The Response of Marine Assemblages to Ocean Acidification

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Abstract

Global industrialisation has led to the anthropogenic raising of global CO_2 concentration from 280 pp to over 380 ppm in the last 200 years causing oceanic pH to drop by 0.1 unit as a result of a processes called ocean acidification. It is expected to further drop by between 0.3 and 0.4 units over the next 100 years. Quantifying the impact of such a pH shift has, to date, largely relied on laboratory studies of model organisms or simple assemblages in mesocosms. Conversely, this work undertook a series of field experiments to examine the effect of predicted pH environmental conditions on a robust marina fouling assemblage and microorganisms through the manipulation of local CO_2 concentration. CO_2 was delivered and controlled above replicated settlement panels that were freely accessible to normal propagule supply. Over 5 months, recruitment and development of macroorganisms and diversity of microorganisms in biofilms was shown to be largely unaffected by low pH. Results of this investigation were contrasted against microbial diversity in biofilms from a low pH volcanic vent site. Molecular analysis of biofilms failed to detect an influence of pH on diversity. The development of an alternative method of CO_2 delivery using silicone membranes is described, which proved to have both antifouling and ocean acidification experimental applications. In conclusion, the marine organisms examined in this study showed little response to pH change of the order that is expected with the progression of ocean acidification. Significant methodological advances to *in situ* pH experimentation have been made, however, which should assist further investigations.

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Chapter 1

Introduction

Ocean acidification

This Chapter introduces the mechanisms that underpin ocean acidification and the challenges faced by marine organisms with the progression of this phenomenon. Gaps in the current knowledge are identified and the structure and aims of this thesis are defined.

1.0 - What is Ocean acidification?

1.1 - Background

Anthropogenic activities, particularly fossil fuel combustion, have increased the partial pressure (p) of CO_2 in the Earth's atmosphere from 280 to 380 parts per million (ppm) in the last two hundred years (Fabry, *et al.,* 2008). The rate of this recent increase is thought to be the fastest for approximately 125 million years (Blackford & Gilbert, 2007). This relatively rapid increase in atmospheric pCO_2 and other carbon rich gases such as methane is now widely considered by environmental scientists to be the major factor contributing to global warming (Intergovernmental Panel on Climate Change (IPCC) 2007). However, an emerging branch of marine environmental science is grappling to understand and evaluate another recently discovered repercussion of rapid increases in atmospheric pCO_2, a phenomenon known as ocean acidification (OA).

1.2 -Mechanism of CO_2 Driven pH Change

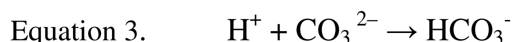
Ocean waters have acted as a sink for atmospheric carbon dioxide over geological timescales. Oceanic pH is thought to have been up to 0.6 pH units lower in ancient oceans than the present day, but has remained above pH 8 for at least the last 20 million years (Pearson & Palmer, 2000). Since the industrial revolution, the ocean has up-taken nearly half of all anthropogenic atmospheric CO_2, approximately 2 Gt per year, reducing the atmospheric pCO_2 (Pearson & Palmer, 2000). However, when CO_2 dissolves in seawater, carbonic acid is initially generated.

Equation 1. $CO_2 + H_2O \rightarrow H_2CO_3$

The carbonic acid is buffered by seawater and dissociates to form bicarbonate and H^+.

Equation 2. $H_2CO_3 \rightarrow H^+ + HCO_3^-$

Some of the H^+ then reacts with carbonate ions existing in the seawater, generating more bicarbonate.



Equation 3. $H^+ + CO_3^{2-} \rightarrow HCO_3^-$

This complex buffering process can be summarised by the following equation which is known as the bicarbonate buffer system in seawater.

Equation 4: $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + H^+$

This series of reactions ultimately leads to a decrease in CO_3^{2-} ions, and an increase in H^+ ions, lowering the pH of shallow ocean waters resulting in OA (Caldeira & Wickett, 2003). OA was first introduced to the wider scientific audience by Richard Feely in the early 1980s

where he identified the link between atmospheric CO_2 and oceanic pH change (Feely & Chen, 1982; Feely, *et al.,* 1984).

During the last 200 years the pH of global ocean water has dropped by 0.1 of a unit as a result of OA, which equates to approximately a 30% increase in H^+ ion concentration (Houghton *et al.,* 2001). A further pH decrease of as much as 0.3 – 0.4 of a unit is predicted by the end of the century, depending on future carbon emissions (Caldeira & Wickett, 2003). A pH decrease of this magnitude equates to an increase in H^+ concentration between 100 - 150 % in three hundred years, and the biggest fluctuation in ocean pH in 25 million years (Figure 1).

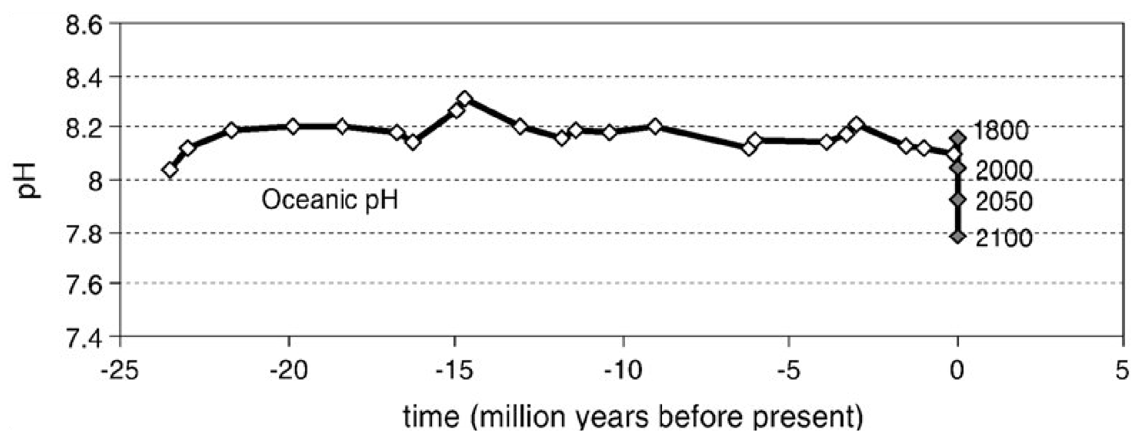


Figure 1: *Past and predicted variability of surface ocean pH from Blackford & Gilbert (2007)*

This widespread decrease in ocean pH may be locally accelerated by CO_2 seepage from carbon capture and sequestration schemes (CCS). These schemes are currently operating and are considered by policymakers as a viable global option for climate change mitigation (Holloway, 2005). For example, the Norwegian energy company StatOilHydro has been injecting waste CO_2 produced during the refinement process of natural gas into an under-ground saline aquifer located in the North Sea called Sleipner, since 1996. (IPCC, 2005**).** Approximately one million tonnes of CO_2 each year are disposed of in this fashion at this site.

However, the environmental consequences of localised OA produced by seepage into deep sea benthic environments are currently unknown.

Preliminary research suggests that some deep-sea organisms like cephalopods have evolved to persist under stable environmental pH conditions, typical of deep sea environments (Drazen, 2002; Seibel & Walsh, 2002). These organisms typically exhibit slow metabolic rates which enables them to cope with sporadic food supplies and cold waters (Drazen, 2002). These adaptations have enabled deep-sea organisms to thrive in what is a stable environment compared to shallow and pelagic waters. However, the trade-off is that many deep-sea species are expected to possess very little tolerance of environmental pH change (Seibel & Walsh, 2002; Pane & Barry, 2007).

1.2.1 -Financial and Social implications of OA

The potential financial implications of OA are vast. The global fishing market was valued at 80 billion dollars in 2008, yet the lower trophic levels which under-pin these fish stocks are considered to be at high risk of depletion as a result of OA. A recent research program supported by United States National Oceanic and Atmospheric Administration (NOAA) suggests that decreased oceanic pH is likely to be underlying the dramatic decrease in pteropod abundance, which can periodically and severely limit Pacific salmon stocks. The United States Forest Service released figures in 2003 valuing the pacific salmon fishing industry in Alaska alone at 195 million dollars and estimated it provided employment for 55 thousand people. The same dependence of fish stocks on zooplankton abundance has been shown to apply across ocean systems (Mueter *et al.,* 2009).

There are also social implications associated with OA. Coral reefs are well described as being biologically valuable ecosystems, but their prosperity also has strong implications for the welfare of the estimated 500 million people who depend directly on coral reefs for food, coastal protection and revenue (Hoegh-Guldberg, 1999). Global goods and services provided by coral reefs are estimated at £375 billion per annum (Hughes *et al.,* 2003; Ammar, 2009). OA threatens this social dependence on coral reef ecosystem services, as the corals themselves are thought to be at considerable risk from decreased calcification, and ultimately dissolution in seas under-saturated in CO_3^{2-} ions (Hoegh-Guldberg, 1999; Gattuso & Buddemeier, 2000; Hughes, *et al.,* 2003; Yates & Halley, 2006; Andersson, *et al.,* 2007; Turley, *et al.,* 2007; Balch & Fabry, 2008; Fabry, *et al.,* 2008; Lough, 2008; Manzello, *et al.,* 2008; Wood, *et al.,* 2008; Wei, *et al.,* 2009).

The link between social welfare and OA is not restricted to coral reefs and large scale fisheries, but also include other practices such as small scale subsistence fishing. Global anthropogenic food security is linked to the functioning and productivity of marine ecosystems as an estimated 2.6 billion people depend on protein derived from marine ecosystems for 20% of their protein intake (Pauly, *et al.,* 2005; Worm, *et al.,* 2006; Brunner, *et al.,* 2008). Current research suggests that OA may result in reduced fitness and survival of species, threatening the functioning of entire food webs which provide goods and services to humans (Fabry, *et al.,* 2008; Wood, *et al.,* 2008). Concerning as these potential threats are, the potential indirect repercussions of global changes in ocean chemistry and geochemical cycling maybe even more far-reaching.

1.2.2 - Global Geochemical Cycling

A decrease in ocean pH produced by dissolution of atmospheric CO_2 in the ocean could alter the chemical speciation of nutrients, affecting their bioavailability (Zeebe & Wolf-Gladrow, 2001; Huesemann, *et al.,* 2002; Blackford & Gilbert, 2007). Therefore, OA has the potential to disrupt biological processes which drive life-sustaining biogeochemical cycles and food webs which most life on earth depends on. Consequently, OA is a major concern not only for marine industries such as fishing, but also on a global scale for policymakers and the general public.

1.2.3 - Global Research Effort

The global implications and scientific uncertainties surrounding OA have been recognised by many of the major research funding bodies around the world that have allocated, or are in the process of allocating, significant proportions of marine research resources towards filling the knowledge gaps and evaluating potential impacts of OA.

NOAA funded research which has been a driving force behind the early exploration and raising awareness of OA. A cruise in 2006 jointly funded by NOAA and the United States National Science Foundation (NSF) was pioneering by actually measuring a decrease in ocean pH across the northern and southern hemispheres, and in doing so supporting the theoretical predictions proposed in 2005 during the National Centre for Atmospheric Research (NCAR) (a sub-division of the NSF) sponsored workshop in Florida, United States, entitled the "Workshop on the Impacts of Increasing Atmospheric CO_2 on Coral Reefs and Other Marine Calcifiers". NOAA produced a series of press releases during 2006 which highlighted the potential implications of OA, including the report entitled "Impacts of Ocean Acidification on Coral Reefs and Other Marine Calcifiers". This document jointly funded by

the NSF and US Geological Survey (USGS) uniquely included a series of recommendations for the direction of future research.

Australia's Commonwealth Scientific and Industrial Research Organisation (CSIRO) have published a series of papers in high ranking scientific journals relating to OA, with particular emphasis on the potential changes to nutrient availability, food web alterations, polar and sub-arctic biology (Poloczanska, *et al.,* 2007; McNeil & Matear, 2008). CSIRO have also funded the production of fact sheets aimed at disseminating the growing scientific concern surrounding OA to the general public.

UK based research agencies such as the Royal Society have also contributed to the global effort by consolidating much of the current knowledge of the time and producing a report entitled "Ocean Acidification Due to Increasing Atmospheric Carbon Dioxide" in 2005. This document was conceived as policy advice for the UK government. The Natural Environment Research Council (of the United Kingdom) (NERC) has also been actively funding OA research such as the Bergen Mesocosm Experiment (Joint, 2006). NERC are also co-funders of a 12 million pound research programme dedicated solely to OA research, a significant proportion of which will be conducted at the Plymouth Marine Laboratory, UK.

International research organisations have also been recently established with the aim of advancing and consolidating OA research efforts. These organisations aim to provide an efficient use of expertise and resources in recognition of the potential severity of the phenomenon and the limited time available before potentially serious global repercussions may be experienced. One such organisation, European Project on Ocean Acidification

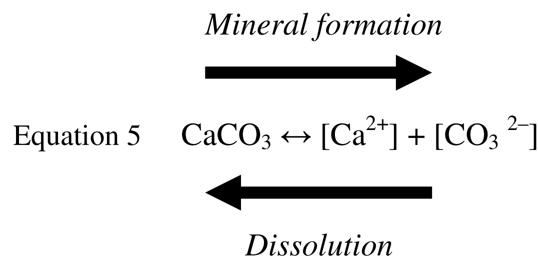
(EPOCA) was established in 2008 and brings together scientists from nine European countries. EPOCA aims to provide an integrated approach to OA research by investigating effects across environment types, and use paleo-records to predict future carbonate availability. EPOCA conducted an experimental program in Svalbard, Norway, during 2009 aimed at identifying benthic responses to OA and followed this with a pelagic experimental regime in 2010. Other international organisations such as United Nations Educational Scientific and Cultural Organisation (UNESCO) have recognised the need for OA research and co-sponsored along with International Geosphere-Biosphere Programme (IGBP), The Second Symposium on the Ocean in a High-CO_2 World in Monaco during 2008 (Orr, *et al.,* 2008). This international conference exhibited the combined efforts of many of the experts in OA research and culminated in the introduction of the Monaco Declaration whereby over 150 scientists highlighted the potential global implications of OA and jointly call for policy makers to reduce CO_2 emissions to reduce the harmful impacts expected to occur if emissions continue at present day levels (Orr, *et al.,* 2008).

1.3 - Challenges faced by marine organisms

1.3.1 - Calcification

Marine calcifying organisms are initially the most obvious group at risk from an increase in environmental acidity due to the dissolution potential of biogenic carbonate at low pH (Gattuso & Buddemeier, 2000; Hallock, 2005; Andersson, *et al.,* 2007; Hall-Spencer, *et al.,* 2008; Hofmann, *et al.,* 2008; Przeslawski, *et al.,* 2008; Hall-Spencer & Rauer, 2009). Although some species are capable of passive carbonate ion concentration, many calcifying organisms exert considerable metabolic effort hyper-concentrating carbonate ions from

seawater for the construction of their calcareous tests (Wood, *et al.,* 2008; Wei, *et al.,* 2009). The effort required for the hyper-concentration of carbonate ions for calcification is dependent on the saturation state of carbonate ions in the seawater. OA reduces carbonate ion availability, continuously lowering the saturation state of carbonate ions in seawater. Theoretically, reducing the saturation state of seawater will increase the metabolic effort calcifying organisms must exert in order to construct their tests. In addition, when the carbonate saturation state reduces to a critical level, calcium carbonate begins to dissolve (Byrne, *et al.,* 1984; Feely, *et al.,* 1988; Raven & Caldeira *et al.,* 2005). Magnesium calcite, a form of calcium carbonate, dissolves first, followed by aragonite and calcite. The difference in their respective dissolution potential is a result of their different mole per cent of magnesium, which is in turn dependant on the Mg / Ca ion ratio in the seawater where the biological mineral was formed (Stanley & Hardie, 1998; Raven, *et al.,* 2005; Andersson, *et al.,* 2008).



Resitance to dissolution is also influenced by the production of other biogenic substances such as chitin. Cummings et al., (2011) demonstrate that the production of the chitin shell structure within which bicarbonate and carbonate is deposited during shell formation is critical for the structural integratiy of a shell.

OA produces a net increase in bicarbonate, a compound also used in the process of calcification by marine organisms like corals. The effect of increasing bicarbonate in seawater has been shown to increase calicifcation rates, even under low pH conditions in some species (Jury *et al*., 2009).

Calcareous shells and tests are critical for the survival of calcifying species such as bivalves, echinoderms and crustaceans (Raven, *et al.,* 2005; Hall-Spencer, *et al.,* 2008; Martin *et al.*, 2008; Przeslawski, *et al.,* 2008; Hall-Spencer & Rauer, 2009). Tests and shells perform vital roles in the life strategies of many marine organisms, frequently in a protective role where their structural integrity reduces predation risk by the formation of a hard defensive barrier between the calcifier and its predators (Gutierrez, *et al.,* 2003). Any compromise in this structural integrity as a result of partial dissolution may significantly increase the success rate of attempted predation events (Gutierrez, *et al.,* 2003).

Laboratory based experimentation designed to measure calcification rates under high CO_2 conditions have produced conflicting results. Some organisms appear able to maintain or increase calcification under experimentally reduced pH levels (Iglesias-Rodriguez, *et al.,* 2008; Wood *et al.,* 2008; Ries *et al.*, 2010) whereas other animals reduce or are unable to maintain calcification to such a degree that their tests disintegrated during sampling (Shirayama & Thornton, 2005). In one recent calcification study, the brittle star *Amphiura filiformis* was seen to intensify calcification under high CO_2, low pH conditions. However, this metabolically costly process resulted in wastage of muscles that controlled arm movements (Wood, *et al.,* 2008). The overall result was a lack of motility that may compromise predator evasion and ultimately the survival of the species (Wood, *et al.,* 2008).

Another group of marine calcifier, coccolithophorids, are unicellular marine algae which produce carbonate discs or liths on their external surfaces. These planktonic organisms appear in vast numbers creating dense blooms under optimal environmental conditions (Turkoglu, 2008; Signorini & McClain, 2009). The accumulated extent of these annual

blooms can be observed in substantial geological formations such as the White Cliffs of Dover, UK which are made up almost entirely of carbonate liths produced by cooclithophorids which have been deposited on the ocean floor.

This deposition of carbonate produced by uni-cellular algae like coccolithophorids constitute between 20 - 80 % of biogenic carbonate in-put from the photic zone into the deep sea (Ziveri, *et al.,* 2007). These highly influential algae also exist in global fossil records dating back over 225 million years (Ziveri, *et al.,* 2007). Because of the relative ease in which coccolithophores can be cultured in the laboratory, together with their calcification and primary production capabilities, they have been the subject of several studies which aimed to measure the effect of calcification under high CO_2, low pH conditions (Burkhardt, *et al.,* 1999; Riebesell, *et al.,* 2000; Berry, *et al.,* 2002; Riebesell, 2004; Riebesell, *et al.,* 2008).

The results of these studies are far from consistant with coccolithophores producing fewer, malformed liths at a reduced rate under elevated CO_2 and low pH conditions in some investigations (Riebesell, *et al.,* 2000), and normal liths at increased rates in others (Iglesias-Rodriguez, *et al.,* 2008). One explanation for the contradictory results may stem from current research that suggests that at an ecosystem level, the processes of calcification and photosynthesis compete for the same pool of dissolved inorganic carbon (DIC) (Berry, *et al.,* 2002). This competition for carbon at an organism level within an individual coccolithophore is not fully understood, but is the subject of on-going research.

It would appear that peculiarities of the particular mechanisms different species and sub populations within species employ to generate carbonate tests are important in determining

their performance under future OA scenarios. However, it is also apparent that further research is required before this susceptibility is understood and can be accurately predicted.

1.3.2 - Primary Production

As DIC is a fundamental requirement for marine primary production, it would be expected that the increase in the availability of CO_2 which is driving OA would result in increased production. Indeed, increased primary production has been observed in studies under elevated CO_2 conditions, microalgae (Riebesell, *et al.,* 1993) macroalgae (Borowitzka & Larkum, 1976) and sea grasses (Zimmerman, *et al.,* 1997). However, as less than one per cent of DIC persists in seawater in the form of dissolved CO_2, most marine algae with the exception of sea grasses use HCO_3^- as a phosphosynthetic substrate (Raven & Caldeira *et al.,* 2005; Rost, *et al.,* 2008). It is likely that many observed increases in primary production may actually result from increased HCO_3^- availability produced by the buffering capacity of seawater in response to OA, an indirect response of elevated CO_2, (see mechanisms section 1.2.).

The main carboxylation enzyme involved in photosynthesis is ribulose-1, 5-bisphosphate carboxylase/oxygenase (RubisCO) which has a low affinity for CO_2 (Rost, *et al.,* 2008). To compensate for this low affinity, marine phytoplankton which are responsible for approximately half the world's primary production, operate CO_2 concentrating mechanisms. These mechanisms can be either passive or metabolically demanding depending on the species (Rost, *et al.,* 2008). Theoretically, both methods would become either more efficient or less energetically demanding respectively, as OA persists and the concentration of HCO_3^- in seawater increases.

Other marine primary producers such as cyanobacteria and diatoms have responded to high pCO_2 conditions in experiments by increasing primary production rates (Bellerby, *et al.,* 2008; Guinotte & Fabry, 2008). Diatoms in particular have also responded to *in situ* pCO_2 increases by shifting species dominance within assemblages (Rost, *et al.,* 2008). However, considerable uncertainty still surrounds the effect of OA on primary production as frequently these experiments are subject to complications associated with laboratory cultures. These constraints include unnatural cell densities and nutrient loads, the use of single strains and also acute pH exposures that differ from the gradual pH change expected to occur with the progression of OA.

1.3.3 - Microbiological Response

The marine microbiological response to OA is poorly understood. Aquatic microbial assemblages in general are highly responsive to fluctuations in environmental conditions (Lindstrom, *et al.,* 2005; Yannarell & Triplett, 2005; Schimel, *et al.,* 2007). Current research suggests that eukaryotic organisms under chronic pH stress often respond by shifting metabolic effort to acid/base regulation, at the expense of other homeostatic functions such as protein synthesis (Seibel & Walsh, 2002). Some prokaryotes also demonstrate short-term low pH tolerance by increasing the rate of metabolically driven active transport of H^+ which increases their intracellular pH (McNeill & Hamilton, 2004).

Contrastingly, other microbes are known to manipulate their local environment by altering the products of their metabolic fermentations. This process usually lowers the extra-cellular pH, and is thought to provide a micro-environmental niche which reduces inter-specific competition with less tolerant microbes (Dilworth & Glenn, 2007). The long term viability of acid/base regulation in nutrient cycling microbes, and ultimately OA tolerance, is not clear.

