *difformis* Williamson, 1858.
Classified as *Brizalina difformis* in Cimerman and Langer (1991).

***Bolivina pseudoplicata* Heron-Allen and Earland, 1930.**

***Bolivina striatula* Cushman, 1922.**
Classified as *Brizalina striatula* in Cimerman and Langer (1991).

Bolivina variabilis **(Williamson)** = *Textularia variabilis* Williamson, 1858.

***Bolivina* (inflated sutures).**
Form with inflated sutures. (Figure i b).

Bolivina **sp. 1.**

Bolivina **sp. 2.**

Bolivina **sp. 3.**

Bolivina **sp. 4.**

Bolivina **sp. 5.**

Bolivina **sp. 6.**
Chambers rounded, final chamber globular.

Bolivina **sp. 7.**

Brizalina **sp. 2 of Cimerman and Langer (1991).**

Buccella tenerrima **(Bandy)** = *Rotalia tenerrima* Bandy, 1950

Bulimina denudata **Cushman and Parker, 1947.**

***Bulimina marginata* d'Orbigny, 1826.** (Figure 2.4 1a).

Bulimina **sp. 1.**

Bulimina **sp. 2.**

Bulimina **sp. 3.**

Calcarina spengleri **(Gmelin)** = *Nautilus spengleri* Gmelin, 1791.
Most authors do not discriminate between *Calcarina gaudichaudii* and *C. spengleri* and use either of the names (Renema and Hohenegger, 2005). *Calcarina spengleri* is used here because Uthicke et al. (2013), who examined foraminiferal assemblages from the same samples, identified specimens as *C. spengleri*.

Cancris auriculus **(Fichtel and Moll)** = *Nautilus auricula* Fichtel and Moll, 1798.

Cassidulina **sp. 1.**

Cassidulina **sp. 2.**

***Cornuspira involvens* (Reuss)** = *Operculina involvens* Reuss, 1850. (Figure ii f).

Cribrostomoides subglobosum (Sars) = *Lituola subglobosa* Sars 1868.
Classified as *Labrospira subglobosa* in Cimerman and Langer (1991).

Cyclocibicides vermiculata (d'Orbigny) = *Planorbulina vermiculata* d'Orbigny, 1826.
Classified as *Cyclocibicides vermiculatus* in Cimerman and Langer (1991).

Cymbaloporetta sp. 1.

Daitrona sp.

Eggerelloides sp. 1.

Elphidium aculeatum (d'Orbigny) = *Polystomella aculeata* d'Orbigny, 1846.

Elphidium crispum (Linné) = *Nautilus crispus* Linné, 1758.

***Elphidium excavatum (Terquem)** = *Polystomella excavata* Terquem, 1875.
A variable species, the variants of which have been given separate species names
(Murray, 1979). (Figure 2.4 3a, 4a).

***Elphidium macellum (Fichtel and Moll)** = *Nautilus macellus* Fichtel and Moll, 1798.
(Figure ii b).

***Elphidium margaritaceum (Cushman)** = *Elphidium advenum* (Cushman) var.
margaritaceum Cushman, 1930.
Pustules covering test which enlarge towards centre of test. Small keel present. (Figure
ii d).

Elphidium cf. *E. advenum.*

Elphidium sp. 5 of Cimerman and Langer (1991).

Elphidium sp. 7 of Cimerman and Langer (1991).

Elphidium sp. 1.

Elphidium sp. 2.
Pustules present on surface.

Elphidium sp. 3.
Large form with carinate periphery.

Elphidium sp. 4.
Medium-sized, keeled form with sub-rounded periphery. Short ponticuli.

Elphidium sp. 5.
Small, keeled form with carinate periphery.

Elphidium sp. 6.
Medium-sized, keeled form with sub-rounded periphery.

Elphidium sp. 7.

Elphidium sp. 8.

***Epistominella bradyana* (Cushman)** = *Pulvinulinella bradyana* Cushman, 1927. (Figure 2.4 2a).

Eponides sp. 1.

Eponides sp. 2.