The microbiological response to OA is particularly critical in the marine sediment environment. In this environment, the relatively rapid change in environmental pH that is occurring with the progression of OA has potential to significantly alter microbial ecology (Dashfield, *et al.,* 2008). Although a large pH gradient exists in coastal sediments (1 unit Δ pH in the top 30 cm of sediment) many organisms operate in a narrow pH niche within the gradient, and are therefore not necessarily tolerant of stress caused by pH fluctuation (Furukawa, 2001).

Marine sediments often appear barren and devoid of life from the surface. However, these environments are the most abundant environment types on earth and frequently support high biomass per unit volume (Webb, 1996) and form a medium for complex biological activity that is vital in the cycling of life sustaining nutrients such as nitrogen (Widdicombe & Austen, 1998). The fixation, metabolism and final reduction of nitrogen back into the atmosphere is largely driven by microbes, and occurs in and around coastal marine sediments (Hunter, 2006). Nitrogen availability partly controls primary production in the oceans and is a fundamental component in DNA and proteins (Charria, *et al.,* 2008). However, alarmingly, scientists currently do not understand how the microbes involved in nitrogen cycling will respond to OA.

Microbes can reproduce extremely rapidly, and have a high rates of mutations in the form of deletions, duplications, inversions, translocations, and insertions (Drake, 1991; Springman, *et al.,* 2010). Microbes also exchange genetic material between individuals during transformation (Herbes & Schwall, 1978), transduction (Jiang & Paul, 1998), and conjugation (Martina & Stefan, 1999). As a result, the potential for adaptation to life under low pH

conditions is high (Springael & Top, 2004). However, there is currently no actual measure of microbial adaptive capacity in relation to OA.

1.3.4 - Generic Response

Most living organisms are dependent on metabolically derived energy in the form of adenosine triphosphate (ATP) to perform homeostatic functions. The allocation of metabolic energy in living organisms can vary in response to short term events like predator evasion (Houston, *et al.,* 1993; Werner & Anholt, 1993; Turner, 1997; Andrew & Thomas, 2002) or chronic environmental stress (Bayne, *et al.,* 1979; Bodar, *et al.,* 1988; Adams & Greeley, 2000; Lodeirosa, *et al.,* 2001; Coen & Janssen, 2003; Smolders, *et al.,* 2004), and also to long term periodic or seasonal events like growth and the production of gametes (Bricelj & Malouf, 1998; John & Parkinson, 2001; Tarjuelo & Turon, 2004). Ultimately, however, the metabolic energy available to an organism is limited by factors like nutrient availability and foraging efficiency, meaning that an organism's capacity to allocate its metabolic resources is fundamental to its fitness and survival.

The resource allocation of metabolically derived energy in marine organisms has evolved over millions of years to enable them to carry out their life strategies and deal with periodic events such as reproduction and environmental stress (Ramirez, 2002). Crucially however, this evolution has occurred while several environmental conditions, including ocean water pH, have remained relatively constant over the last 20 million years (Blackford & Gilbert, 2007, Figure 1.).

As the pH of ocean waters decreases with the progression of OA, marine organisms are expected to face acidification stress as the pH of their environment drops below ambient

conditions. The most common mechanisms currently known to be used by marine organisms for intracellular acid/base regulation are the metabolic inter-conversion of acids into bases, intracellular buffering and the active transport of H^+ ions across and the cell membranes (Seibel & Walsh, 2002). All of these processes are accompanied by a metabolic cost, which is further exacerbated by a reduction in oxygen binding capacity of respiratory proteins caused by elevated extra cellular pH (Ramirez, 2002; Seibel & Walsh, 2002; Wood, *et al.,* 2008).

Perhaps the greatest concern of marine scientists investigating OA is how marine organisms will cope with the increased metabolic demand of dealing with pH stress over chronic or indefinite timescales. Many marine animals, particularly those living in estuarine (Howland, *et al.,* 2000) or tidal environments routinely deal with acute pH stress (Gnaiger, *et al.,* 1978), and the pH of the shallow oceans vary daily as an indirect result of primary production (Gnaiger, *et al.,* 1978; Andersson, *et al.,* 2007). However, these events are temporary or periodic and allow the afflicted organism’s periods of time where they are exposed to ambient pH in which to recover and revert to normal metabolic loads. These normal environmental shifts present a very different situation compared to the advance of OA. OA is effectively a one-way process in relation to biological timescales where the environmental pH is decreasing, but will not revert back to ambient conditions, possibly for thousands of years depending on the rate of ocean water mixing and flux in the carbon cycle.

1.3.5 - Gaps in the current knowledge

Although OA research is a relatively new field, 372 experimental works currently exist in peer reviewed journals and data from these studies have been subjected to a recent meta-analysis (Hendriks, *et al.,* 2010). The aim of the meta-analysis was to evaluate the threat posed to global biodiversity by OA, and the results suggest that the biological responses in general are variable and are not always negative. For example, growth rates across different phyla were seen to increase under some high CO_2 exposures (Hendriks, *et al.,* 2010). Calcification and fertility were seen to be generally negatively influenced by high CO_2 treatments across all groups of organisms (Hendriks, *et al.,* 2010). The meta-analysis results provide some evidence of a heterotrophic / autotrophic divide, with responses to high CO_2 being generally more positive in autotrophs, and more negative in heterotrophs.

Hendriks, *et al.,* (2010) interpret their findings to indicate that the potential threat posed by OA is perhaps less extreme than was originally predicted. Crucially however, the meta-analysis is based on a collection of studies that routinely fail to look at multiple cohorts, down-stream reproductive viability, ecological responses and synergistic responses with other factors such as temperature. It seems premature to evaluate the threat OA poses to global biodiversity, when it is clear that most current experimental approaches are failing to fully represent the effect of OA on marine organisms, such as multi species responses over chronic timescales.

The results of the meta-analysis conducted by Hendriks, *et al.,* (2010) contrast with other similar meta analysis, and there is concern that this paper does not offer a fair representation of the results of individual studies carried out to investigate the effects of OA on marine organisms to date (Dupont, *et al*., 2010; Kroeker, *et al*., 2010). What is questionable is the

ability of the majority of OA related experiments used in the meta-analysis to accurately reflect OA, given the lack of attention paid to processes like recruitment and succession. This need for a more robust form of experimentation with an emphasis on measuring whole assemblage responses to OA is recognised by Hendriks, *et al.* (2010) which further adds to the justification for the direction of this thesis. It is my view that the review by Hendriks, *et al.* (2010) serves more to highlight the need for a new approach to OA experimentation, rather than as a weighting of the relative importance of OA as a threat to global diversity.

OA is a global phenomenon with the potential to disrupt or alter the most crucial and fundamental aspects of life in the oceans and on earth including primary production, nutrient speciation, nutrient cycling, calcification and metabolism. Early efforts to measure and predict these potential impacts have been made and are reviewed by Hendriks, *et al.* (2010). However, significant gaps in our knowledge remain.

These knowledge gaps have now been internationally identified as research priorities following the first and second symposiums on The Oceans in a High CO_2 World (Orr, *et al.,* 2008). These key gaps were identified as the need to understand ecological responses, long term responses over multiple generations, together with evaluating the adaption potential of marine organisms. The proceedings from these landmark conferences discuss the need for an urgent inter-disciplinary approach to allow quantification of the magnitude of the impacts posed by OA. In addition, they call for greater investigation into ecosystem wide responses to decreased pH and mention *in-situ* perturbation experiments as a possible but economically costly way of gaining such knowledge.

Experimentally investigating the responses of marine organisms to OA *in situ* is difficult due to the pH buffering capacity of seawater. As a result, the number of true *in situ* studies are limited and almost exclusively conducted by the Montaray Bay Aquarium Research Institute (MBARI). These experiments focus on the concept of the terrestrial Free Air CO_2 Enrichment (FACE) experiment (Figure 2) which was subsequently adapted for marine applications in 3 different designs (Figure 3).

Figure 2. *Terrestrial Free Air CO_2 Enrichment (FACE) experiment which was adapted for marine application. The diametres of each circular array is 27 m. Source www.bnl.gov/face/images/GEORGE_sm.jpg*

The circular arrays used in the FACE experiments were made up of a series of vertical vent pipes or VVPs. The CO_2 supply to each VVP was independently controlled at the control centre shown in the top left of Figure 2 to ensure that gas was correctly supplied to the treatment plot to take into account the effects of variation in wind speed and direction.

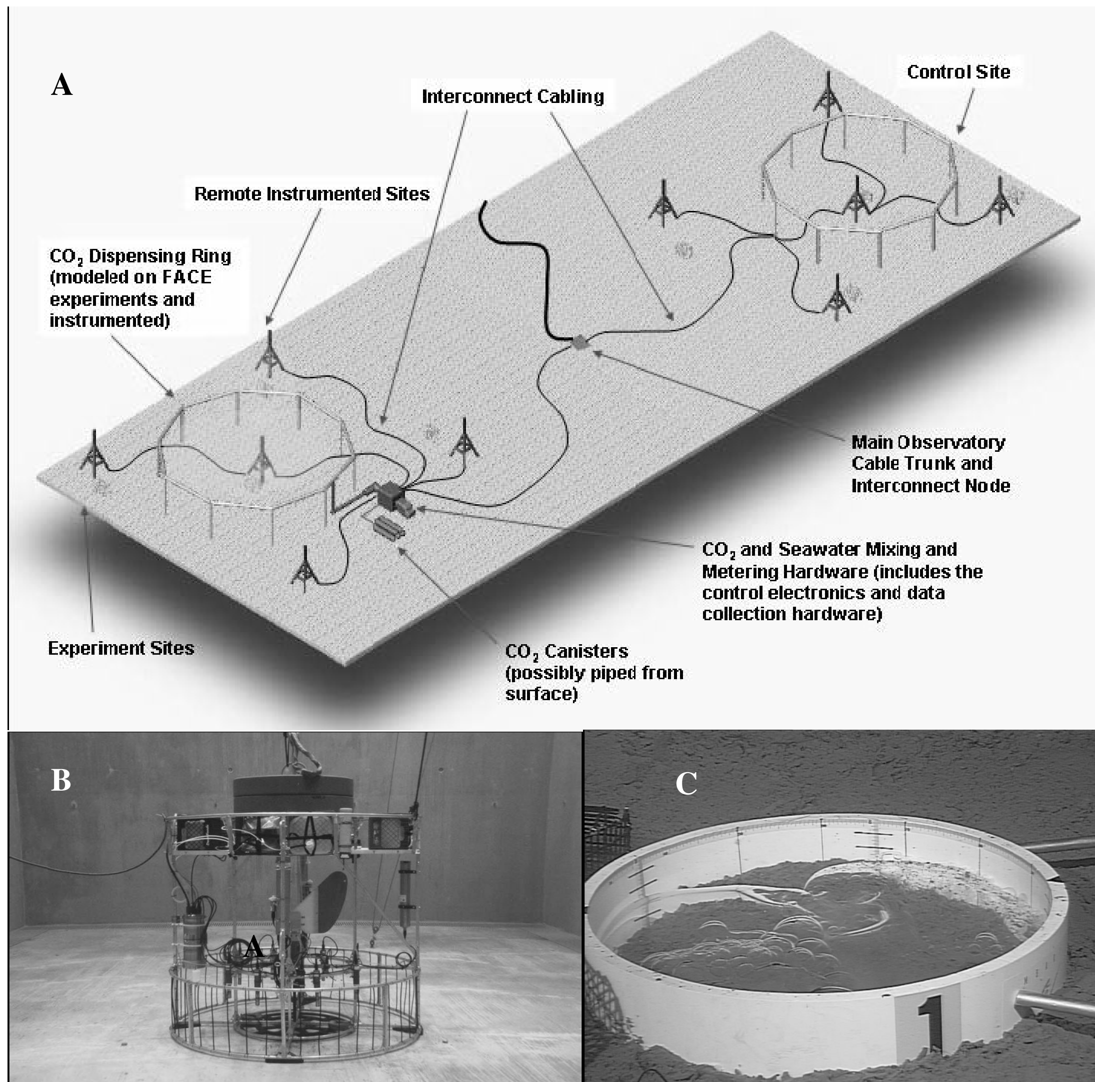


Figure 3. *MBARI funded in situ OA experiments. A & B: Prototypes of the Free Ocean CO_2 Enrichment (FOCE) method, adaptations of the terrestrial (FACE) experiment. No scale is provided for the prototype in image A. C: Plastic rims containing liquid CO_2 set into the benthos. A cage containing test organisms can be seen in the far left of the image. See references in above text for scale, dimensions and full methods associated with these techniques. Images A and B are sourced from MBARI and image C is from Barry et al (2004).*

These *in situ* approaches are ambitious and if fully developed would allow the response of assemblages of organisms to be measured in response to reduced pH, a significant advance on single species laboratory studies. However, the considerable costs and logistics involved

with deep sea experimentation on this scale, together with the inherent challenges of controlled *in situ* manipulation of carbonate chemistry in the ocean, make achieving the balance of obtaining useful data at a justifiable cost difficult to achieve (Barry *et al.,* 2004; Barry & Drazen, 2007; Barry *et al*., 2010).

Due to the difficulties associated with *in situ* experimentation, the vast majority of the biological response studies conducted to-date have been carried out using laboratory or mesocosm approaches. Laboratory and mesocosm experiments under manipulated pCO_2 conditions have identified species and processes that appear vulnerable to OA. However, even the most rigorous of this style of experiment is inherently constrained by attempting to measure complex natural processes in a simplified artificial environment. These approaches are simply not capable of realistically mimicking the complex assemblage interactions and nutrient fluxes that exist in ocean ecosystems.

Field-based CO_2 enrichment experiments involving the introduction of large CO_2 plumes into the ocean were discussed by the panel of experts at the recent Ocean in a High-CO_2 World conference in Monaco (Orr *et al.,* 2008) as one way of addressing the current knowledge gaps. However, this type of experimentation is fundamentally flawed by being highly transient and difficult to generate and monitor (Brewer *et al.,* 2004). In addition, they are frequently incapable of mimicking chronic ocean acidification conditions (Brewer, *et al.,* 2004), and are probably illegal under the United Nations Convention on the Law of the Sea (UNCLOS) 1994. Orr (2009) suggests that the scientific advance achieved by long term *in-situ* CO_2 perturbation studies may not balance the considerable research costs involved. In addition, the amount of CO_2 required to perform such experiments may be perceived by the general public to be contrary to the underlying research cause.

Large scale field CO_2 enrichment approaches also offer little capacity for rigorous experimental design, incorporating adequate replication of treatments and appropriate controls. The necessity of adhering to rigorous principles of experimental design have been reiterated frequently when attempting to meaningfully measure and understand isolated aspects of inherently complex systems such as ocean ecosystems (Underwood, 2000) .

Sites that naturally experience low pH waters, such as cold volcanic CO_2 seeps have been used as natural laboratories to measure the effects of OA on multi species assemblages (Hall-Spencer, *et al.,* 2008; Hall-Spencer & Rauer, 2009) This approach is very useful for discovering the biological implications of relatively long term, low pH exposure. Uniquely, the vent site described by Hall-Spencer, *et al.,* (2008) was very rich in CO_2, and very light in other volcanic gases, making it a rare but valuable experimental site. However, other vent sites frequently extrude volcanic gases such as sulphur dioxide, which are not related to OA induced stress. This can present a challenge when trying to find comparable site with which to compare results with.

Despite this limitation, the use of such sites provides an exciting yet concerning glimpse at ecosystems that have been subjected to low pH conditions over multiple life-cycles. An investigation into microbial diversity at the site described by Hall-Spencer, *et al.,* (2008) is presented in Chapter 6 of this thesis.

Until now, few if any suitable methods for experimental *in situ* pH manipulation were available for OA research. As a result, we currently have a limited understanding of how crucial, life-supporting, biologically driven processes may be affected by changes in oceanic

pH. What is needed to address this short-fall in our knowledge is an adaptable experimental system that:

A: allows the user to locally manipulate the environmental pH around a target response assemblage whilst maintaining the assemblage in the field where it is fully exposed to natural process and conditions.

B: allows the user to generate, and crucially, maintain pH exposure treatments that closely mimic predicted OA scenarios.

1.4 - Aims of current study

The principal aim of this study was to measure the response of assemblages of marine microbes, recruiting macro-invertebrates and established macro-invertebrates to environmental conditions that simulate ocean acidification, whilst maintaining these assemblages in otherwise natural conditions in the field. In order to achieve these aims, the following research questions were addressed;

1: Is it possible to experimentally manipulate the pH seawater in the field to represent ocean acidification conditions of the future?

2: How are marine microbe diversity, macro-invertebrate recruitment and macro-invertebrate succession affected by *in situ* pH manipulation that simulates OA?

To address question 1, it was necessary to design and test a novel device which allows the user to manipulate the pH of a small body of water around a test assemblage. This had to be achieved whilst maintaining the assemblage in the field where it is subjected to unrestricted

natural processes such as recruitment and nutrient flux. In addition, the device had to be capable of producing experimentally rigorous data resulting from adequate replication of treatments and suitable controls. Incorporating pH control into the system was crucial to ensure that the environmental pH surrounding the test assemblage was not simply manipulated, but manipulated in a fashion that corresponds to oceanic conditions that are predicted to occur with the progression of OA.

To address question 2, the experimental apparatus developed to address question 1 was applied in a series of experimental applications, where different assemblages of marine organisms were measured for their response to pH stress. After exposure to manipulated pH treatments, assemblages were analysed for response variables considered to be susceptible or indicative of pH change, namely diversity, relative abundance, and in the case of established macro-fouling organisms, unique aspects of their morphology or physiology such as shell thickness or gonad to somatic tissue ratios. To complement the traditional experimental approach described above, the natural *in situ* pH manipulation provided by the vent site described by Hall-Spencer *et al.,* (2008) was applied in Chapter 6 to investigate how microbial diversity in biofilms was affected by exposure to low pH.

This work is not intended to present a total methodological solution for field based OA research. However, it is hoped that this work presents an alternative direction for OA research to follow. This new direction leads away from simplistic and potentially confounded laboratory based experimentation, towards a goal of accurately simulating OA in the field and therefore predicting the full biological repercussions with greater accuracy.

1.5 - Thesis Structure

Chapter 1: Introduction and Literature Review

Chapter 2: Methodological Development and Evaluation

Chapter 3: Ocean Acidification and Biofilms I. Experimental Manipulation.

Chapter 4: Macro Invertebrate and Algal Recruitment

Chapter 5: Macro Invertebrate Succession

Chapter 6: Ocean Acidification and Biofilms II. Diversity at a Low pH Vent Site

Chapter 7: Industrial Applications

Chapter 8: Discussion

Chapter 2
Methodological Development and Evaluation

2.0 - Methodological Requirements

In order to measure responses of marine assemblages to environmental pH change that simulated ocean acidification, it was necessary to design, evaluate and apply a novel experimental unit capable of:

1: exposing a target assemblage to manipulated pH treatments, whilst maintaining the assemblage in the field where it is exposed to natural processes such as larval supply and nutrient flux.

2: generating and maintaining pH treatments that closely mimic the environmental conditions that are predicted to occur as a result of ocean acidification.

3: performing rigorous experiments where treatments and suitable controls are adequately replicated.

4: ensuring independence of treatments between replicate experimental units.

5: encompassing all these specifications in one unit that could be constructed, deployed and operated by an individual.

2.1 - Conception

Sea water readily buffers pH fluctuation depending on temperature, salinity, pCO_2, carbonate ion saturation state and total alkalinity (Zeebe & Wolf-Gladrow, 2001). As a result of this rapid buffering potential, the volume of CO_2 required to lower the pH around a settlement panel in order to mimic OA scenarios in an unrestricted open ocean environment was considered prohibitively expensive and logistically difficult. This opinion is shared by other reviews of open ocean perturbation experiments (Orr *et al.,* 2008).

Semi-containment of a body of water provided a potential solution to this problem. Conceptually, smaller volumes of CO_2 could be used to lower the pH of a restricted body of water, and the influence of the CO_2 would be prolonged as the mixing of ambient and treated water would be reduced. Theoretically, this would all be possible while still allowing sufficient water exchange between the semi-contained and ambient waters to maintain natural processes such as larval recruitment. To clarify, the water exchange and larval access would be identical between treatment and controls, the only difference would be the introduction of CO_2.

The incorporation of a feedback system that controlled the introduction of CO_2 into the semi contained body of water, and subsequently the pH, was also considered to be achievable. This design was inspired by the principle used by Gonzalez-Fernandez, *et al.* (2008). In this application, a partially open structure was used as a terrestrial mesocosm, allowing scientists to measure the response of plants to artificially increased ozone concentrations. Ozone was introduced into the mesocosms and the walls of the structure prevent the gas from mixing

fully with the ambient atmosphere. This system maintains an artificially high concentration of ozone around the test organisms whilst still maintaining normal gaseous exchange with the atmosphere. When ozone concentration measurements in the mesocosm fall below a predetermined level, more gas is introduced and the cycle is repeated. The terrestrial application developed by Gonzalez-Fernandez, *et al,* (2008) was modified for marine applications and a pilot study was conducted to investigae the potential of this concept by providing data to satisfy the following questions;

i) can the pH of a semi-contained body of water be manipulated in the field?

ii) is the range of any pH manipulation produced sufficient to simulate future OA scenarios?

iii) is the stability of any pH manipulation produced sufficient to simulate future OA scenarios?

iv) is the concentration of dissolved oxygen in the sea water affected by the introduction of CO_2?

v) what is the effect range and duration of any pH treatment effect produced?

2.2 - Design

The equipment constructed for pilot testing (Figure 4) consisted of two parts; a base frame that supported three horizontally orientated settlement panels, and a top frame which supported three plastic chambers. When the top frame was fitted on the base frame, each chamber was supported above each settlement panel. The whole unit was adjustable. The distance between each chamber, settlement panel height and distance between each panel and chamber pair could be altered. The unit was immersed in seawater at Hartlepool marina (site

description to follow) and fixed under a pontoon with the panels at a constant depth of 30 cm. When submerged, the chambers acted as hoods, semi enclosing the body of water above the panels (Figure 5).

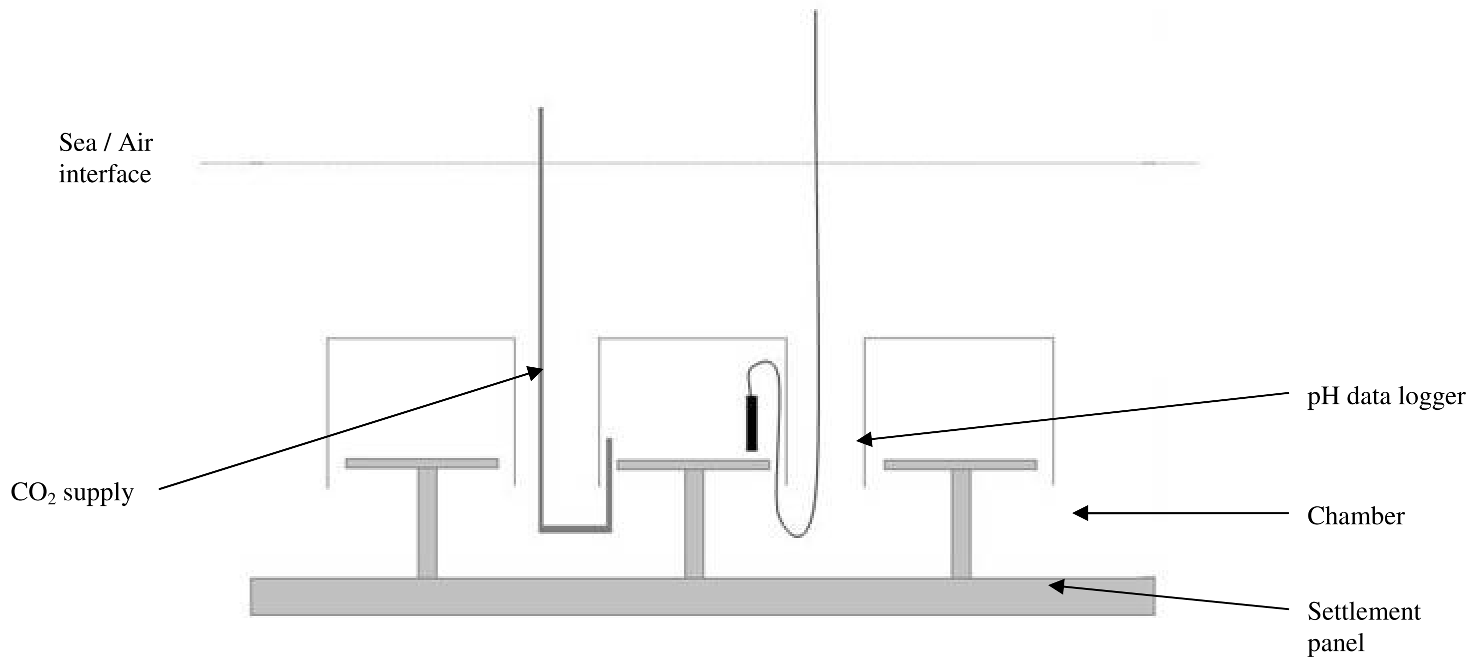


Figure 4. *Diagram of the pilot experimental setup consisting of settlement panels and accompanying semi containment chambers. CO_2 was introduced into the chamber and a data logger recorded the resulting environmental conditions within the chamber. Not to scale*

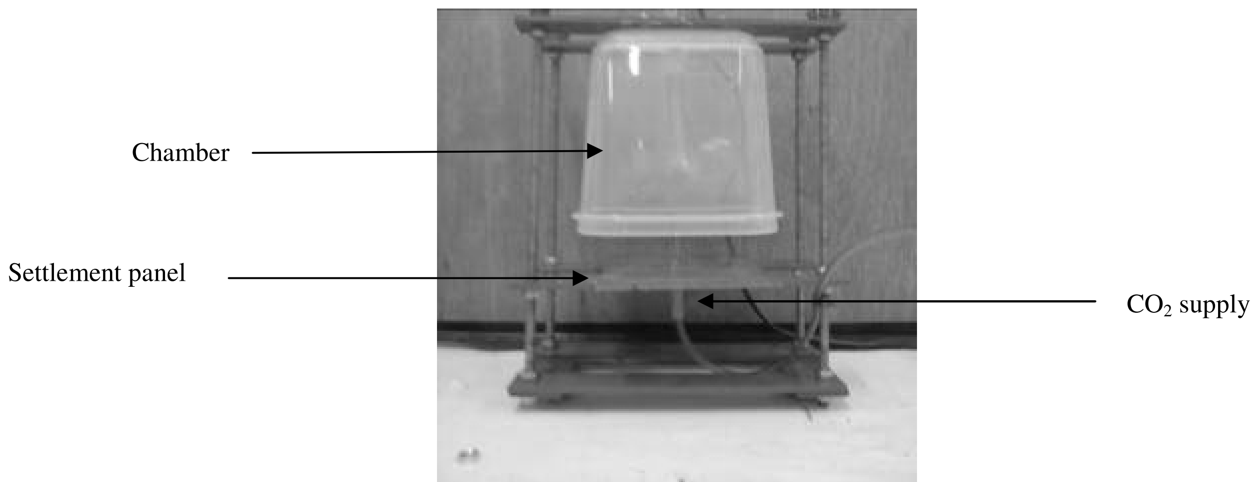


Figure 5. Prototype of the panel and chamber design. Both the settlement panel and chamber are secured with nuts on threaded bars to allow fine tuning of their relative positions to control the influx of ambient water. The chamber is 150 x 150 cm square at the open end.

2.3 - Evaluation

2.3.1 - Dissolved Oxygen and pH range

CO_2 (99.0 % industrial grade, BOC Plc.) was introduced into the chamber from a 5,000,000 Pa cylinder stored on an overhead pontoon. CO_2 was introduced via an open ended tube (0.5 cm diametres, Tygon™) at a pressure of 13700 Pa, at rate of approximately 0.3 l min^{-1}. Exhaust vents (25 mm diametres holes) were made in the top of each chamber to allow the CO_2 to escape after the gas head space in the chamber had reached a height of 2 cm. A data logger (Yellow Springs Instruments, Multi Probe Metres 556) was fixed inside one chamber where it recorded the internal environmental conditions generated by the introduction of the CO_2.

The data logger was attached so that the probes were positioned 1 cm above the settlement panel. The data logger recorded pH (± 0.02 units) and dissolved oxygen (DO ± 0.02 mg/l) during the introduction of CO_2 into the chamber (Figure 6).

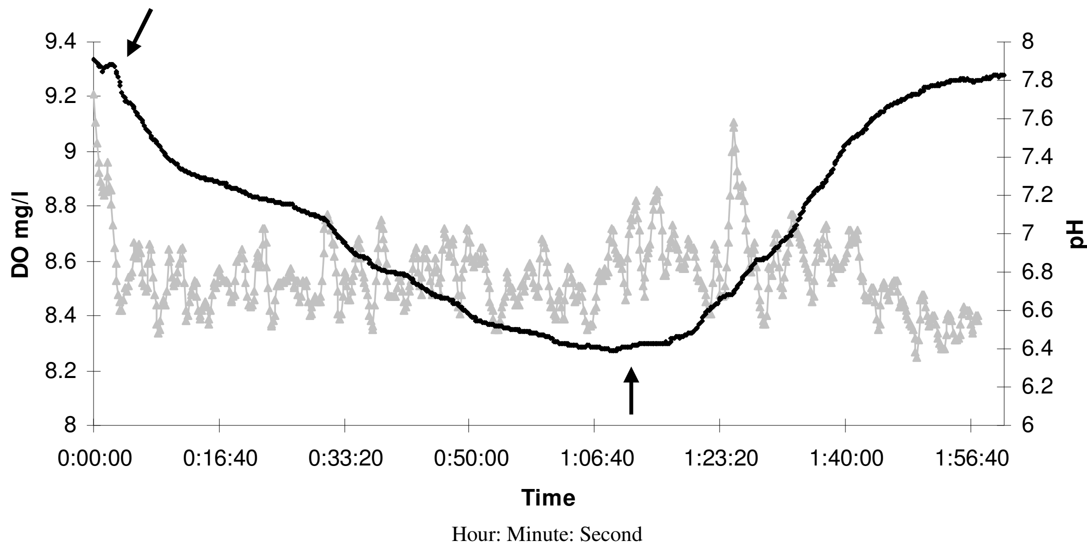


Figure 6: *Dissolved oxygen (grey line) and pH (black line) measured at the height of the settlement panel during the introduction of CO_2 into the chamber. The CO_2 supply was started (↓) 10 seconds after submersing the unit and stopped 1 hr. 10 minutes into the trial (↑).*

This pilot study provided data to satisfy research questions i), ii), iv) and v). Figure 6 shows that it is possible to semi-contain a body of water in the field and temporarily manipulate the pH of that water. The minimum pH produced during this trial was 6.59, which is approximately one whole pH unit lower that the worst case prediction scenarios for OA in the next 300 years (Caldeira & Wickett, 2003), meaning that this system was capable of producing pH conditions that correspond to the full range of conditions expected to result from the advance of OA over the next three centuries.

The concentration of DO did not significantly fluctuate beyond normal parametress ($\bar{x}$ DO = 8.54 ± 0.12 mg l^{-1}) in response to the introduction of CO_2 (Ramosa *et al.,* 2009). In contrast, the pH would have to be controlled if it were to be considered to be representative of future OA conditions ($\bar{x}$ pH = 7.23 ± 0.48) (Caldeira & Wickett, 2003). The maximum effect range of the pH treatment was determined to be 13 cm. Beyond this distance, the effect of CO_2 introduction was buffered by the ambient sea water, meaning that as long as chamber and panel pairs were deployed at least 13 cm apart, and the introduction of gas kept constant, they could be considered independent experimental units in terms of pH treatment effect.

2.3.2 - pH stability

To satisfy research question iii) it was necessary to incorporate control of the internal pH of the chamber into the design, thereby simulating the predicted effects of OA. One possible way of achieving this was the introduction of a specific fixed blend of atmospheric air and CO_2 that would create the desired pH environment by adjusting the pCO_2 of the semi contained environment by a consistent degree.

This approach was attempted by using pre-mixed air and CO_2 blends, and by manually mixing air and CO_2 with pressure regulators. Both methods however were rejected, firstly because of the prohibitive cost of pre-mixed gas blends (£450 per cylinder) and secondly, a fixed pCO_2 was not capable of creating the required pH decrease in an environment that was highly variable with respect to localised hydrodynamics and resulting fluctuations in buffering potential. It became apparent that variable pCO_2 manipulation was required to deal with the varying environmental conditions encountered in the field.

The incorporation of a pH controller into the design provided this desired variability in pH treatment. pH control was achieved by regulating a supply of CO_2 that was stored in 5,000,000 Pa cylinders on the overhead pontoon (Figure 7). The gas was delivered via a network of tubes (0.5 cm diametres Tygon^{TM}) that ran through the base frame, through a 0.7 cm diametres hole in the settlement panels and up into the head space of the chambers. This CO_2 supply was controlled by a feedback loop that incorporated a pH probe, solenoid and the pH controller.

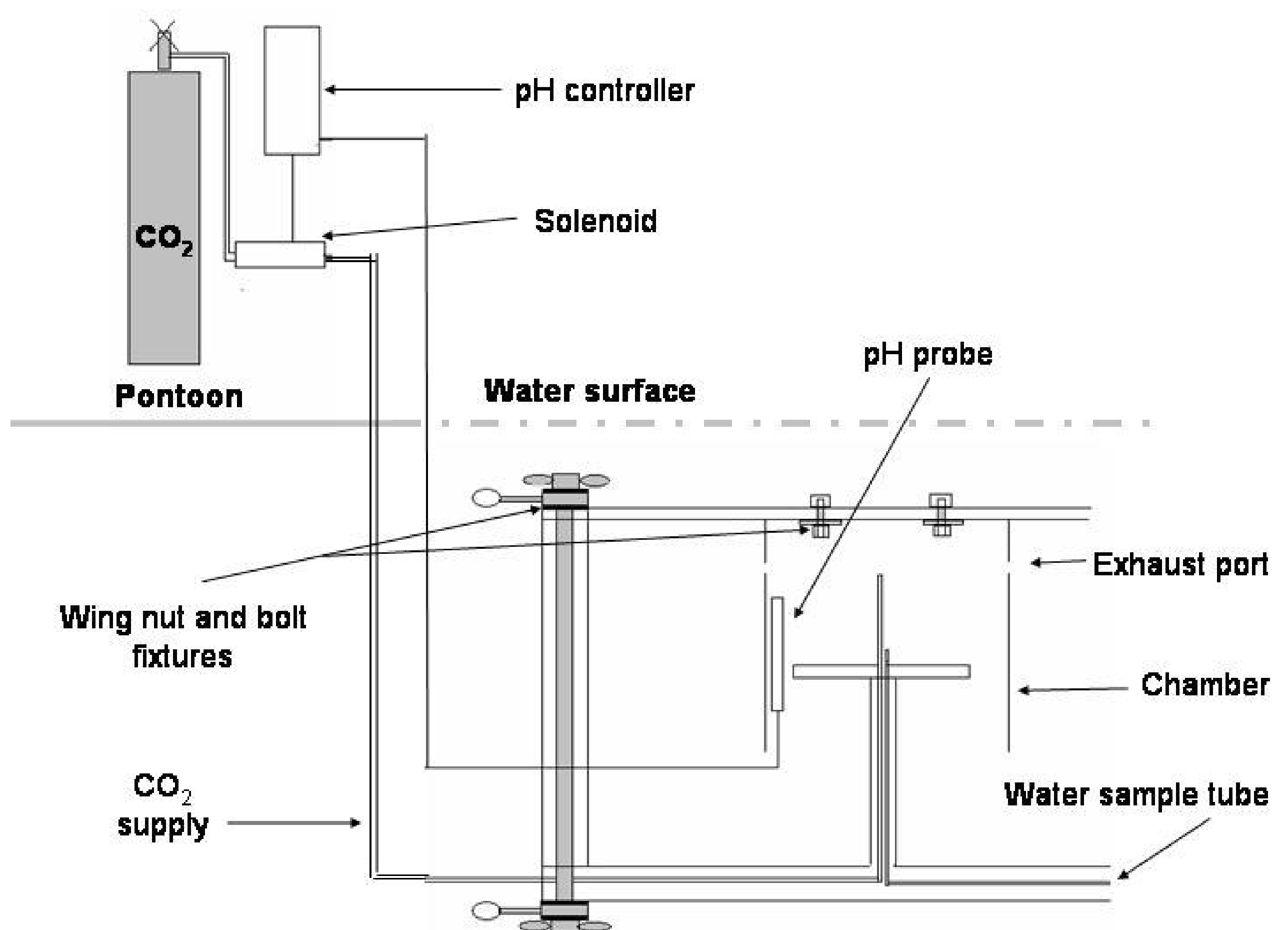


Figure 7. *Diagram to show method of pH control within the test chamber using a feedback loop incorporating a pH controller, gas supply, solenoid and pH probe. Only one chamber shown, not to scale.*

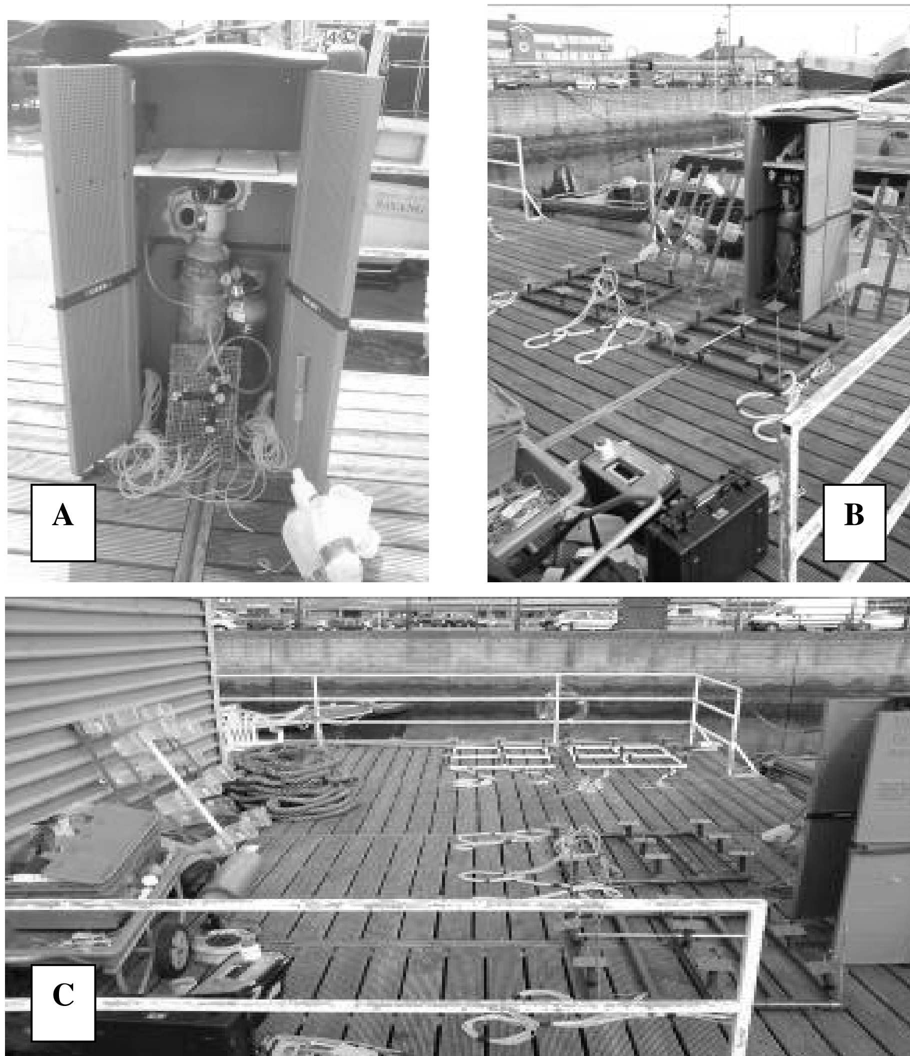


Figure 8. *A: Picture of plastic box that housed cylinders, data logger, gas and pH control equipment. B and C: Pictures showing frames and accompanying gas tubing ready for deployment*

The pH controller (Aqua Medic pH Controller 2001) was housed in a plastic control box on an overhead pontoon (Figure 8), and was connected to a pH probe fixed on the inside of a reference chamber via an extended BNC connection. The pH probe was fitted in the seawater 2 cm below the gas head space of the chamber.

When the pH of the water in the chamber rose above a pre-programmed value, the controller automatically opened a solenoid valve (part of kit supplied with Aqua Medic pH Controller 2001) to allow CO_2 into the appropriate gas chamber, lowering pH of the water. When the probe measured a pH 0.03 units lower that the pre-programmed value, the solenoid valve was automatically shut by the controller, preventing any further release of $CO_{2.}$

Once the seawater in the chamber had buffered the pH decrease resulting from the initial release of gas, the pH would slowly rise until it reached the pre- programmed value. When this value was exceeded by 0.03 units, the solenoid would again allow gas into the chamber, and the cycle would be repeated. The time period between cycles varied constantly in response to fluctuating local hydrodynamics.

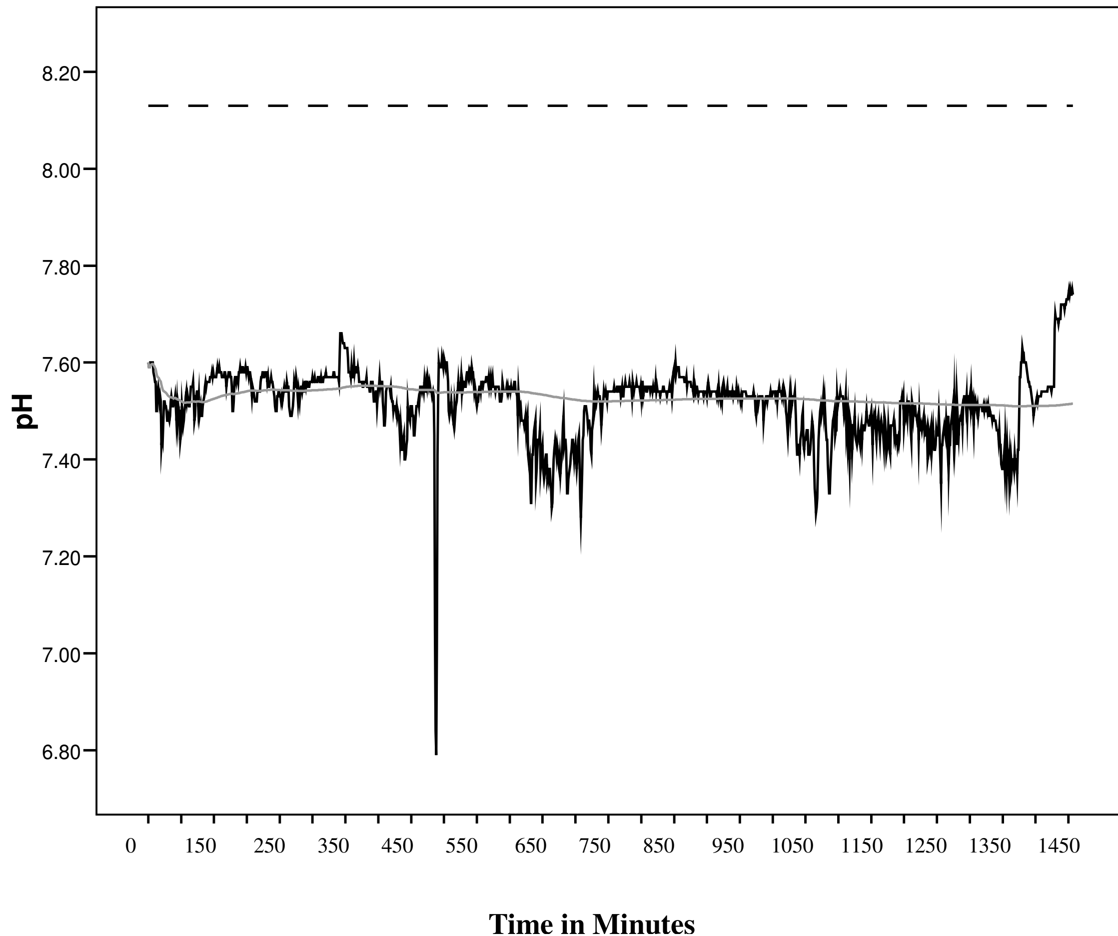


Figure 9. *pH controller performance. The dotted line indicates* $\bar{x}$ *ambient pH in Hartlepool marina during the trial. The solid black line indicates the actual pH measured in the chamber, 1 cm from the settlement panel. The grey line indicates the running* $\bar{x}$ *pH in the chamber. The very large low pH spike between 450 and 500 minutes was a result of the solenoid used in the pilot work sticking open. An upgraded solenoid was used for the actual experiment.*

The gas supply tubes inside the chamber were 2 cm tall and vertical. All gas bubbles rose through the water above the settlement panel and did not subject the panel itself to any mechanical bubble disturbance. Exhaust gas was automatically expelled via exhaust holes 2 cm from the top of the chamber when the CO_2 was replenished by the feedback loop.

The gas supply tubes inside and outside the frame were all the same length and secured at the same height. As a result, tubes that supplied chambers close to the gas supply were the same length as the tubes that supplied the more distant chambers. The extra length was loosely

coiled and secured at a constant height within the base frame. By ensuring all gas delivery tubes were the same length and at the same depth, all gas supplied at a constant pressure reached each chamber equally. The chambers acted as hoods which semi-contained seawater within the chamber around the settlement panels, preventing it from fully mixing with the surrounding water. The resulting semi containment allowed the water to be manipulated with regards to pH, while still exposing the settlement panel to water exchange with the ambient environment allowing larval supply to continue.