***Fursenkoina acuta* (d'Orbigny)** = *Polymorphina acuta* d'Orbigny, 1846.

***Hanzawaia nitidula* (Bandy)** = *Cibicides basiloba* (Cushman) var. *nitidula* Bandy, 1953.

***Haplophragmoides canariensis* (d'Orbigny)** = *Nonionina canariensis* d'Orbigny, 1839.

***Haynesina depressula* (Walker and Jacob)** = *Nautilus depressulus* Walker and Jacob, 1798. (Figure 3.22 c).

***Haynesina* sp. 1 of Cimerman and Langer 1991.**

***Laevipeneroplis karreri* (Wiesner)** = *Peneroplis karreri* Wiesner, 1923.

Lagena sp. 1.

Lagena sp. 2.

Lagena sp. 3.

Lenticulina sp. 1.

***Lobatula lobatula* (Walker and Jacob)** = *Nautilus lobatulus* Walker and Jacob, 1798. Classified as *Cibicides lobatulus* in Cimerman and Langer (1991).

***Loxostomum pseudobeyrichi* (Cushman)** = *Bolivina pseudobeyrichi* Cushman, 1926.

***Massilina gualtieriana* (d'Orbigny)** = *Quinqueloculina gualtieriana* d'Orbigny, 1839.

***Miliolinella subrotunda* (Montagu)** = *Vermiculum subrotundum* Montagu, 1803. (Figure 3.22 e).

***Miliolinella webbiana* (d'Orbigny)** = *Triloculina webbiana* d'Orbigny, 1839.

Miliolinella sp. 1.

***Nonionella basispinata* (Cushman and Moyer)** = *Nonion pizarrense* var. *basispinatum* Cushman and Moyer, 1930.

***Nonionella* sp. 1.**

***Nubeculina divaricata* (Brady)** = *Sagrina divaricata* Brady, 1879.

***Patellina corrugata* Williamson, 1858.**

***Peneroplis pertusus* (Forskål)** = *Nautilus pertusus* Forskål, 1775.

***Peneroplis planatus* (Fichtel and Moll)** = *Nautilus planatus* Fichtel and Moll, 1798.

****Pileolina patelliformis* (Brady)** = *Discorbina patelliformis* Brady, 1884.
Synonymised under *Pileolina patelliformis* in World Register of Marine Species, but according to Loeblich and Tappan (1987) *Pileolina* remains unrecognisable based on the type species. Classified as *Conorbella patelliformis* in Cimerman and Langer (1991).
A very abundant species in samples from the Mediterranean Sea. Large variation in the height of specimens. (Figure 3.22 a).

****Pileolina patelliformis* showing plastogamy.**
Plastogamous pairs of *Pileolina patelliformis*, alternatively this could be a specimen with a float chamber. An abundant type in the Mediterranean Sea found as epiphytes. (Figure 3.22 g).

****Planorbulina mediterranensis* d'Orbigny, 1826.** (Figure ii c).

***Pseudotriloculina laevigata* (d'Orbigny)** = *Triloculina laevigata* d'Orbigny, 1826.

***Pseudotriloculina rotunda* (Schlumberger)** = *Triloculina rotunda* Schlumberger, 1893.

***Pseudotriloculina* sp. 1 of Milker and Schmiedl (2012).**

***Quinqueloculina annectens* (Schlumberger)** = *Massilina annectens* Schlumberger, 1893.

***Quinqueloculina auberiana* d'Orbigny, 1839**.

***Quinqueloculina bosciana* d'Orbigny, 1839.**

***Quinqueloculina disparilis* d'Orbigny, 1826.**

***Quinqueloculina limbata* d'Orbigny, 1826.**

***Quinqueloculina parvula* Schlumberger, 1894.**

***Quinqueloculina stelligera* Schlumberger, 1893.**

Quinqueloculina sp. 1.
Smooth-walled form. Slightly raised lip around aperture.

Quinqueloculina sp. 2.
Surface has striations.

Quinqueloculina sp. 3.
Rough surface. Rim around aperture.

Rectuvigerina elongatastriata (Colom) = *Angulogerina elongatastriata* Colom, 1952.

Reophax sp. 1.

Reophax sp. 2.