2.4 - Application

Following the successful pilot study, the experimental device was scaled up for long-term field deployment. The full scale device consisted of two parts; a square base frame that supported nine horizontally orientated settlement panels, and a top frame which supported nine plastic chambers.

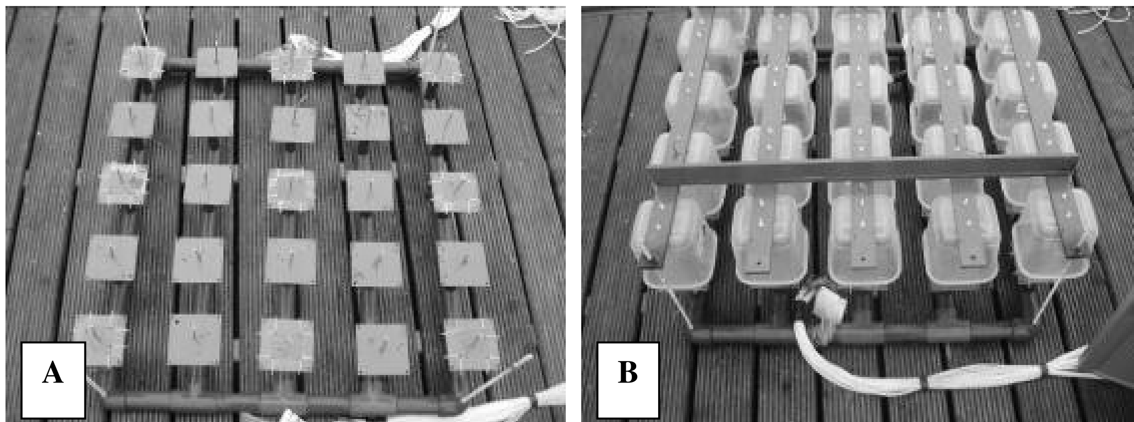


***Figure 10**. A: settlement panels mounted on frames and gas tubing. B: Settlement panels with the chambers fitted on top, gas supply tubes and manifold in the foreground. Note: pictures show prototype version with 25 chamber and panel pairs, this was later reduced to 9 pairs per frame to minimise weight and technical complexity. Scale – each settlement panel is 120 mm square and the whole frame is 1200 mm square.*

The base frame was 1200 mm square and constructed from polyvinyl chloride (PVC) pipes which housed a network of tubes (Tygon^{TM} OD 0.8mm) that supplied either 99.9% industrial

grade CO_2 or air (BOC industrial gases), through a central hole (20 mm diametres) in the settlement panel, and into the overhead chambers (Figure 10). The settlement panels were 120 x 120 mm square and made from 3mm thick grey PVC sheet. The panels were modified by sanding or by the addition of microscope slides for the different experiments and these details are provided in the methods section of each respective Chapter. The settlement panels were glued using a solvent adhesive to 120 mm long lengths of PVC pipe that slotted into the base frame. The chambers are 150 x 150 mm square at the open end, 170 mm high and made from food grade polypropylene. The chambers were fixed to the top frame (120 mm square, made from PCV lengths 1200mm x 800 mm x 150 mm) with nylon cheese head slotted screws (Radio Spares Ltd part number: 527-993) and solvent adhesive.

2.4.1 - Treatments

Each chamber and panel pair was randomly assigned one of four treatments that included pH values corresponding to predicted OA scenarios of the future and ambient pH controls. pH 100 years (7.80-8.00), pH 300 years (7.80-7.50), air control (AC) (pH 8.08-8.45), and no-gas control (NGC) (pH 8.08-8.45), (Figure 11). The pH conditions were maintained by adjusting the pH controllers pre-set control mechanism to the desired pH value.

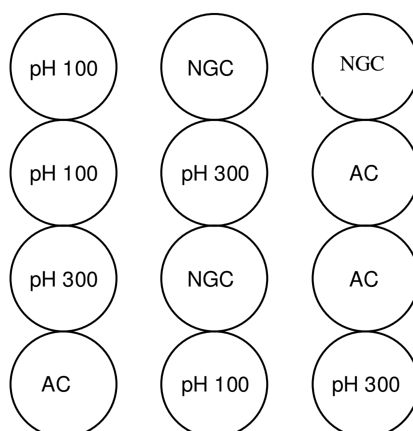


Figure 11. *Plan to show arrangements of treatments and controls within the experimental block. NGC = no gas control, AC = air control.*

AC and NGC panel and chamber pairs served as controls against the specific introduction of CO_2, and the introduction of gas in general, respectively. Compressed atmospheric air was delivered continuously to treatment AC panel and chamber pairs from a 20,000,000 Pa cylinder on an overhead pontoon, in the same fashion as the CO_2 but without the control mechanism. NGC panel and chamber pairs were not supplied with any gas.

2.4.2 - Water sampling

In addition to the tubes that supplied gas to the panels, a separate network of tubes of the same specification also ran through the base frame, and emerged though the middle of each panel where they were fixed 1 cm above the panel height. This second network of tubes allowed water samples to be slowly withdrawn using a 30 ml plastic syringe from immediately above the panel, up to an overhead pontoon for analysis (Figures 7-8, 10).

To ensure the samples represented environmental conditions within the chamber and not the tube at the moment of sampling, the internal volume of the sample tube (37 ml) was drawn up and discarded and another 37 ml was drawn up from the chamber for analysis. Water samples

were analysed for pH using a Hanna Instruments pH metres and probe (HI 9125) calibrated with National Institute of Standards and Technology (NIST) standardised buffers supplied by Fisher Scientific Ltd.

2.4.3 - Maintenance

Maintenance was required in order to keep the unit functioning. This included the replacement of CO_2 and compressed air cylinders. Compressed air was used at a constant rate, making it simple to calculate how long each cylinder would last. CO_2 usage in the feedback loop varied, particularly in response to wind speed. Increased wind speeds created small waves or chop on the marina surface. In some cases, these disturbances appeared to enhance the dissolution of the CO_2 in the seawater, increasing the duration of the pH treatment. In other more severe cases, winds produced a rocking motion which increased the rate at which the treated water exchanged with the ambient water outside the chamber, raising the internal pH and subsequently increasing gas consumption. This variation was not problematic in terms of treatment effect as the feedback system on the pH controller was designed to deal with such fluctuations. However, varying gas usage made judging how often to replace CO_2 difficult, so site visits were made every two days during experimental run times to avoid running out of gas.

In addition to changing gas cylinders, regular calibration and cleaning of the pH probes used in the pH control mechanism was required to prevent the accumulation of biofilms on the sensor surface which may have produced drift in the measurements. During experimental run times, the probes were removed and cleaned every two days before calibration in NIST buffers and replacement. Cleaning consisted of rapidly swirling the probe tip in calibration buffers to remove any residual slime. Probes were used for the duration of one experiment

and then discarded. To minimise the effect of heterogeneous shading the whole unit was submerged and fixed under a pontoon with the panels at a constant depth of 30 cm.

This method was applied to perform three different experiments, the results of which are presented in the subsequent Chapters. In each experiment, minor alterations to the methods were made to increase replicate numbers, and these modifications are detailed in the preface of the respective Chapters.

2.4.4 - Study Site

All experiments were conducted at Hartlepool Marina on the North East coast of England (54°41'31.34"N 1°12'00.28"W). Hartlepool is a large marina with approximately 450 births for pleasure craft and a small commercial fishing fleet. Pontoons span the marina dividing up the individual births. The pontoons are attached to fixed pilings in a fashion that allows them to rise and fall with the water height which is controlled by a lock system that operates in the East of the marina. The lock moderates the tidal influence in the marina and is operated variably to allowing the entry and exit of vessels.

Figure 12. Site map of Hartlepool marina showing lock system, experimental site and proximity to the North Sea. Map supplied by Google Earth ™.

Hartlepool marina is uniformly 5-7 metress deep depending on tide and lock position. Hard substrata in the marina are characteristically inhabited by fouling assemblages dominated by mussels (*Mytilus edulis*) solitary tunicates (*Ciona intestinalis, Ascidiella aspersa, Clavelina lepadiformis),* colonial tunicates (*Botrylloides schlosseri*, *Botrylloides leachii*, *Diplosoma listerianum,* and barnacles (*Balanus crenatus*) (Sugden, *et al.,* 2007). Benthic substrata are typically represented by soft sediments which are dominated by hydroids and a thick biofilm where undisturbed.

Invertebrate larval recruitment occurs typically from April to October, peaking in September and August (Prendergast, 2007). Environmental parametress of the marina water vary considerably in contrast to the North Sea some 100m away (Table 1). Water in the marina is influenced both by freshwater run off from the surrounding man-made developments during

wet weather, and evaporation in dry weather. Being only 5-7 metres deep, the water warms and cools quickly in comparison to the open ocean. Relatively high temperatures together with frequent sea breezes often produce hyper-thermal and hyper-saline conditions in comparison to the North Sea, particularly during the summer.

Table 1. *Environmental parametress and annual maximum and minimum values for 2008. Hartlepool data was collected by the author using a calibrated YSI data logger (see methods). North Sea data representing Red Car and Hartlepool Bay were obtained from CEFAS and the Environment Agency.*

	Salinity (PSU)	**Temperature (°C)**	**pH**
Hartlepool Marina	33.13 - 36.08	4.46 - 19.11	8.08 - 8.45
North Sea	34 - 35	5.80 - 14.06	8.1 - 8.2

The marina perimetres is fenced and public access is restricted, reducing the likelihood of disruption to long-term field experiments. Although the marina is not a fully open sea environment, it was considered a suitable site to conduct the present work for a number of reasons. Firstly, the volume of water in the marina provided, in terms of this work, an effectively infinite buffering capacity against localised pH change. Secondly, the site is bathed in its own larval supply which is complemented by open ocean larval supply periodically introduced by the opening and closing of the lock system. Thirdly, the site was close enough to allow the necessary frequency of trips required in order to conduct the work.

Chapter 3

Ocean Acidification and Biofilms I. Experimental Manipulation

3.0 - Introduction

The overall aim of this thesis is to investigate the responses of different assemblages of marine organisms to low pH conditions that simulate OA scenarios of the future. In order to achieve this aim it seemed reasonable to start with marine biofilms for two reasons. Firstly, marine biofilms are the primary colonisers of all substrata immersed in seawater, and secondly, marine biofilms settle, develop and grow quickly. In this Chapter the apparatus described in Chapter 2 was applied to grow marine biofilms and measure their diversity under experimentally manipulated low pH conditions that simulate future OA scenarios.

Marine biofilms can be characterised as complex polysaccharide matrixes inhabited by varying ratios of marine bacteria, diatoms, fungi, and protozoa that quickly form on the surface of most substrata, often within seconds of immersion in seawater (Characklis, 1981; Cooksey, 1995; Maki, 1999; Marshall & Bowden, 2000; O'Toole, 2000; Qian *et al.,* 2007). Biofilm formation is initiated by the interaction of planktonic microbes with organic and inorganic particles on an un-colonised surface (Qian *et al.,* 2007). This primary recruitment is followed by secondary and tertiary settlement of microbes, culminating in a mature biofilm wherein microbes interact with each other either synergistically or competitively (Qian *et al.,* 2007). As with all living assemblages, even mature biofilms are thought to be temporally and spatially plastic in terms of diversity and relative abundance, and the extent of this plasticity varies between environments (Dobretsov *et al.,* 2006; Lindstrom, 2005; Aguilera, 2007; Anderson-Glenna, 2008).

The structure of a biofilm can also vary from a primary monolayer one cell thick, to a fully developed film comprised of both microbial cells and extra-cellular polymeric substances (EPS), frequently in the form of polysaccharides (Qian *et al.,* 2007). Fully developed films often possess internal pH and DO gradients, as well as networks of water channels which hydrate the film and supply nutrients to its inhabitants (Qian *et al.,* 2007). This structure provides biofilms with many advantages, including the potential to communicate with other microbes via quorum sensing. This mechanism of microbial cell to cell communication is considered important in the organisation and distribution of vital microbial processes such as gene expression and population density (Nealson, 1979; Surette, 1999; Huang *et al.,* 2007 Dobretsov *et al.,* 2009). Other advantages of microbial existence in biofilms include protection from undesirable environmental exposure (Davey, 2000; Daims, 2006) and increased nutrient supply compared to open water (often in the form of metabolic waste products from other film co-habiting microbes) together with the lack of demand for metabolic expenditure required for motility (Jin-Woo *et al.,* 2008). Existence within a biofilm is clearly potentially beneficial for an inhabiting microbe (Costerton, 1995; Donlan, 2002).

Biofilms and biofilm associated microbes, however, also exert a much wider influence on ecosystem structuring and functioning in marine environments. Unicellular organisms commonly found within marine biofilms are directly involved in the most fundamental of life sustaining biogeochemical processes in the ocean such as carbon, silica and nitrogen cycling (Paerl & Pinckyney, 1996). In addition, because of the ability of biofilms to colonise virtually all moist surfaces, the microbiologically active surface area of a marine environment is substantially increased by the formation of biofilms on and in marine substrata.

Biofilm coatings on marine substrata also serve a more direct service to marine ecosystems by providing a valuable food source for grazers. Historically, motile macro-invertebrates such as molluscs, crustaceans and polychaetes were considered to be the primary beneficiaries of this resource (Lam, *et al.,* 2005b; Lau, *et al.,* 2005; Hung, *et al.,* 2005a; Qian, *et al.,* 2007). More recent research has produced evidence to suggest that larger mega-fauna such as birds like the Western Sandpiper also have evolved specialised bill and tongue morphology which assists them in harvesting biofilms from marine substrata. These biofilms form significant proportions of their diet (Tomohiro, *et al.,* 2008).

Biofilm associated microbes also significantly influence the structure of benthic assemblages. Many marine species capitalise on the larval or gametic dispersal potential presented by living in a fluid environment by incorporating a planktonic stage in their life cycle (Scheltema, 1971). Marine larvae in particular, have been shown to respond to physical or chemical cues produced by biofilms when recruiting (Pawlik, 1992; Wieczorek, 1996; Hadfield, 1998; Olivier, 2000; Zhao, 2002; Hung, *et al.,* 2005a; Lam, *et al.,* 2005b; Lau, *et al.,* 2005; Bao, 2007; Qian, *et al.,* 2007). This is of great importance given the inextricable link between recruitment and the structuring of marine assemblages. These cues include biofilm density, metabolic activity, origin and crucially, composition (Wieczorek, 1996; Grasland, *et al.,* 2003; Chiu, 2008). The latter is of particular relevance to OA as biofilm assemblage composition in aqueous environments have been shown to shift in response to environmental factors, particularly pH (Lindstrom, *et al.,* 2005; Yannarell & Triplett, 2005; Gieseke, *et al.,* 2006; Green, *et al.,* 2006).

OA is predicted to lower the environmental pH in shallow ocean waters by approximately 0.7 pH units, possibly for thousands of years (Caldeira & Wickett, 2003). pH variation of less

than 0.7 of a unit has been reported as being either the primary or secondary factor in determining biofilm species composition in fresh water systems (Lindstrom, *et al.,* 2005). As pH change is known to affect biofilm composition, there is real potential for OA to alter current assemblage structuring processes such as recruitment and nutrient cycling. Currently, the likelihood or magnitude of this alteration is unknown.

To date, no previous work has investigated the response of marine biofilms to low pH conditions designed to simulate future OA conditions, meaning that the potential impact of OA on biofilms and biofilm associated process are unknown. In order to assess how marine biofilm associated microbes might respond to low pH environments produced by the advance of OA, biofilms were grown in the field under experimentally manipulated pH conditions to test the following hypothesis: biofilm diversity will change in response to low pH treatments. These conditions were produced using the method and protocol and described in the previous Chapter.

3.1.1 - Methods

Biofilms were grown under two manipulated pH treatments; pH 100 year = (median pH 7.75) and pH 300 year = (median pH 7.45), control conditions; Air control = (median pH 8.1), and No gas control = (median pH 8.10). The treatments and control conditions were created using the apparatus described in Chapter 2. Each treatment or control chamber was replicated three times. The broad pH range of the treatments reflects the challenges associated with controlling pH in the field. These challenges are explored and discussed throughout the thesis.

All biofilms were grown in the field on autoclaved glass microscope slides (ground edges, plain glass 76mm x 26mm 0.8mm to 1.0mm thick, Fisherbrand) that were attached with cable ties to the settlement panels of the apparatus (Figure 13). Gloves were used for the attachment process and care was taken to avoid contact with the slides. Four slides were attached to each panel (Figure 13). The experimental frames were deployed in Hartlepool marina for 12 days from the 02.02.08 – 14.02.08 with the panels and attached slides at 30 cm depth. A 12 day run time was used as substantial biofilm formation was expected to occur during this time (O'Toole, 2000). Also, at this stage in the development of the method it was not known if the apperatus was suitable for long term deployment. Every two days during the run time, water samples were taken from panel height and analysed for pH to determine the long term carbonate conditions the biofilms were being exposed to. During this time the gas supplies were maintained as described in the previous Chapter.

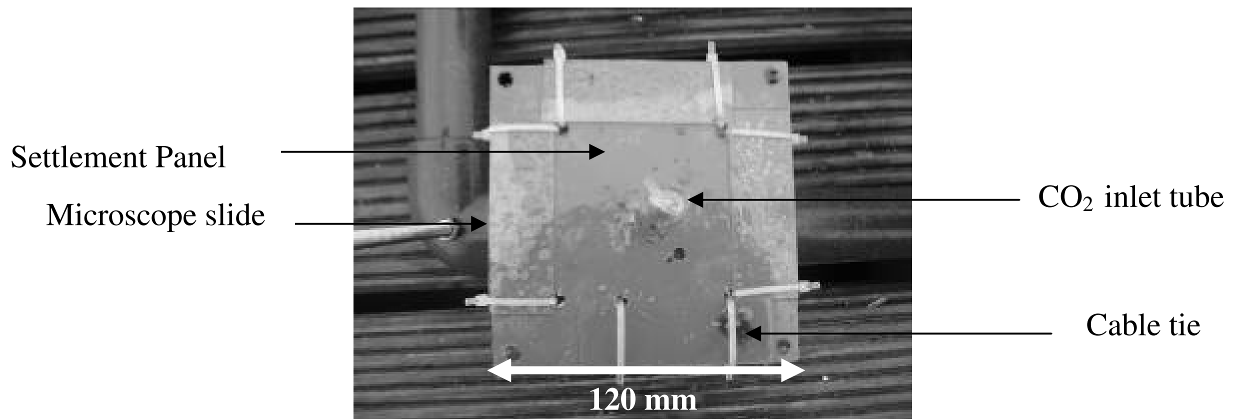


Figure 13. *Attachment of microscope slides to experimental pH exposure apparatus.*

After 12 days the slides were aseptically recovered from the frames and placed in autoclaved containers. The containers were placed in a cool bag with ice packs and transported back to Newcastle University where they were stored at -80° C within 90 minutes of removal from the marina.

3.1.2 - Overview of Analytical Procedures

One slide out of the four slides per chamber was randomly selected for molecular analysis, so that for each respective treatment or control n = 3. DNA was extracted from the biofilm samples and amplified using PCR (polymerase chain reaction techniques). The amplified DNA was run on DGGE gels (Denaturing Gradient Gel Electrophoresis) to provide a coarse measure of diversity both between and within samples. Individual bands were extracted from the gels and sequenced in an attempt to establish the functional roles of the isolated microbes.

3.1.3 - DNA Extraction

Genomic DNA was extracted from the biofilm samples following the protocol supplied with the extraction kit, DNAeasy Blood and Tissue spin column kit produced by QIAGEN**.** 16S rRNA fragments were amplified for DGGE analysis using PCR which was conducted in 30µl reactions with 1µl of target DNA at approximately 12 µg per µl . The following program was used for all PCR reactions in a Thermoscientific Hybaid Px2 thermal cycler: 94 °C for 5 minutes, 94 °C for 30 seconds, 53 °C for 30 seconds, 72 °C for 45 seconds and final extension at 72°C for ten minutes (30 cycles).

3.1.4 - DGGE

Denaturing gradient gel electrophoresis was performed as previously described by Muyzer *et al.,* (1993). Approximately 280ng of PCR product was loaded in each well of a 10% polyacrylamide gel, with a linear denaturing gradient of 30 – 60%. Electrophoresis was performed for 5 hours at a constant 150 V. Gels were incubated for 20 minutes in Cybr Gold before imaging on a Fluor-S Multimager transluminator. DGGE gels were run with each sample repeated three times to ensure consistency between gels. Once gels of suitable quality

were obtained, individual gels were placed between two acetate sheets, carefully wrapped in cling film and stored at -20°C until the picking of bands for sequencing.

3.1.5 - Statistical Analysis of DGGE Data

Bionumerics software version five was used to analyses the DGGE gel images and to generate data representing the presence or absence of each band identified in each gel. The presence or absence of bands on the DGGE gels was considered a proxy for microbial diversity, as each band represents strands of genomic microbial DNA of a particular frequency of base pairs, indicating a particular species or sub-species. This proxy data was analysed using binary logistic regression to determine whether or not the environmental pH can be used to predict microbial diversity in marine biofilms.

3.1.6 - Sequencing

Bands were selected from the DGGE gels for sequencing for two reasons. Firstly, this provided confirmation that the technique worked correctly and the different bands did actually represent different species or sub-species of microbes. Secondly, sequencing provided the oppertunity to identify microbes within the biofilms with the aim of interpreting how any fluctuations in their abundance may influence the wider marine environment.

Immediately prior to the picking of bands, the gels were removed from the freezer, carefully unwrapped and generously bathed in 1 x TAE (Tris – acetate) buffer solution to prevent fragmentation during thawing. Gels were placed on a UV light box and the appropriate band was identified. The band was picked using a 200 µl pipette tip, and the resulting plug of gel was extracted and placed in 100 µl of clean water and stored overnight. One µl of this product

was then used as the target DNA in a PCR reaction conducted with the same method as previously described.

Samples were prepared for sequencing by purifying 30µ of PCR product (DNA concentration of approximately 12 µg per µl) with a Zymo Research DNA + Concentrator -5. Samples were eluted in 10µl of clean water. Following purification, 40µl clean water was added to each sample and the final DNA concentration in all samples varied between 10 and 18 µg per µl. Samples were sent to Geneius Labs, Newcastle, UK for sequencing and were supplied as 1 µl of purified sample mixed with 1µl of 357 forward primer without the GC - clamp.

3.1.7 - Diatom counts

One slide from each panel and chamber pair was chosen at random and placed under a Leica DMRB microscope and viewed under an x 100 objective lens. The slide was placed on the staging and the lens moved to approximately the middle of the slide. After focusing, a digital image of the slide was taken and the staging moved using random coordinates generated in Minitab Software version 15. Eight images of each slide were obtained in this manner. The images were imported into Image J image analysis software and all visible diatoms were counted and identified using to the lowest taxonomic level possible.

3.2 - Results

pH data were collected during the experiment which represented the internal environmental conditions in the test chambers (see Chapter 2 for sampling methodology). These data were analysed using a repeated measures ANOVA test with between subject factor of treatment and subsequent Ryan-Einot-Gabriel-Welsch F (REGWF) post hoc analysis performed in SPSS version 15. Prior to the ANOVA tests, all data were tested for normality using the

Anderson darling test performed with Minitab software. All results produced $p > 0.05$ and so data were not transformed.

3.2.1 – pH treatments and controls.

Initial plotting of the raw data suggested that a) the pH controllers produced two distinct pH treatments within their respective chambers and b) the environmental pH in the chambers acting as both types of control were very similar (Figure 14).

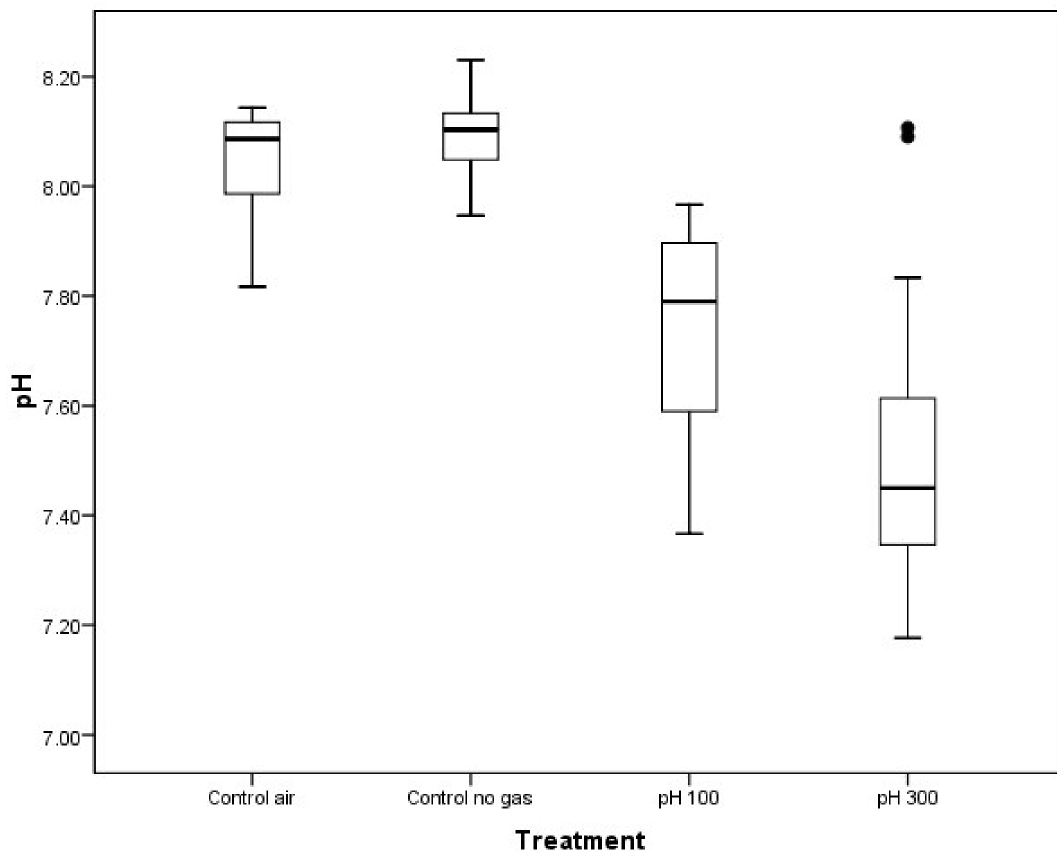


Figure 14. *Box plot displaying the internal pH of the experimental chambers, summarised over the 12 day duration of the experiment. n = 3, Boxes represent the interquartile range, horizontal lines indicate median pH and whiskers indicate 95% confidence interval. The asterisk represents an outlier, likely to result from the temporary failure of the solenoid causing the pH to rise above the target range.*

The treatment effect shown by the box plot in Figure 14 was confirmed statistically by performing a final repeated measures ANOVA test. This test encompassed all treatment and control pH data, complete with a REGWF post hoc test to indicate the subset grouping of the treatment or controls, relative to the pH values measured in each type of chamber. The Mauchly's Test of Sphericity confirmed the data were spherical, with a Greenhouse-Geisser value of 0.435. A highly significant difference (p <0.001) was produced by the repeated measures ANOVA confirming, as already suspected, a clear difference between treatments and controls.

Table 2. *Repeated measures ANOVA table showing a highly significant difference (p <0.001) between the pH in control chambers and treatment chambers.*

Source	Type III Sum of Squares	df	$\bar{x}$ Square	F	Sig.
Intercept	5147.442	1	5147.442	150400.067	p <0.001
Treatment	5.830	3	1.943	56.779	p <0.001
Error	.274	8	.034		

REGWF post hoc analysis demonstrated that, as intended, the pH 300 years treatment was significantly different and lower that the pH 100 years treatment. In addition, both controls were very similar to each other at around pH 8.01 (ambient), but significantly less acidic than either treatment.

Table 3. *REGWF post hoc table showing distinct and highly significant subsets between the environmental pH in control and treatment chambers, with treatment pH 300 years being distinct and significantly lower than treatment pH 100 years, which were in turn both distinct from the controls.*

Treatment	N	Subset		
	1	2	3	1
pH 300 years	3	7.45		
pH 100 years	3		7.70	
Control Air	3			8.06
Control no gas	3			8.08
Sig.		1.00	1.00	.94

.

The fluctuation of the internal pH in treatment and control chambers throughout the entire experiment is illustrated in Figure 15. A general trend of decreasing pH in all chambers was detectable at the end of the experiment, with the exception of treatment pH 100 years which increases between days ten and twelve.

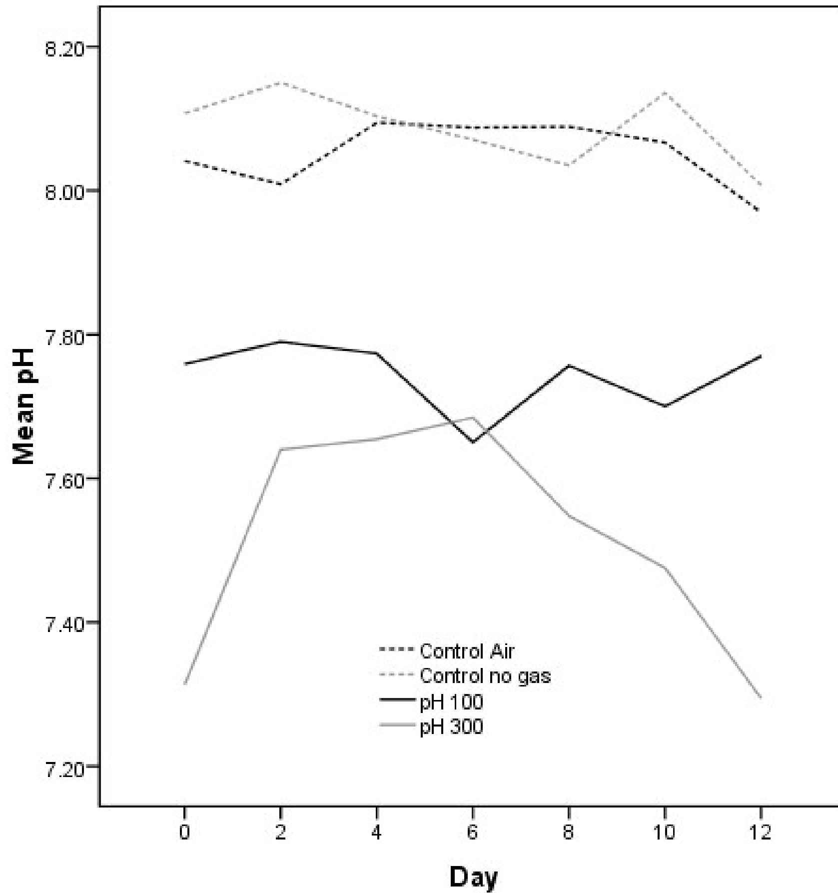


Figure 15. *$\bar{x}$ pH (n = 3 per treatment or control) measured at panel height inside experimental chambers where biofilms were cultured. Treatments represent the predicted ocean pH in 100 and 300 years time respectively, and with the continuation of OA. Error bars are removed for clarity. Variance around the $\bar{x}$ values is shown in Figure 14.*

The fluctuation of pH conditions generated inside treatment and control chambers were as follows: Control Air $\bar{x}$ pH = 8.06 ± 0.55, Control No Gas pH $\bar{x}$ = 8.08 ± 0.61, pH 100 year $\bar{x}$ = pH 7.70 ± 0.19, pH 300 year $\bar{x}$ = pH 7.455 ± 0.148. The range of fluctuation in both types of control is low, and corresponds to normal pH fluctuations in Hartlepool marina generated by water exchange in the lock system, evaporation and surface run off. The range of fluctuation in both treatments is nearly three times higher than the controls, but is consistent amongst the treatments. Fluctuation of the controls is expected as both pH controllers used to maintain the treatments were using the same type of microprocessor, which was set to cut in

and out when a deviation of ± 0.03 of a pH unit from the pre-programmed control value was measured.

3.2.2 - Molecular Results

All biofilm samples, irrespective of the environmental pH in which they developed, produced between 24 and 28 discrete bands when run on DGGE gels. A consistent pattern was evident in all gels, with little variation in the number or position of bands indicating genetic similarity between samples.

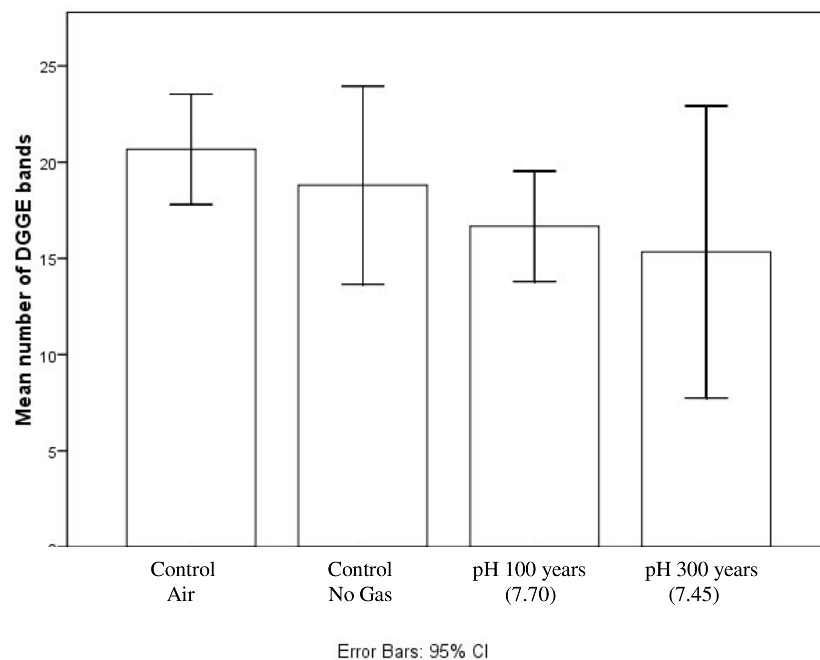


Figure 16. *Chart to show the* $\bar{x}$ *number of different DGGE bands on gels from each treatment or controls, n=3. According to the theory of DGGE and as confirmed by the sequence results, each band represents a different species or sub species of microbe (Muyzer, Dewaal et al., 1993).*

Table 4. *Univariate Analysis of Variance table indicating no significant difference between the $\bar{x}$ number of bands on DGGE gels representing biofilm microbial diversity from films grown under different pH regimes. df = degrees of freedom, F = F ratio, and p = probability of significance*

Tests of Between-Subjects Effects

Source	Type III Sum of Squares	df	$\bar{x}$ Square	F	p
Corrected Model	51.20[a]	3	17.06	1.83	.20
Intercept	4256.23	1	4256.23	458.64	.00
Treatment	51.20	3	17.06	1.83	.20
Error	92.80	10	9.28		
Total	4680.00	14			
Corrected Total	144.00	13			

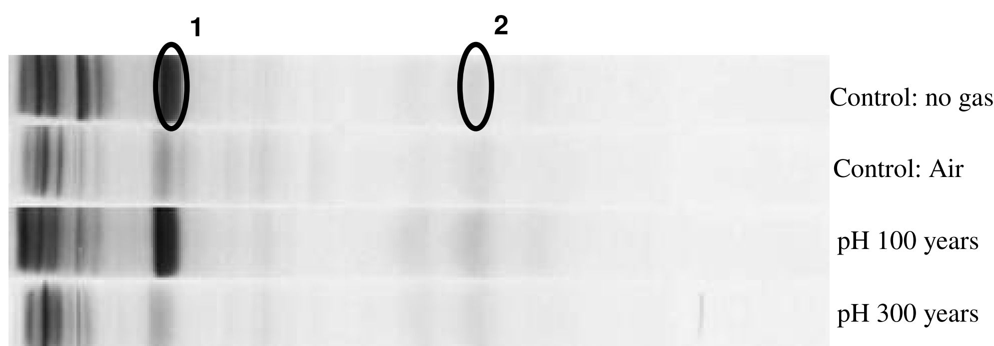


Figure 17. *DGGE gel showing bands produced by DNA extracted from biofilm samples cultured under control and treatment conditions. Ovals indicate bands extracted and successfully sequenced.*

Multiple bands were selected from the DGGE gels for sequencing, however only two of the bands came back from the sequencing process with sufficiently long and clean sequences to enable them to be blasted on the National Centre for Biotechnology Information (NCBI) microbes database. The results of from the NCBI data base are displayed in Table 5.

Table 5. *Sequencing results. DNA extracted from selected bands on the DGGE gel were sequenced and blasted on the NCBI microbes database.*

DGGE band #	NCBI access #	Closest relative/NCBI Ref	Similarity	Phylogenetic group
1	Z3573VRU016	Nodularia spumigena *CC*Y9414 1099428179654	*92 %*	*Cyanobacteria*
2	Z36RB2W3016	*Escherichia coli* B7A gcontig_1112495752718	88 %	Proteobacteria

Binary logistic regression analysis of the data obtained from the presence or absence of bands on DGGE gels confirms the impression of visual observation of the gels. A non-significant odds ratio (p= 0.29) and a concordant association value of 38.6 % suggest that treatment type (or control type) is not a suitable predictor for the presence or absence of DGGE bands, a proxy for microbial diversity within biofilms. The test also shows that the slopes of the data are not significantly different from zero (p = 0.290).

Table 6. *Binary Logistic Regression Table for presence or absence of bands on DGGE gels run with DNA extracted from biofilm samples grown under control pH (~ pH 8.01) and treatment one = pH 100 years (7.80-8.00) and treatment two = pH 300 years (7.80-7.50) conditions.*

Predictor	Coef	SE Coef	Z	P	Odds Ratio	95% CI Lower	95% CI Upper
Constant	-0.56	0.23	-2.37	0.01			
Treatment	-0.09	0.09	-1.05	0.29	0.91	0.75	1.09

Log-Likelihood = -215.77. Test that all slopes are zero: G = 1.11, DF = 1, p = **0.29**

Goodness-of-Fit Tests

Method	Chi-Square	DF	P
Hosmer-Lemeshow	0.03	2	**0.98**

Measures of Association between the Response Variable and Predicted Probabilities.

Pairs	Number	%
Concordant	10054	**38.6**
Discordant	8343	32.0
Ties	7654	29.4
Total	26051	100.0

3.3 - Diatom Abundance

Analysis of the biofilm samples using conventional light microscopy produced diatom abundance data representing 6 different genera; *Nitszchia sp*, *Achnanthes* sp, *Cocconeis* sp, *Pinnularia* sp, *Tabellaria* sp, *Navicula* sp.

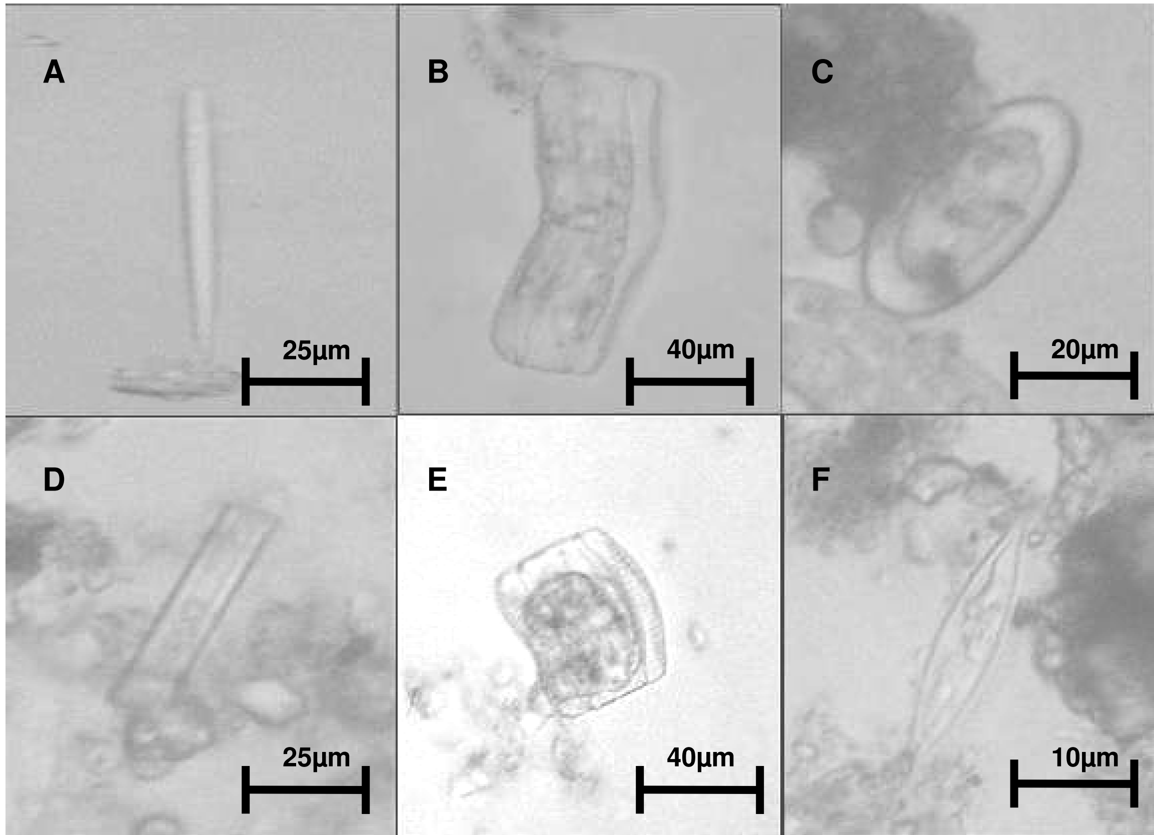


Figure 18. *Typical examples of diatoms identified from biofilm samples. A: Nitszchia sp B: Achnanthes sp, C: Cocconeis sp, D: Pinnularia sp, E:Tabellaria sp, F:Navicula sp.*

All these organisms occurred at low abundances (Figure 20) and there was no clear pattern of distribution in relation to treatment or control type. ANOSIM analysis of the diatom abundance data produced a sample statistic Global R = -0.004 with a significance level of the sample statistic: $p = 0.78$ indicating no significant difference of diatom abundance when compared between treatment type.

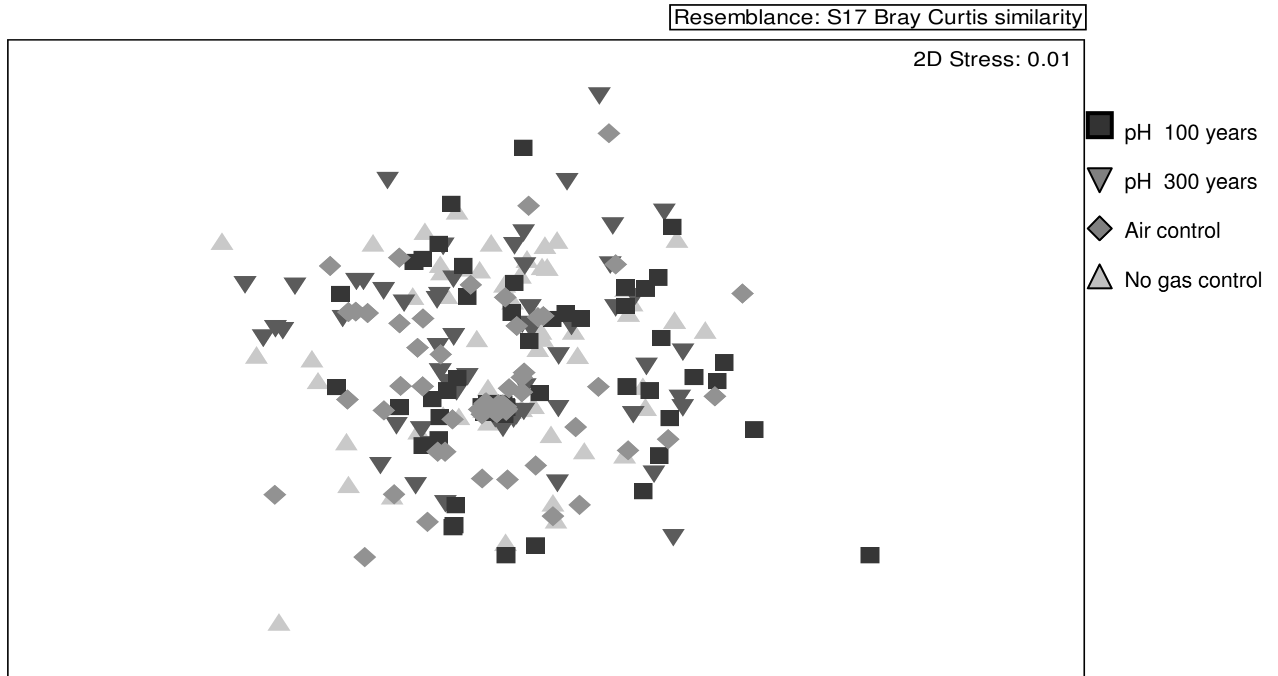


Figure 19. *MDS plot showing abundance of 6 species of diatoms (Nitszchia sp, Achnanthes sp, Cocconeis sp, Pinnularia sp, Tabellaria sp, Navicula sp.) identified from samples exposed to different treatment types pH 100 years 7.7, pH 300 7.45 years, Air control (pH 8.2) No gas control (pH 8.2). Each point represents a sub sample of diatom abundance in one of eight fields of view from three different sides exposed to each treatment type, n= 3. ANOSIM analysis: sample statistic (Global R): -0.004, significance level 78.2%.*

Pinnularia sp was the most abundant diatom species regardless of treatment. *Pinnularia* sp was also more abundant in the controls than treatments. However, the actual abundance of the diatom was very low in all samples (approximately one individual per sub sample). ANOSIM (Global R: - <0.01, p = 0.78) and ANOVA analysis (p = 0.54) confirm that no statistically significant difference between the abundance of *Pinnularia* sp occurred across the range of pH treatments. The post hoc analysis from the ANOVA is presented in Table 7.