Reophax sp. 3.

Rosalina globularis d'Orbigny, 1826.

***Rosalina* sp. 1.** (Figure ii a).
Coarsely perforate form.

Sigmoilina costata Schlumberger, 1893.
Classified as *Sigmoilinita costata* in Cimerman and Langer (1991).

Siphonaperta agglutinans (d'Orbigny) = *Quiqueloculina agglutinans* d'Orbigny, 1839.

Siphonaperta sp. 1.

Spiroloculina excavata d'Orbigny, 1846.

Spiroloculina ornata d'Orbigny, 1839.

Textularia sp. 1.

Textularia sp. 2.

Textularia sp. 3.

Textularia sp. 4.

Textularia sp. 5.

Tortoplectella rhomboidalis (Millett) = *Textularia rhomboidalis* Millett, 1899.
Classified as *Abditodentrix rhomboidalis* in Cimerman and Langer (1991).

Tretomphalus bulloides (d'Orbigny) = *Rosalina bulloides* d'Orbigny, 1839.

Trifarina angulosa (Williamson) = *Uvigerina angulosa* Williamson, 1858.
Classified as *Angulogerina angulosa* in Cimerman and Langer (1991).

Triloculina adriatica Le Calvez, J. and Y., 1958.

Triloculina marioni Schlumberger, 1893.

Triloculina plicata Terquem, 1878.

Triloculina sp. 1.

Triloculina sp. 2.

Triloculinella dilatata (d'Orbigny) = *Quinqueloculina dilatata* d'Orbigny, 1839. Classified as *Miliolinella dilatata* in Cimerman and Langer (1991)

Trochammina inflata (Montagu) = *Nautilus inflatus* Montagu, 1808.

Trochammina sp. 1.

Trochammina sp. 2.

Trochammina sp. 3.

Trochammina sp. 4.

Trochammina sp. 5.

Uvigerina excellens Todd, 1948.

Uvigerina sp. 1 of Cimerman and Langer (1991).

Veleroninoides sp. 1.

**Vertebralina striata* d'Orbigny, 1826. (Figure ii e).

Wellmanellinella striata (Sidebottom) = *Planispirina striata* Sidebottom, 1904.