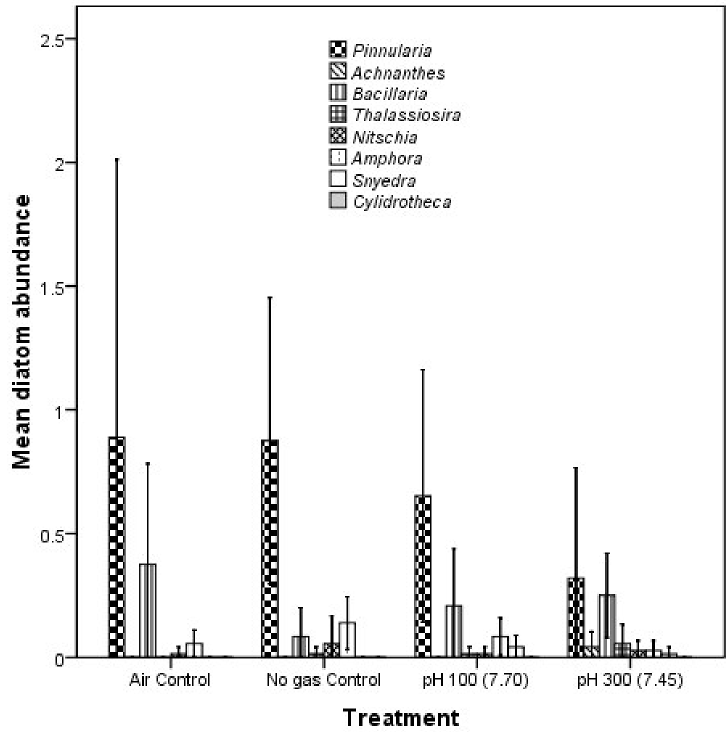


Figure 20. *$\bar{x}$ diatom abundance measured in biofilms grown under Air control and No gas control conditions (~ pH 8.01) and pH 100 (7.70) and pH 300 (7.45). n = 3, Error bars show 95% CI*

Table 7. REGWF post hoc analysis of Pinnularia sp abundance in biofilms grown under different pH regimes; pH 300, pH 100, Air control and No gas control conditions, n = 3.

Treatment	N	Subset
		1
pH 300 years	72	1.3194
pH 100 years	72	1.6528
No gas control	64	1.9844
Air control	65	1.9846
Sig.		.545

3.4 – Discussion

This Chapter aimed to investigate the microbial diversity associated with marine biofilms that had been grown under pH environments that simulate future OA conditions. The first requirement of achieving this aim was to use the newly conceived method described in Chapter 2 to generate pH treatments and controls that simulate OA predictions. Analysis of the pH data obtained throughout the duration of the experiment confirmed that discrete pH treatment and control conditions were created by the apparatus, and were maintained during the experimental run time.

The internal pH of the pH100 year and pH300 year chambers was being controlled by pH controllers set at 7.80 and 7.50 respectively. The pH measured during the run time of the experiment was lower than anticipated for both treatments. The observed $\bar{x}$ value for pH 100 years treatment being 7.70, and the pH 300 years treatment being 7.45 compared to the expected values of pH 7.80 and pH 7.50 respectively. This discrepancy between the pre-programmed pH value and the observed pH value is likely to be a result of the internal calibration of the pH controllers, as accurate calibration of the pH probes supplying the controllers with environmental data was conducted every two days. However, the variation between the pre-programmed pH values and the observed $\bar{x}$ pH values are still within the range predictions of future ocean pH conditions in 100 and 300 years time. As a result, they can still be considered suitable experimental treatments (Caldeira & Wickett, 2003). Both types of control remained at near ambient pH during the experiment suggesting that the no gas control and the introduction of compressed atmospheric air had no detectable effect on the environmental pH. Therefore, these conditions can be accepted as suitable controls,

designed to control for any effects on biofilm processes caused by any non pH related artefact of gas introduction.

Overall analysis of the pH profiles generated and maintained by the novel exposure apparatus are extremely encouraging. The pH data collected during this experiment suggest that this equipment and method is capable of filling its design brief, which was to create and maintain low pH micro environments in the field, where the response of marine assemblages to OA simulations can be measured.

Analysis of the DGGE gels suggests that in this instance, experimental manipulation of environmental pH had no measurable effect on biofilm associated microbial diversity. DNA extracted from biofilm samples grown under control and treatment conditions all shared highly similar patterns in terms of presence, absence and the position of bands, which were considered a proxy for microbial diversity (Muyzer, *et al.,* 1993). The DNA sequenced from two different bands produced good Blast matches with two different microbes, so it is reasonable to presume that the different bands on the DGGE gels do indeed represent different microbial species or sub-species, and therefore can be considered a proxy for microbial diversity (Muyzer *et al.,* 1993). Similarly, there was no effect of pH treatment detected in the diatom data with the exception of *Pinnularia* sp abundance under the pH 300 treatment. This leads to the acceptance of the null hypothesis: ocean acidification driven pH change will not significantly affect the microbial diversity of biofilms in temperate marinas in the next 300 years.

Initially it is surprising that there was not a greater effect of pH manipulation on the response variables measured in this study considering the pH decrease generated in the pH 100 year

chambers equates to ~75 % increase in acidity or [H^+] compared to ambient conditions. However, there are several possible explanations for this apparent lack of influence. The biofilms that recruited and developed during the two week experiment can be considered early colonisers of the substratum. Primary marine colonisers are frequently tolerant of a range of environmental conditions that allow them to perform their opportunistic strategies in a variety of environments (Peckol & Rivers, 1995; Karol, 2004). It is possible that the biofilms and associated microbes that recruited during this experiment are also characteristically tolerant to environmental fluctuations, and therefore were able to grow to the same extent, irrespective of experimental pH manipulations.

It is possible that a greater response to the treatment conditions created during this experiment would have been detected if the same experiment was conducted in a more stable, fully open ocean system. The treatments produced during these experiments produced pH conditions that were lower that the 100 year and 300 predictions for future OA conditions. Although unlikely, had the pH change produced by the pH treatments been closer to the predicted values, a different effect on diversity may have been measured. It is equally possible that the biofilm associated microbes measured in this study are simply able to tolerate what can be fairly described as considerable pH stress, equivalent to the most severe conditions expected to occur as a result of ocean acidification in the next 300 years. If the latter is the true we may expect to see an equally lush biofilm assemblage in the face of environmental pH change. Crucially however, this does not take into account any synergistic effects of other environmental factors such as temperature change and nutrient availability.

Another possible explanation for the lack of effect seen in this study is the fact that Hartlepool marina is subject to substantial variation in environmental conditions as a result of

water exchange from the lock mechanism, run off from surrounding man made surfaces, storm drains and evaporation. By definition, any organism capable of reproducing and growing in Hartlepool is tolerant of this variation in environmental conditions, and may therefore be relatively unsusceptible to the pH manipulations generated during this experiment. This underlying variation in environmental conditions at Hartlepool brings in to question its suitability as an experimental site for this type of work. However, the main aim of this study was to experimentally lower the pH of a body of water *in situ*, by approximately 0.7 of a pH unit compared to ambient, to simulate predicted OA scenarios. Hartlepool proved to be secure enough to maintain the necessary experimental rig and equipment, and also had the required utilities, space, permission and location to fulfill this aim.

Biofilms are three dimensional matrixes that restrict the flow of water within the film and are frequently inhabited by large numbers of respiring microbes (Qian, *et al.,* 2007). These conditions often create internal pH gradients to which the inhabiting microbes must be tolerant in order to survive (Qian, *et al.,* 2007). This inherent pH tolerance may also explain some of the lack of response detected by this study.

Alternative methods of microbial assemblage analysis such as Fluorescence *In Situ* Hybridisation (FISH) should be considered when advancing this area of research as any positional alterations of microbial distribution within the film induced by the external modification of pH could be detected in this way (Pernthaler, 2002). The distribution of microbes within a film may well alter its function by affecting respiration and primary production rates as distance from the oxygenated surface layers of the film increases.

The conclusion reached from analysis of this experiment is that environmental pH decreases of up to 0.7 of a unit will not have a major effect on the diversity of microbes in two week old biofilms. Minor changes in relative diatom abundance may have been induced by manipulation of environmental pH, yet these changes were not strong.

Research on the effect of low pH on eukaryotes has demonstrated that short term exposure to conditions that simulate OA one hundred years in the future can result in subtle changes to physiological parametress such as muscle tissue ratios (Wood, *et al.,* 2008) and calcite integrity (Michaelidis, *et al.,* 2005). These responses are very important in terms of the long term persistence of a species. However, short term experiments, such as this study, may miss these more intricate responses if only coarse response variables such as presence and absence or diversity are measured. It is possible that analysis of physiological performance of the microbes that grew during this experiment would indicate an effect of the low pH exposure.

However, caution must be exerted before robust predictions can be made concerning marine biofilm associated processes and their response to OA environmental conditions. It will be necessary to perform trials similar to this study, but in fully open ocean systems where the biofilm associated microbes are recruiting from, and are acclimatised to, the stable pH conditions that currently persist in most oceans environments. Results from this study suggest that this method, if applied in an open ocean system may be capable of providing data which would form the basis of such predictions.

Chapter 4

Macro-invertebrate and Algal Recruitment

4.0 - Introduction

Marine biofilms are primary colonisers of vacant substratum immersed in seawater and are the first stage of marine fouling assemblage development. Secondary colonisation involves the recruitment of macro-invertebrate larvae and algal spores which develop into juvenile organisms. Chapter 4 investigates this secondary stage, and attempts to measure recruitment and dynamics of assemblages subjected to manipulated pH treatments that simulate future OA scenarios.

Many marine organisms, particularly benthic macro-invertebrates, incorporate a larval stage in their life cycle. This allows an organism to achieve several objectives in one life stage, promoting the survival and fitness of both the individual and the population (Thorson, 1950; Scheltema, 1972; Vance, 1973a; Vance, 1973b; Underwood, 1974; Graham, 1985; McEdward, 1997; Pechenik, 1999). An obvious advantage of a larval stage is to facilitate the dispersal of viable genetic material, with the aim of either maintaining or increasing the abundance and distribution of a population. The range and duration of this larval distribution stage is highly variable between species and can be as little as a few centimetres in a few minutes in tunicates or hundreds of kilometres over several months or even years in crabs (Scheltema, 1972; Pawlik, 1992; Cowen, 2000; Hadfield, 2001; Barber, 2002; Sotka & Palumbi, 2006).

Larval dispersal strategies can be broadly categorised as either direct development, where larvae are fully formed and move only a short distance from the adult, lecithotrophic where larvae are usually equipped with a finite food supply frequently in the form of an egg sack or planktonotrophic where the larvae are fully adapted to survive in a planktonic mid-water stage where it feeds from the water column until recruitment (Thorson, 1950; Pawlik, 1992; Pechenik, 1999). These strategies vary between groups of organisms and are not species or even phylum specific.

The dispersal of larvae away from the adults also provides the next generation of individuals with the opportunity to escape parasites which may have established themselves on the parent population. In addition, this life cycle phase provides the opportunity to avoid demersal predators associated with the parent population, together with seeking out environmentally beneficial settlement sites with superior nutrient supplies (Marshall & Bolton, 2007; Visser, 2007; Visser, *et al.,* 2009). The dispersal of offspring also reduces the genetically negative effects of intra-specific competition (Scheltema, 1972; Graham, 1985; Pechenik, 1999).

Although larval stages provide clear advantages and benefits to the survival and fitness of an organism, there are considerable risks to individuals during this stage (Visser, 2007; Visser, *et al.,* 2009). Frequently, marine invertebrate larvae are released by an adult in response to environmental cues. These cues include day length, optimum environmental conditions or nutrient supply (Marshall & Bolton, 2007). These larval releases often involve vast numbers of individuals (Marshall & Bolton, 2007) and although some larvae are capable of small scale predator avoidance behaviour (Kiørboe, 1999; Navarrete, 2000; Metaxas, 2006), larval releases represent an abundant and relatively defenceless food supply to a whole host of predators.

Marine invertebrate larvae can be considered to perform a dual role, contributing to both the survival of their own kind, but also as sustenance for other species. It is the importance of this dual role in virtually every marine ecosystem type which concerns marine biologists, as the response marine larvae and their ability to recruit whilst enduring the advance of OA is currently unknown. Marine invertebrate larvae contribute extensively to the diet of many marine organisms from large and small pelagic and demersal fish, massive cetaceans and even to larger larvae (Frederiksen, 2006; Visser, 2007; Visser, *et al.,* 2009). This source of nutrients, which is routinely pumped into the oceans forms important lower trophic levels which often underpin entire ecosystems.

Marine invertebrate larvae are potentially vulnerable to low pH environments produced by the progression of OA for several reasons. Many larvae, particularly of crustaceans, possess a test or carapace formed from calcite, aragonite, or high magnesium calcite. As a result, they are at risk of dissolution if carbonate ion concentration dips below the saturation state in sea water. This critical reduction in saturation state, a direct effect of OA, is predicted to occur in some regions as early as 2038 (McNeil & Matear, 2008). The second factor that puts marine larvae at high risk from negative impacts resulting from OA is their limited capacity for large-scale avoidance, partly a result of their small individual size. Many marine invertebrate larvae are often highly motile on a micro scale and are able to exert chemo or photo-taxis together with small scale predator evasion (Chia, *et al.,* 1984; Navarrete, 2000; Marshall & Bolton, 2007; Visser, 2007). This degree of taxis allows larvae to avoid unfavourable substrata when settling, and select beneficial environmental conditions. However, the extent of this motility is usually limited to small three dimensional movements and/or slow diurnal vertical migration in the water column to allow nocturnal feeding (Chia, *et al.,* 1984). This

capacity of motility does not allow the larvae to avoid large scale areas of low pH or under saturated water. As a result, many larvae are at the mercy of oceanic currents which may bring them into contact with potentially corrosive waters, in contrast to animals such as adult pelagic fish which may be able to avoid such areas (Moel, 2009).

Although marine invertebrate larvae are generally not capable of active large scale horizontal motility, they do exert rapid small scale moments and, when viewed under magnification, often appear highly energetic. This level of activity is frequently combined with fast rates of growth which together mean that larvae generate high metabolic demands, supported by fast metabolic rates (Hendrickx, 2008). This high metabolic rate puts them at further risk of negative effects caused by the progression of OA, and one of the most common methods of dealing with pH stress in marine invertebrates in to increase active ion transport across cell membranes, a metabolically costly process (Seibel & Walsh, 2002). This extra metabolic demand could mean reduced energy allocations available for protein synthesis together with other vital homeostatic processes, ultimately threatening the survival of the larvae.

As discussed in Chapter 2, on completion of the larval stage, many marine invertebrates settle out of the water column. Once settled, the new recruits begin a sessile adult life stage. During the process of settlement, larvae are able to exert taxis in response to a variety of stimuli, allowing them to actively select optimum environmental conditions including neighbouring assemblage composition (Kon-Ya, 1994; Rittschof, 1998; Maki, 1999; Hadfield, 2001; Steinberg, 2002; Dobretsov, *et al.,* 2006; Dobretsov & Qian, 2006a; Qian, *et al.,* 2007; Prendergast, 2008). This process of selective settlement is frequently a result of chemical cues (Maki, 1999; Dobretsov, *et al.,* 2006; Dobretsov & Qian, 2006a; Qian, *et al.,* 2007).

As OA alters the chemical composition of seawater, it has the potential to disrupt the chemically mediated process of selective recruitment of marine larvae. The effect of OA on invertebrate recruitment has not received much attention (Cigliano *et al.,* 2010). Any effect of OA on recruiting marine larvae has great potential to alter benthic environments, as the interaction between larvae and a recruitment substratum is a major component determining assemblage structure and composition in benthic systems (Gaines, 1985; Butman, 1987; Ólafsson, 1994).

It is very difficult to measure the responses of larvae to OA in the field where they behave naturally. As a result, the only studies aimed at measuring larval settlement and recruitment together with early assemblage development under conditions that simulate future OA have been conducted in mesocosms with the exception of Cigliano *et al.* (2010). Kuffner *et al.,* (2007) and Jokiel *et al.,* (2008) provide two such accounts where a through-flow mesocosm system was used to measure invertebrate recruitment under low pH conditions that simulate OA. The proximity of the mesocosm system to the ocean allowed unfiltered ocean water to be pumped through chambers in which the pH had been experimentally manipulated to mimic OA. Invertebrate and algal recruitment on plastic substrata was measured, and while coral recruitment continued under low pH conditions, crustose coralline algae showed a marked reduction in recruitment and growth rates.

These studies represent a substantial advance on traditional laboratory based techniques as the recruitment under low pH conditions can be measured without altering larval densities, nutrient flux and environmental parametress such as temperature and light. However, one significant deviation from realistic OA conditions remains with this methodology as the low pH conditions were created with the controlled addition of H_2SO_4.

The biological response to low pH environments created with mineral acids differs to the response to the same pH conditions created by the addition of gaseous CO_2. Kikkawa, *et al.,* (2004) demonstrated the different response with flounder and suggest that the difference in effect is thought to be a result of the greater ability of CO_2 to permeate cell membranes compared with H_2SO_4. Because of this difference in severity of effect, it can be considered that experiments that use mineral acids as a proxy for OA induced pH change might be under-estimating the true implications of the phenomenon. This is concerning as even these underestimations still produced results suggesting that important assemblage structuring species such as coralline algae were less able to recruit under low pH conditions.

A review of the literature conducted for Chapter 1 suggests that a method is needed which would allow recruitment to be measured under low pH conditions that simulate OA, without altering natural larval densities, nutrient flux and environmental conditions. In addition, this method should ideally create the low pH conditions with the addition of CO_2 rather than mineral acids. The technique developed for Chapter 2 of this thesis provided an opportunity to achieve these objectives. In this Chapter, the same methodology as described in the two previous Chapters is used to expose settling marine invertebrate larvae to experimental pH manipulations that represent future OA scenarios. This experiment was conducted to test the following hypotheses:

1: Macro-invertebrate and algal settlement and recruitment is affected by *in situ* environmental pH reduction.

2: Early assemblage composition is altered by decreased environmental pH.

4. 2 - Methods

Recruitment and early succession was measured under a manipulated pH treatment, pH 100 years (7.74) as well as control conditions at ambient pH in the form of an air control (pH 7.95), and a no gas control (pH 7.94). The pH 100 years treatment was selected to simulate predicted pH conditions of open waters in 100 years as a result of OA (Caldeira & Wickett, 2003). The control values are lower than could be expected of fully oceanic waters, but reflect the background pH during the summer in Hartlepool marina. Each treatment or control chamber was replicated three times, n= 3. All recruitment took place on settlement panels attached to the apparatus as described in Chapter 2. The settlement panels were 12 x 12 cm square, three mm thick and made from grey PVC supplied by Bay Plastics of North Shields, Tyne and Wear, U.K. All panels were evenly roughened for one minute with coarse sand paper (grit size 60) on an electric orbital sander, in order to ensure homogeneous rugosity between panels.

The set-up, gas delivery and sampling regime were identical to that described in Chapter 2, the only exception being that one pH treatment (pH 100 years (7.74) was used instead of two. The experimental apparatus complete with settlement panels was deployed in Hartlepool marina from the 1st of May until the 19th September 2008 with all the panels maintained at 30 cm depth. During this time, water samples were collected and analysed from panel height every two days, and the gas supplies were maintained as described in Chapter 2.

The same data logger described in the methods, Chapter 2 was fixed on a supporting beam of the experimental frame at 30 cm depth, but 30 cm away from the nearest chamber. This data

logger was left *in situ* to log ambient pH in the marina. Every four days the data logger probe was cleaned by vigorously swirling it in calibration buffer. This used buffer was discarded and the probe was re-calibrated with fresh buffer and fixed back in place.

During the first month of the deployment the settlement panels were left to develop without any interference. During this time, any larvae that recruited on the panels were left to grow and increase in size until such a point where they could be identified to species level from photographs and examination during short periods of emersion during sampling. After this month the whole experimental unit was removed from the water, and high quality digital photographs were taken of each panel (Canon G4, 4 $x10^6$ pixels, RAW file format). Photographs were repeated in this manner every two weeks for the duration of the experiment. For a full description of this method see Sugden *et al.,* (2008).

The digital photographs were cropped to 11 x 11 cm using Photoshop CS software, which served both to standardise the size of the image and remove a 1 cm wide perimetres from each image to reduce the influence of any edge effects in further analysis (Underwood, 1997). The images were then analysed using ImageJ image analysis software (ImageJ, 2007) by creating a grid arrangement of 100 dots as a layer on each image. The particular species under each dot was identified and used to represent 1% cover. Where one species had overgrown another, and the underlying species' identity was obvious, both layers of assemblage were counted to provide a coarse measure of stereology, occasionally giving rise to values of percentage cover that exceeded 100%.

After nearly five months in the field, the experimental apparatus was recovered from the water and the settlement panels were detached and placed in labelled freezer bags. The bags

were taken back to the laboratory where they were stored at - 20°C until further analysis. Back in the laboratory, the panels were carefully defrosted and individual mussels (*Mytilus edulis*), and solitary tunicates (*Ciona intestinalis*) were removed from each panel (for a full species list see Table 13). Only individuals that had recruited and grown on the top of the settlement panel were selected for analysis as these animals had been exposed to the full treatment effect. Other animals had grown on the underside of the panel, but as these were not directly exposed to the full treatment effect they were removed and discarded. To further reduce the influence of edge effects, only the individuals that had grown in the central 11 x 11 cm area of the panel were analysed (Underwood, 1997).

4.2.1 - Biogenic Ratios

This method was conducted in order to compile a data set representing the ratio between somatic tissue weight and shell or gonad weight, depending on the species. This response variable was chosen as increased active pH buffering by test organisms may have resulted in a reduced energy allocation for calcification or protein synthesis, leading to alterations in gonad/somatic tissue/shell ratios.

The somatic tissue was removed from *M. edulis* individuals and placed in Petri dishes for drying, along with the organism's accompanying shell. *C. intestinalis* individuals were dissected immediately after defrosting while still wet, and their entire gonad was removed. The somatic tissue or tunic of the squirt was then dried in an open Petri dish that also contained its extracted gonad in an Epindorf tube, which was left open to aid drying.

Following dissection, all tissues along with their accompanying shell or gonad were dried in ovens set to 50°C for 48 hours or until repeated weighing showed no further decrease in

weight. An electric balance (Satorious, BA210) was used to weigh the somatic tissues of larger animals and shells. A micro balance (Mettler Toledo, MX5) was used to weigh the tunicates gonads.

In addition to the somatic tissue measurements, mussel shell length and thickness was measured in all *M. edulis* individuals extracted from the settlement panels. Shell length was measured on the longest dimension of the shell using electronic callipers (RoHS accurate to ± 0.03 mm). Thickness was measured at three separate areas of the shell depending on overall shell size using a calibrated micrometres (Mitutoyo, accuracy ± 1 µm, flatness ± 0.3 µm, parallelism ± 1 µm). The edge was measured if the shell was too small for accurate measurements to be made at dorsal scar or the centre.

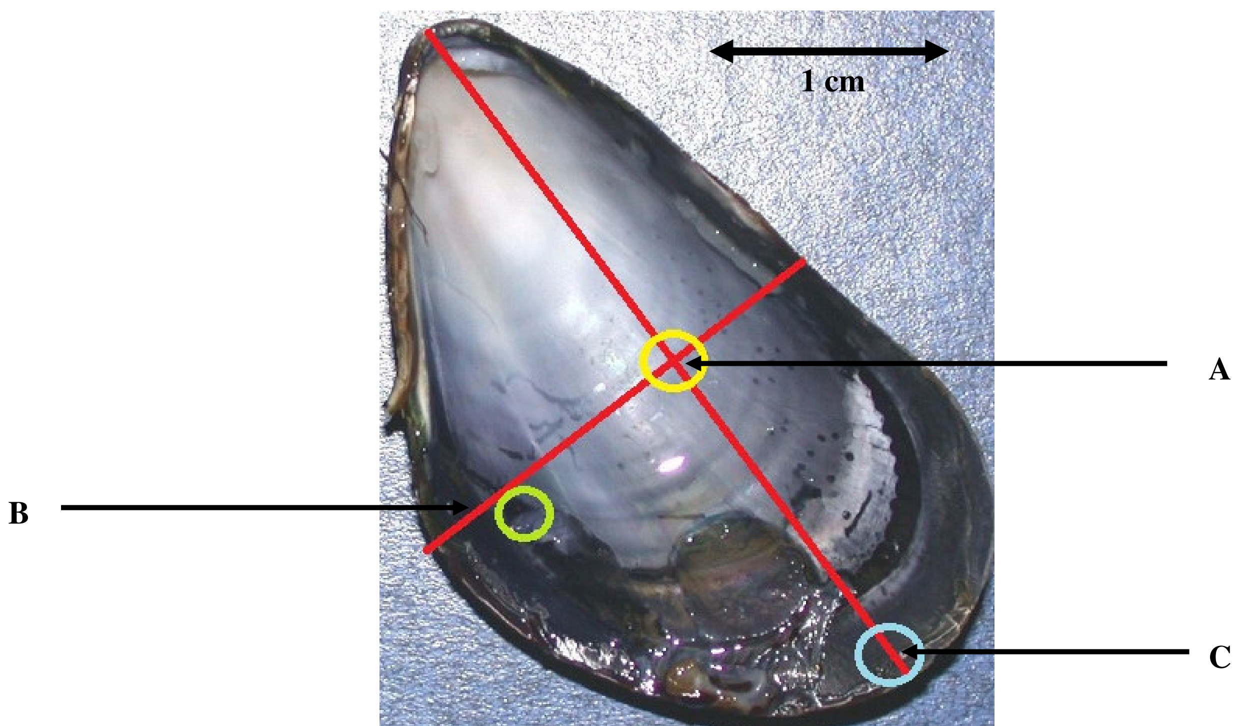


Figure 21. *Image showing measurement points on mussel shells, centre, dorsal scar, and edge. A is the centre of the shell found at the intersection between the longest and widest dimension of the shell. B indicates the base of the dorsal scar and C indicates the edge measurement at the end of the longest dimension of the shell.*

4.2.2 - Statistical Analysis

pH data obtained from water sampling conducted during the experiment was analysed using a repeated measures ANOVA and a Ryan-Einot-Gabriel-Welsch F post hoc test both conducted in SPSS v. 15. All environmental pH data were statistically normal with Anderson-Darling tests of normality conducted in Minitab v. 15 producing $p = 0.05$ in all cases.

In order to test for differences between the species composition in the early developing assemblages, the percentage cover data were arc-sin transformed and analysed using Primer Software version 6. Bray Curtis similarity matrixes were calculated representing the percentage cover of all species on all settlement panels at each month. Resemblance matrixes were calculated with the inclusion of a dummy variable to down weigh the influence of sparse species. These matrixes were analysed using Non Metric Multidimensional Scaling (NMDS) and Analysis of Similarity (ANOSIM) to test for similarity between assemblage composition between panels exposed to reduced pH and the two controls. If significant differences were detected by the ANOSIM test, further analysis was conducted with Similarity Percentages (SIMPER) to determine the rank percentage contribution of the different species to the total dissimilarity, n = 3 for both treatment and control.

Additional multivariate exploration of the percentage cover data was conducted with Principal Response Curve (PRC) analysis to test for the effect of the introduction of CO_2 on percentage cover of the resident species over time. Significance of the PRC analysis was produced by a Monte Carlo test. Both tests were performed using R statistical software (R, 2008).

The response variables *M. edulis* shell thickness; shell length and shell to somatic tissue ratio were measured as end point responses after the full five months of exposure to treatment and control conditions. *C. intestinalis* gonad to somatic tissue ratios were also measured as an endpoint response variable. These multiple response variables were first analysed using MANOVA, followed by a one way GLM ANOVA, in SPSS v. 15. If any significant difference was detected, a Ryan-Einot-Gabriel-Welsch post hoc test was used to further separate the proportion of the variance attributed to each treatment. Prior to analysis, data were transformed if they failed to meet the Anderson-Darling test of normality, and all ratio data was arc-sin transformed. A summary of the response variables and any transformations is shown in Table 8.

Table 8. *End point response variables, transformations performed and results of normality testing.*

Response Variable	**Transformation**	**Anderson Darling Test of Normality** ***p*** **=**
Ciona tissue	None	0.20
Ciona gonad	None	0.25
Ciona tissue to gonad ratio	Arc-sin	0.58
Mytilus tissue	None	0.23
Mytilus shell	None	0.20
Mytilus tissue to shell ratio	Arc - sin	0.93

4.3 - Results

4.3.1 - Test of experimentally produced low pH environmental conditions

Statistical analysis of the water sample data confirmed that the apparatus and technique used in this experiment produced an experimental pH treatment that was significantly ($p<0.001$) different from both the controls. Post hoc testing revealed the introduction of CO_2 produced environmental conditions around the settlement panels which were 0.2 of a pH unit lower than the controls. A pH decrease of this magnitude is predicted to occur as a result of OA by the year 2100 according to the models produced by Caldeira & Wickett, (2003).

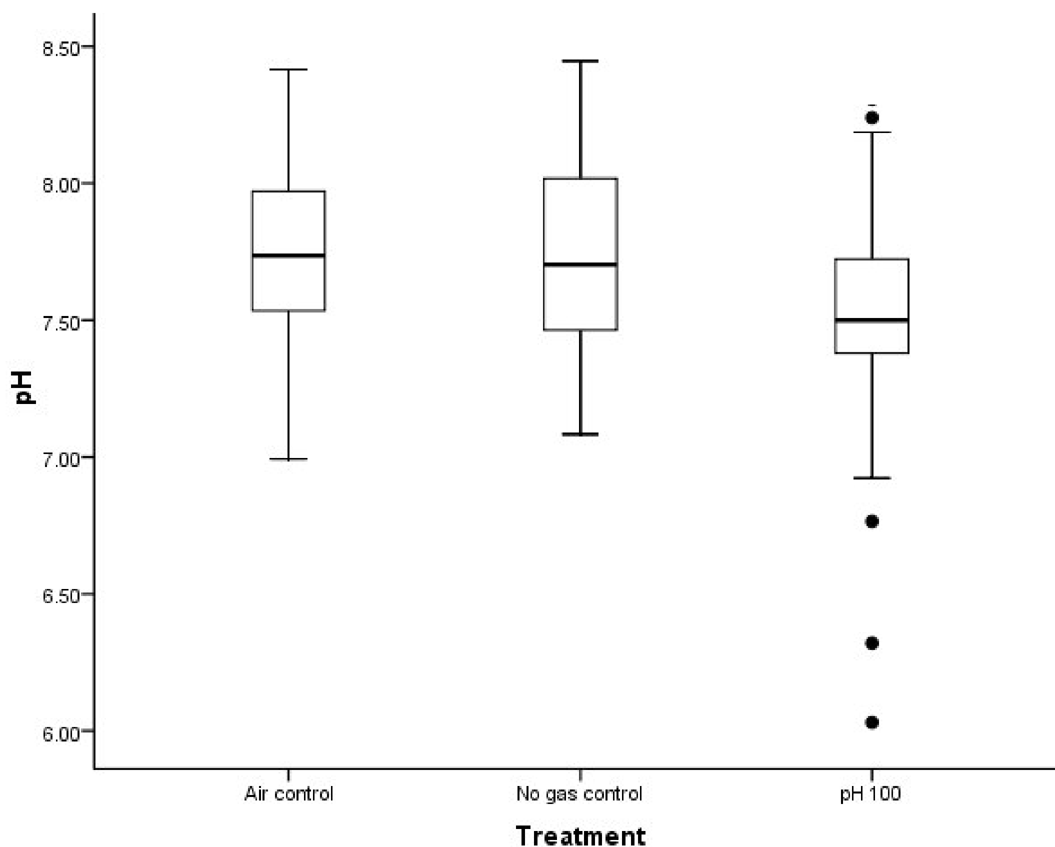


Figure 22. *Box plot of water sample pH taken from experimental chambers. Horizontal lines indicate the median and bars indicate 95 % confidence intervals, black dots indicate outliers .*

***Table 9**. Repeated measures ANOVA table showing a test for difference between the experimentally reduced pH conditions produced by the introduction of CO2 and an air control and no gas control. Data were spherical, Greenhouse geisser p = 0.28.*

Source	Type III Sum of Squares	df	$\bar{x}$ Square	F	p
Intercept	19650.61	1	19650.61	112776.89	<0.01
Treatment	2.94	2	1.47	8.44	.01
Error	1.04	6	.17		

***Table 10.** Post hoc analysis of experimentally reduced pH conditions produced by the introduction of CO_2 compared to an air control and no gas control.*

Treatment		N	Subset	
		1	2	1
Ryan-Einot-Gabriel-Welsch F(a)	pH 100 years	3	7.74	
	No gas control	3		7.94
	Air control	3		7.95
	Sig.		1.00	.80
Ryan-Einot-Gabriel-Welsch Range(a)	pH 100 years	3	7.74	
	No gas control	3		7.94
	Air control	3		7.95
	Sig.		1.00	.80

A running $\bar{x}$ value of pH 8.07 was calculated from the ambient pH data collected by the data logger (data in appendix 1). This value deviates from the $\bar{x}$ control pH values by ± 0.13 and deviates from the $\bar{x}$ low pH treatment value by ± 0.33 of a unit.

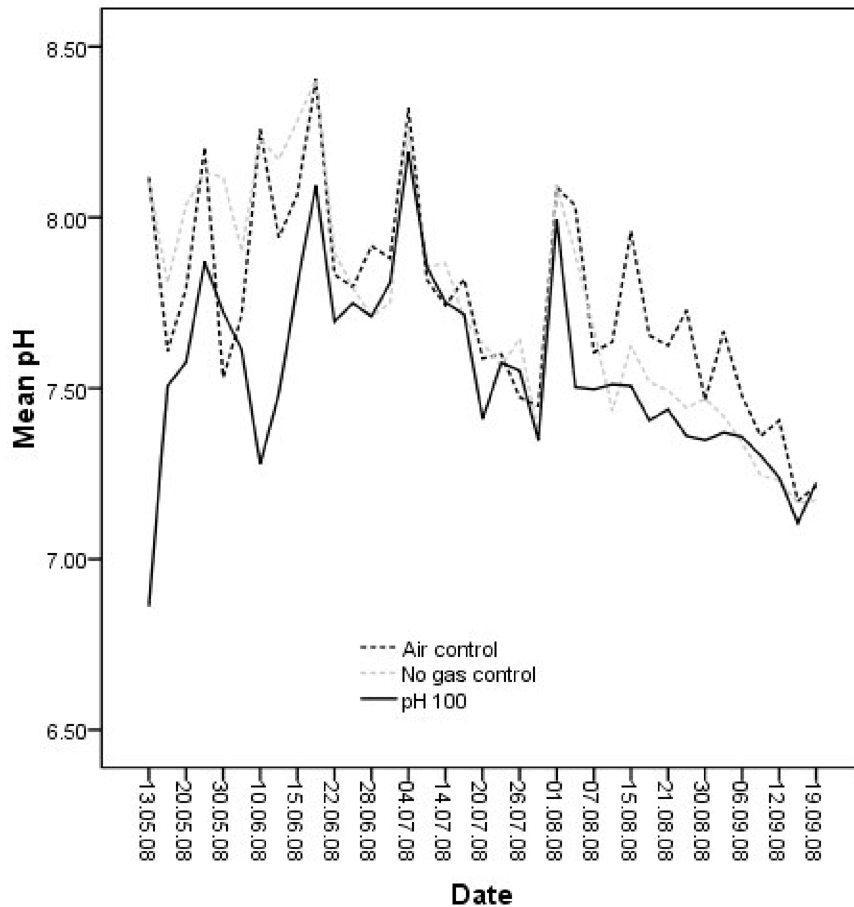


Figure 23. *$\bar{x}$ pH of water from experimental chambers. Error bars are removed for clarity. The variance around the $\bar{x}$ values is shown in Figure 22 and Table 9.*

The time series pH data from the contols (Figure 23) and the data logger (appendix 1) show variable but comparable pH values until the 4th of July. From this point onwards a clear drift can be seen between the data logger and the treatments and controls. This drift is attributed to a measurement error and is addressed in the final discussion, Chapter 8. The water samples taken before the drift occurred were analysed with a separate repeated measures ANOVA and again the treatment effect was significant ($p = 0.05$), Table 9 – 10. Data after this point (post 4th of July) were not analysed separately as the extent of the drift is not possible to separate from the background data.

The considerable variation in pH treatments is in part attributed to fluctuation of the background pH in the shallow waters of Hartlepool Marina, this is also discussed in Chapter 8.

Table 11. *Repeated measures ANOVA table showing test for difference between the experimentally reduced pH conditions produced by the introduction of CO2 and an air control and no gas control data from before drift occurred at 4*th *of July only.*

Tests of Between-Subjects Effects

Source	Type III Sum of Squares	df	$\bar{x}$ Square	F	Sig.
Intercept	8397.986	1	8397.986	69082.511	.000
Treatment	3.515	2	1.757	14.456	.005
Error	.729	6	.122		

Table 12. *Post hoc analysis of experimentally reduced pH conditions produced by the introduction of* CO_2 *compared to an air control and no gas control.*

	Treatment	N	Subset 1	Subset 2
Ryan-Einot-Gabriel-Welsch F[a]	pH 100 years	3	7.6637	
	Air control	3		7.9587
	No gas control	3		8.0390
	Sig.		1.000	.316
Ryan-Einot-Gabriel-Welsch Range[a]	pH 100 years	3	7.6637	
	Air control	3		7.9587
	No gas control	3		8.0390
	Sig.		1.000	.316

4.3.2 – Assemblage responses

During the five month run of this experiment, macro-invertebrate and algal assemblages recruited and developed on the settlement panels. In general, these assemblages consisted of thirteen macro-fouling species and biofilm. The assemblage development over time can be seen for a typical replicate of each treatment type in Figure 26.

Table 13*. Species list from all panels excluding biofilm*

Mytilus edulis	Bivalve mollusc
Balanus crenatus	Arthropod crustacean
Ectocarpus siliculosus	Filamentous brown algae
Punctaria plantaginea	Brown algae
Pomatoceros triqueter	Tube dwelling polychaete worm
Obelia dichotoma	Erect bryozoan
Bugula flabellata	Erect bryozoan
Membranipora membranacea	Encrusting bryozoan
Ciona intestinalis	Solitary tunicate
Clavelina lepadiformis	Solitary tunicate
Ascidiella aspersa	Solitary tunicate
Botrylloides leachii	Colonial tunicate
Diplosoma listerianum	Colonial tunicate
Botryllus schlosseri	Colonial tunicate

The assemblages were initially dominated during the first two months by colonial tunicates (*Botrylloides leachii, Diplosoma listerianum, Botryllus schlosseri*) algae (*Ectocarpus siliculosus*) and a solitary tunicate (*Clavelina lepadiformis*). Subsequently, species dominance shifted towards larger solitary tunicates such as *Ciona intestinalis* and *Ascidiella aspersa* (Table 13).

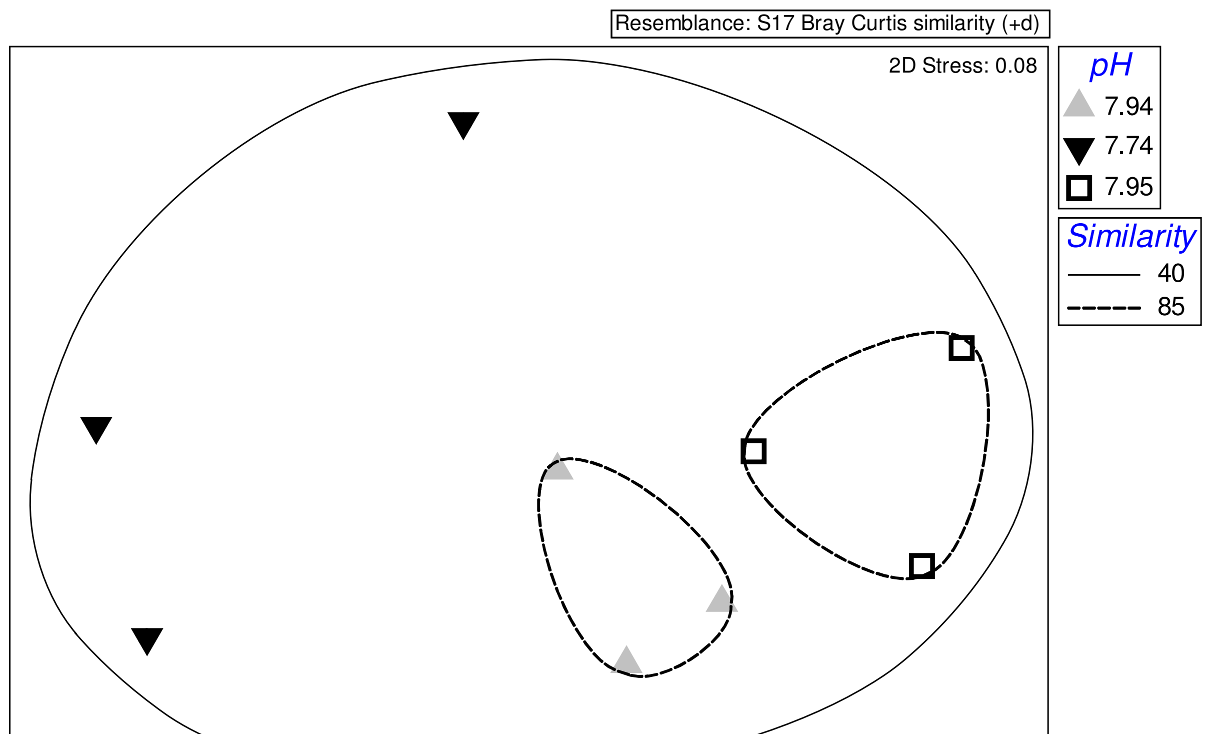


Figure 25. *nMDS diagram showing relationship between recruitment of fouling species and pH treatment in July. The pH 100 year panels support a different and more highly variable assemblage structure compared to the two control treatments.*

ANOSIM of the percentage cover data in Table 10 shows significant differences in species cover occurring between pH 100 years (7.74 pH) panels and control panels during July (p =0.04).

Table 14. *ANOSIM results used to test for differences between percentage coverage of species on panels exposed to either pH 7.94 (no gas control) 7.95 (air only control) and 7.74 (pH 100 years) at each sampling point.*

Date	Global R	*p*
June	-0.01	0.5
July	0.51	0.04
August	0.23	0.06
September	0.11	0.14

Pairwise ANOSIM analysis of percentage cover data from all panels in July revealed that the greatest dissimilarity occurred between the low pH panels and both the controls, whereas the least dissimilarity occurred between the two controls.