APPENDIX II: SEM images
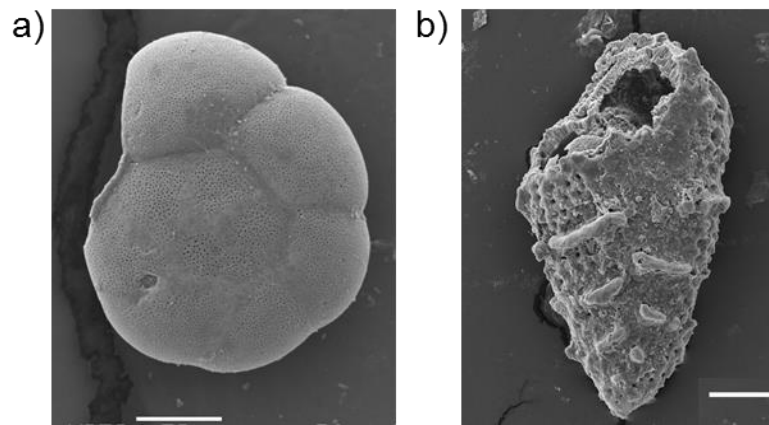
Figure i: Scanning electron microscope images of some benthic foraminifera from the Gulf of California. a) *Ammonia* sp. 1 from station 31, b) *Bolivina* (inflated sutures) from station 2. The scale bar is 50 µm.
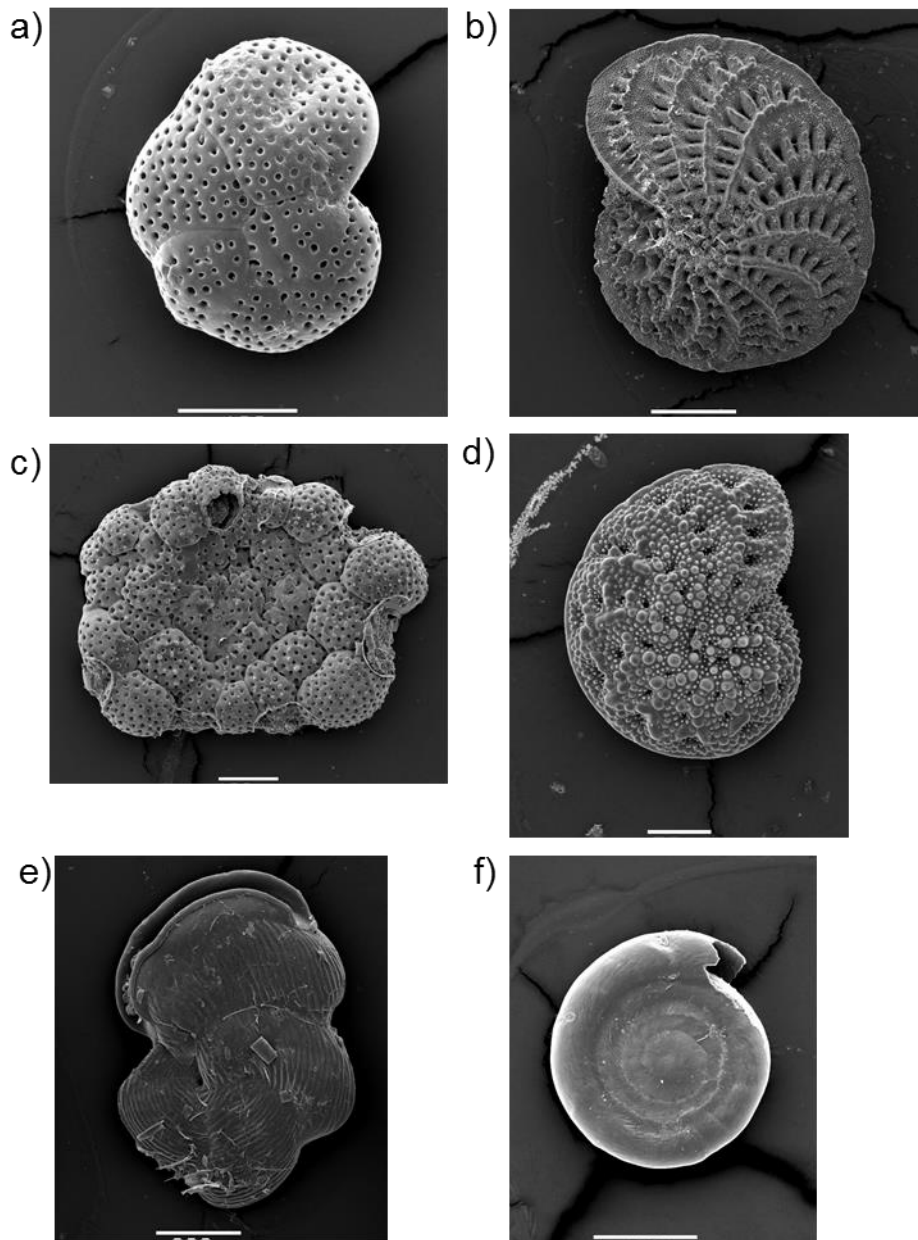
Figure ii: Scanning electron microscope images of some benthic foraminifera found as epiphytes offshore Vulcano. a) *Rosalina* sp. 1 from air dried *Padina pavonica* thalli, May 2012 sample site Ref 2, b) *Elphidium macellum* from air dried *P. pavonica* thalli, May 2012 sample site Ref 2, c) *Planorbulina mediterranensis* from formalin preserved *P. pavonica* thalli, May 2012 sample site Ref 2, d) *Elphidium margaritaceum* from air dried *P. pavonica* thalli, May 2012 sample site Ref 2, e) *Vertebralina striata* from air dried *P. pavonica* thalli, September 2012 sample site Ref 2, f) *Cornuspira involvens* from air dried *P. pavonica* thalli, May 2012 sample site Ref 2. The scale bar for b) and e) is 200 µm, the scale bar for a), c) and f) is 100 µm, and the scale bar for d) is 50 µm.

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