Pair	Pairwise R	Significance *p* =
Air control / pH 100 years	0.48	0.10
No gas control / pH 100 years	0.81	0.10
Air control / no gas control	0.37	0.20

SIMPER analysis (Table 15) also allowed the rank percentage contribution to the dissimilarity to be identified and attributed to individual species. During July, the percentage cover of biofilm, *Clavelina lepadiformis* and *Ectocarpus siliculosus* contributed most to the dissimilarity between the low pH panels and the controls. During August, the top three contributors to the dissimilarity between low pH panels and controls was the percentage cover of biofilm, *Botrylloides leachii* and *Ciona intestinalis.*

Table 15. *SIMPER analysis showing the ranked percentage contribution to the dissimilarity between panels exposed to either pH 7.94 (No gas control) 7.95 (Air only control) and 7.74 (pH 100 years) Av.Abund = Average abundance, Av.Sim = Average similarity, Sim/SD = Similarity standard deviation, Contrib% = Contribution to dissimilarity, Cum.% = Cumulative dissimilarity.*

Sample Date: July

Air control, pH 7.95, Total Average similarity: 83.45

	Av.Abund	**Av.Sim**	**Sim/SD**	**Contrib%**	**Cum.%**
Diplosoma listerianum	0.74	23.20	59.30	27.80	27.80
Ectocarpus siliculosus	0.72	19.12	15.29	22.91	50.71
Biofilm	0.51	15.15	54.17	18.15	68.86
Clavelina lepadiformis	0.35	8.59	9.57	10.30	79.16
Bugula flabellata	0.24	6.11	5.93	7.33	86.48
Ascidiella aspersa	0.17	3.97	3.10	4.76	91.24

pH 100 years (7.74) Average similarity: 70.33

	Av.Abund	**Av.Sim**	**Sim/SD**	**Contrib%**	**Cum.%**
Diplosoma listerianum	0.76	21.25	4.42	30.21	30.21
Ectocarpus siliculosus	0.52	16.91	7.73	24.04	54.25
Clavelina lepadiformis	0.54	13.70	2.97	19.47	73.73
Bugula flabellata	0.25	6.88	5.74	9.79	83.51
Botrylloides leachii	0.29	6.80	1.26	9.66	93.17

No gas control, pH 7.95 Average similarity: 85.13

	Av.Abund	**Av.Sim**	**Sim/SD**	**Contrib%**	**Cum.%**
Ectocarpus siliculosus	0.77	24.30	22.44	28.55	28.55
Biofilm	0.71	22.15	17.00	26.01	54.56
Diplosoma listerianum	0.64	19.47	10.24	22.87	77.43
Clavelina lepadiformis	0.30	8.13	5.62	9.55	86.98
Bugula flabellata	0.27	8.10	22.44	9.52	96.50

No gas control pH 7.95 & pH 100 years(7.74) Average dissimilarity = 29.98

	Group 7.94	Group 7.74				
	Av.Abund	**Av.Abund**	**Av.Diss**	**Diss/SD**	**Contrib%**	**Cum.%**
Biofilm	0.51	0.00	8.75	8.83	29.18	29.18
Clavelina lepadiformis	0.35	0.54	4.08	1.23	13.62	42.79
Ectocarpus siliculosus	0.72	0.52	3.54	1.10	11.79	54.59
Diplosoma listerianum	0.74	0.76	3.15	1.49	10.50	65.08
Ascidiella aspers	0.17	0.10	2.70	2.30	9.00	74.09
Botrylloides leachii	0.20	0.29	2.64	1.17	8.82	82.90
Ciona intestinalis	0.16	0.19	1.92	1.14	6.41	89.31
Bugula flabellata	0.24	0.25	1.26	1.16	4.21	93.53

Air control pH 7.94 & No gas control pH 7.95 Average dissimilarity = 20.45

	Group 7.94	Group 7.95				
	Av.Abund	**Av.Abund**	**Av.Diss**	**Diss/SD**	**Contrib%**	**Cum.%**
Biofilm	0.51	0.71	3.14	2.65	15.34	15.34
Ascidiella aspersa	0.17	0.00	2.79	2.74	13.65	28.99
Ectocarpus siliculosus	0.72	0.77	2.76	3.65	13.50	42.49
Botrylloides leachii	0.20	0.20	2.68	1.66	13.08	55.57
Ciona intestinalis	0.16	0.03	2.11	1.53	10.30	65.87
Diplosoma listerianum	0.74	0.64	1.71	1.98	8.35	74.22
Clavelina lepadiformis	0.35	0.30	1.64	1.27	8.01	82.23
Bugula flabellata	0.24	0.27	1.05	1.41	5.11	87.35
Membranipora membranace	0.00	0.07	1.03	0.67	5.06	92.41

pH 100 years (7.74) & No gas control pH 7.95 Average dissimilarity = 36.57

	Group 7.74	Group 7.95				
	Av.Abund	**Av.Abund**	**Av.Diss**	**Diss/SD**	**Contrib%**	**Cum.%**
Biofilm	0.00	0.71	12.10	16.92	33.10	33.10
Clavelina lepadiformis	0.54	0.30	4.43	1.32	12.12	45.22
Ectocarpus siliculosus	0.52	0.77	4.36	3.19	11.92	57.14
Diplosoma listerianum	0.76	0.64	3.43	1.19	9.37	66.51
Botrylloides leachii	0.29	0.20	3.10	1.32	8.47	74.98
Ciona intestinalis	0.19	0.03	2.69	1.10	7.35	82.33
Ascidiella aspersa	0.10	0.00	1.65	0.67	4.51	86.84
Botryllus schlosseri	0.07	0.07	1.50	1.48	4.10	90.94

During July significant dissimilarity (p = 0.04) occurred between the treatment and the controls. The percentage cover of biofilm was the greatest contributor to the dissimilarity. In all cases it was nearly twice as important in determining the dissimilarity of assemblage composition compared to the next highest contributor.

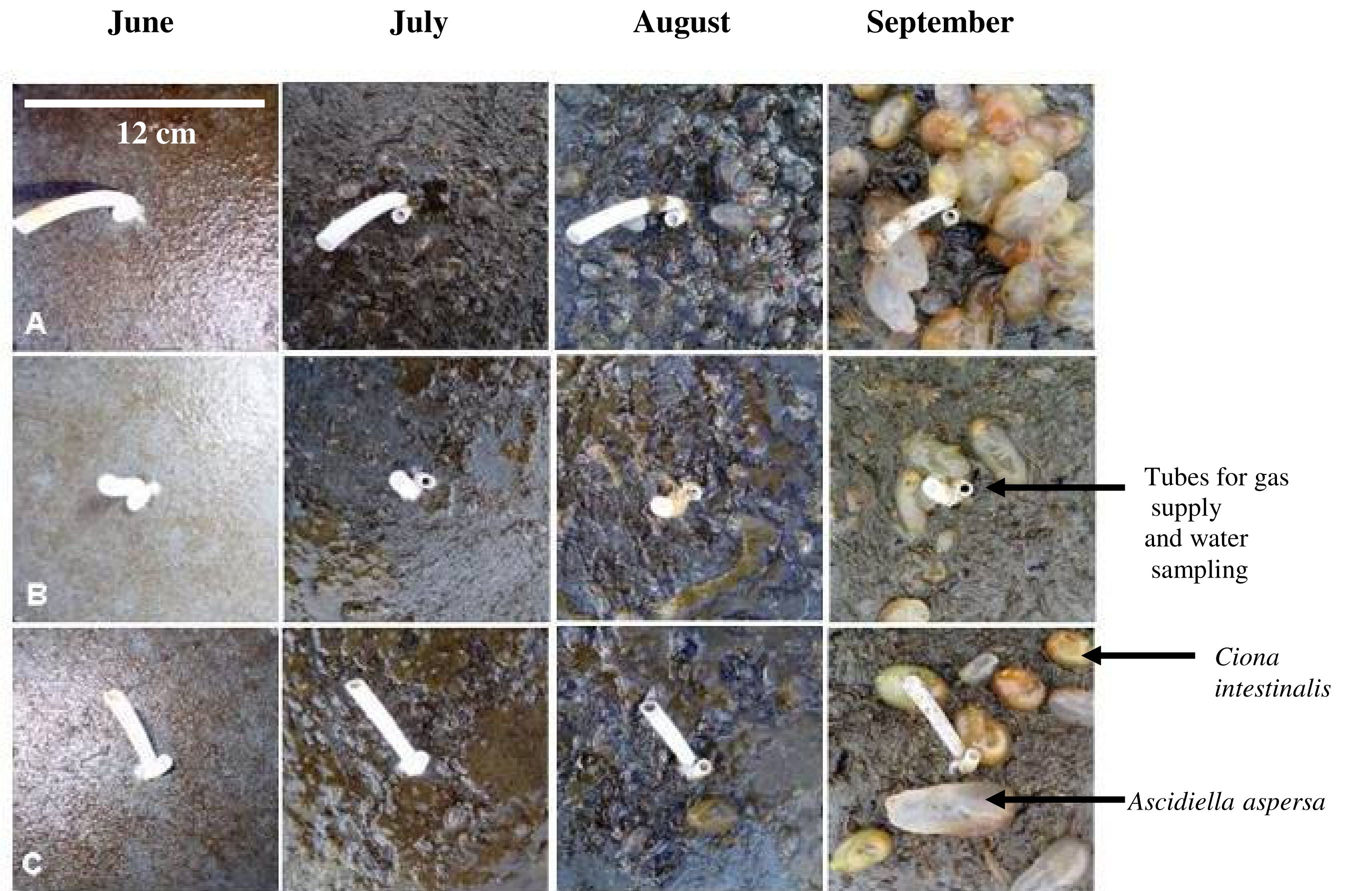


Figure 26. *Assemblage development from left to right. Row A = pH 100 years (7.74), row B = Air control (pH 7.95), and row C = No gas control (pH 7.94). All images are scaled at 11 x 11 cm*^{2}.

4.4 – Principal Response Curve Analysis

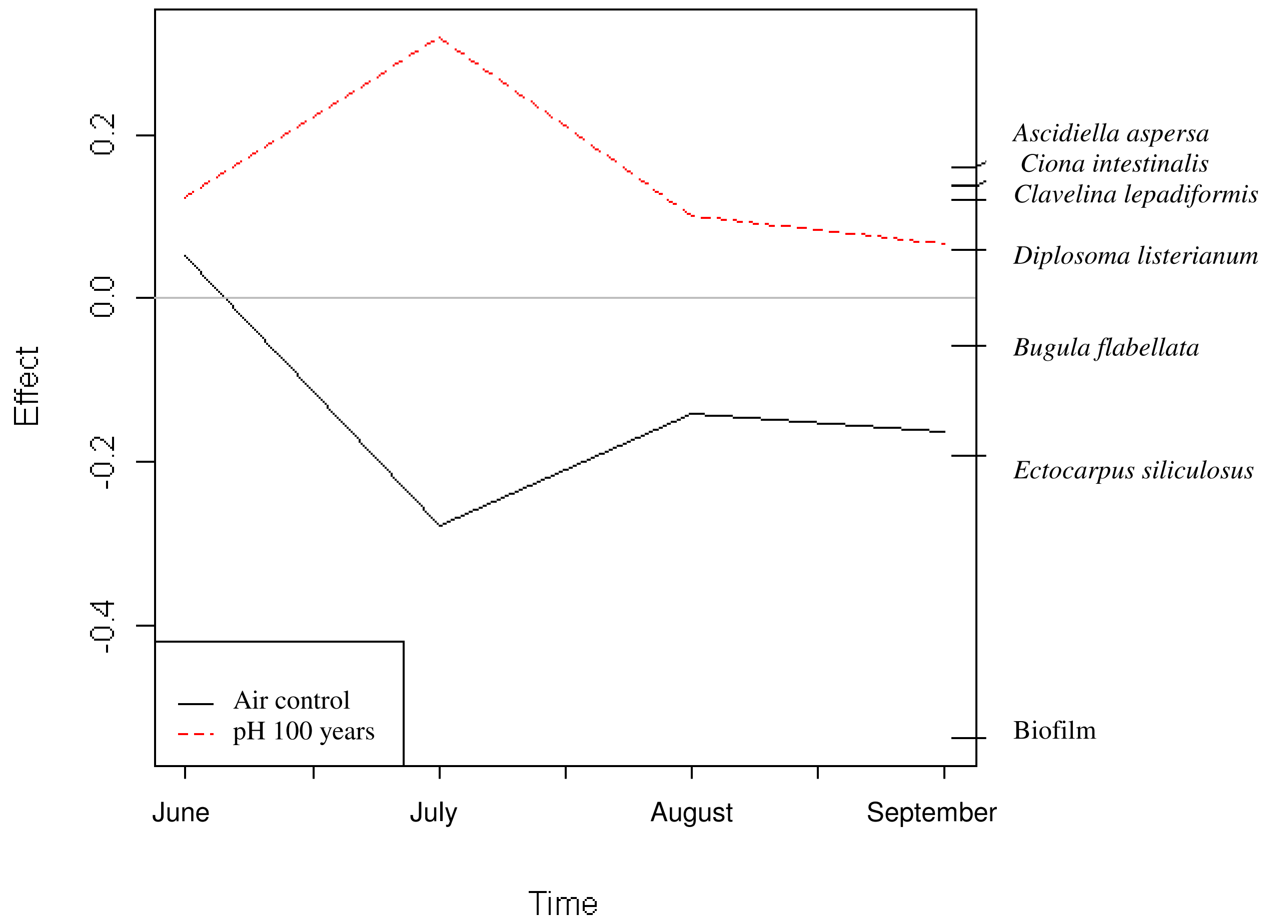


Figure 27. *Principal Response Curve Analysis (PRC) showing effect of introduction of CO_2 (treatment pH 100 years) on the percentage cover of species on settlement panels over time. Species weights for species contributing more that 10% coverage are shown on the right of the plot.*

Table 16. *Model: PRC (response = spp, treatment = pH, time = Time)*

	Df	**Variance**	**F**	**N.Permutations**	**P**
Redundancy Analysis	1	0.042649	10.870	199	0.005
Residual	24	0.094170			

MANOVA was used to test for differences between the following end point response variables; *M. edulis* shell thickness in the centre of the shell and the bottom of the dorsal scar, *M. edulis* shell weight, *M. edulis* somatic tissue weight, *M. edulis* shell to somatic tissue ratio, *C. inestinalis* somatic tissue weight, *C. inestinalis* gonad weight and *C. inestinalis* gonad to somatic tissue ratio (data in Appendix 9.2). All data were tested for normality and transformed if required, Table 8. In all cases, no significant difference (p = 0.05) was detected between these characteristics measured from individuals that had been growing under pH 7.94 (no gas control) pH 7.95 (air control) and pH 7.74 (pH 100 years), Table 12.

4.4 - Discussion

This study suggests that recruitment success of invertebrates and algae on vacant substrata is not affected by a pH decrease of 0.2 of a unit during the first month of development. However, early assemblage cover during the second month was affected by a pH decrease of 0.2 of a unit. These findings lead to the acceptance of null hypothesis one: macro invertebrate recruitment is not affected by *in situ* environmental pH reduction, and acceptance of hypothesis two: early assemblage composition is altered by decreased environmental pH.

The implication of these results is that environmental pH exerts a weak and transient influence on assemblage composition at early stages of development, specifically at two months after emersion, but not at month one, four and five. This is surprising as the actual act of recruitment, which is known to be largely chemically mediated (Dobretsov, *et al.,* 2006; Dobretsov & Qian 2006a; Maki, 1999; Qian, *et al.,* 2007) appears to be unaffected by a 0.2 unit drop in pH which represents a significant alteration of seawater chemistry, i.e. a 120 % increase in H^+ ions. It is possible that recruiting larvae responded to the initial contact with

the low pH conditions in such a way that was not measured by this study, but even if this was the case, there was no detectable effect of low pH on a one month old assemblage.

During July, significant differences between assemblage composition on low pH and control panels were found. Further SIMPER analysis revealed that during July, the significant difference was attributed to a total lack of open space or biofilm on the low pH panels. In addition, the percentage coverage of the tunicate *C. lepadiformis* was considerably higher on low pH panels compared to the controls. These results suggest that the growth rate of *C. lepadiformis* is facilitated by low pH conditions, allowing it to overgrow areas of the panel that would be otherwise covered by biofilms or thin mats of *E. siliculosus*.

It is also possible that both biofilms and *E. siliculosus*, in common with many other marine organisms, normally possess un-described antifouling capabilities which were suppressed by the low pH environment, allowing increased dominance by *C. lepadiformis*. The direction of effect is not clear; does *C. lepadiformis* respond positively to low pH and overgrow *E. siliculosus* or is the capacity of *E. siliculosus* to resist overgrowth reduced by exposure to low pH (a negative response), or a combination of the two? Further experiments with artificially manipulated assemblages selected to contain only the pH responsive species (*C. lepadiformis* and *E. siliculosus*) may help clarify the direction of the responses seen here.

Complementary multivariate analysis using PRC, also shows an effect of the low pH treatment that is greatest during July. This finding is interesting on two counts. Firstly, the effect of low pH appears to have a greater effect on community composition during early development, rather than recruitment which is the intuitively more chemically receptive stage. Secondly, as a comparative exercise between SIMPER, ANOSIM and PRC, it is clear that

both ANOSIM and PRC are consistent in their ability to detect the greatest influence of the low pH treatment over time.

The weighting of species to show their respective contribution to the treatment effect is also consistent between SIMPER and PRC, with each analysis type both indicating 2 of the 4 most influential species as being *C. lepadiformis* and *D.listerianum.* The consistancy between the PRC, ANSIM and SIMPER analysis gives added confidence that the true patterns in the multi-variate data were detected.

The assemblage inhabiting the low pH panels during the final sampling session in September is arguably the most indicative of future ecological states. At this stage these panels had been exposed to a full five months of environmental conditions at an average pH of 0.2 units below the controls. It is interesting that six of the fourteen species present on the panels were calcifiers, which are generally considered to be at high risk from OA (see Chapter 1), yet none of these calcifying species displayed measurable effects to low pH exposure. These findings suggest that the future of benthic marine calcifying organisms is not threatened if they respond in the wild in the same as they did during this experiment.

One of the most dominant species on all panels during July was *Diplosoma listerianum.* This species is known to exert incredibly fast rates of growth, including the overgrowth of other species and vastly alter assemblage structure where it takes hold (Vance, *et al.,* 2008). It could be considered that variation in assemblage structure resulting from growth under low pH conditions may have been masked by the effect of such a dominant and vivacious competitor and that different outcomes may prevail in ecosystems devoid of *D. listerianum* or species with similar exceptional ecological traits.

The end point measurements of *M. edulis* shell thickness, shell weight, tissue weight to shell weight ratio were similar, regardless of pH exposure. Based on previous research it was expected that calcifiers existing in seas that were under saturated with respect to carbonate ions would exhibit either reduced shell thickness attributed to corrosion or reduced internal biomass resulting from increased metabolic activity being diverted towards carbonate ion concentration (Andersson, 2008; Hall-Spencer, *et al.,* 2008; Wood, 2008; Moel, 2009). It was also expected that an organism stressed by reduced pH would divert energy towards homeostasis rather than gamete production in the gonads. However, *C. intestinalis* tissue weight, gonad weight and tissue to gonad ratios were also similar regardless of pH exposure. As these end point measurements failed to detect any difference between organisms growing under low pH and control conditions, several possibilities remain.

Firstly it is possible that *M. edulis* and *C. intestinalis* possess highly efficient acid/base tolerant homeostatic potential, giving them the capability to tolerate low pH exposure (Michaelidis, *et al*., 2005; Gazeau, *et al*., 2007; Thomsen, *et al*., 2010). This is reasonable given that these species often inhabit highly plastic environments with respect to water chemistry parametress like as estuaries. Secondly, the duration of this experiment may simply have been insufficient to elicit measurable responses of this kind. Thirdly, the method applied in this study works by manipulating the pH of a semi-contained body of water which is eventually exchanged with ambient water. A short lag time exists in which the inhabiting organisms may be bathed in ambient pH water prior to the dissolution of the introduced CO_2. It is possible that this access to ambient pH water, although minimal, is sufficient to dampen any effect of low pH stress that would otherwise occur.

The method developed for this thesis proved successful at generating low pH micro environments around settlement panels. During this experiment, three panels were maintained in a low pH environment that was approximately 0.2 of a unit lower than the control panels and ambient conditions. This decrease in pH corresponds to the 100 year ocean pH predictions produced by Caldeira & Wickett, (2003). The unique design of the apparatus used in this study ensured that factors other than pH which could have influenced recruitment such as light, temperature, nutrient availability and larval densities were not manipulated. Larvae were presented to restricted access to the settlement panel caused by the hood, but this restriction was common to both treatments and controls and provided no greater restriction than a natural crevice in a rocky reef.

Uniquely, this study was able to closely recreate future OA carbonate conditions without manipulating these other important factors. It is reasonable therefore to consider the findings of this study to be similar to what can be expected to occur with the increase in OA. No significant difference in water pH was measured between the two types of controls. The introduction of gas (other than CO_2) into the chamber surrounding the settlement panel had no detectable effect on either the pH of the micro environment surrounding the panel or the recruitment and early development of the assemblages. As a result, it is reasonable to assume that any detectable differences between treatment and control panels originates from the experimental manipulation of the pH caused by the controlled introduction of CO_2.

The implications from this study suggest that a pH decrease of 0.2 units does not affect initial recruitment success and assemblage composition except during month two. Low pH conditions appear to favour the growth of *C. lepadiformis* which subsequently overgrows patches in the assemblage previously inhabited by biofilms and *E. siliculosus*. At five months

there are still fewer biofilm patches in the low pH assemblages, although this difference is slight and statistically non-significant. *M. edulis* shell thickness, shell weight, tissue weight to shell weight ratio and *C. intestinalis* tissue weight, gonad weight and tissue to gonad ratios were unaffected by five months exposure to the low pH conditions generated by this study. A similar response of keystone calcifying species such as mussels to pH decreases of the same magnitude were also measured by Thomsen, *et al* (2010) giving confidence to the conclusions reached here.

Chapter 5

Macro-invertebrate Succession

5.0 - Introduction

The establishment and development of fouling assemblages is well described (Bailey-Brock, 1989; Altman & Whitlatch, 2007; Nydam & Stachowicz, 2007). Vacant substrata are colonised within minutes by microbial biofilms (Characklis, 1981; Costerton, 1995; Cooksey, 1995; O'Toole, 2000; Acuna, 2006; Qian, *et al.,* 2007; Jin-Woo, 2008). The films provide cues which influence subsequent macro invertebrate and algal larval recruitment (Pawlik, 1992; Joint, 2002a; Dahms *et al.,* 2006; Dobretsov & Qian, 2006a; Bao, 2007; Dobretsov & Qian, *et al.,* 2007). The previous two Chapters describe an investigation of the effects of OA on these preliminary stages of assemblage development, and this Chapter follows the developing assemblage and reports on the response of mature marine assemblages to OA as they undergo succession.

The settlement panels deployed in Hartlepool for the previous Chapter followed a typical pattern of benthic assemblage development (Bailey-Brock, 1989). Initially, fast growing opportunistic species established themselves quickly on vacant substrata in the form of the alga *Ectocarpus siliculosus* and the tunicate *Diplosoma listerianum.* These opportunistic species were gradually overgrown by slower growing, larger species (*Botrylloides leachii*, *Ciona intestinalis* and *Clavelina lepadiformis*) which displayed competitive exclusion over the large patches of the initial colonisers. It was only at the end of the experiment, after five

months deployment, that recruitment was evident from the species which eventually dominated the mature assemblages in Hartlepool (*Mytilus edulis* and *Balanus crenatus*).

Mature assemblages are historically referred to as climax assemblages (Whittaker, 1953). A climax assemblage is a somewhat ambiguous term which infers a static stage of development. In practice, living assemblages are never static but are in a constant state of flux with respect to structure and composition. Despite this ambiguity, benthic assemblages on hard substrata in Hartlepool over twenty four months in age, display many common characteristics and are generally dominated by similar ratios of *M. edulis*, *B. crenatus*, *C. intestinalis* and *Ascidiella aspersa* (Predergast, 2007; Sugden, 2007). In this text, assemblages over 24 months old will be referred to as mature assemblages. The mature assemblages at Hartlepool comprise largely of the same species as the younger assemblages described in the previous Chapter. However, the ratio and developmental stage of these species differs considerably to that of the younger assemblages. As a result, the form and function of the mature assemblages also differ considerably.

Mature assemblages are generally different from newly recruited assemblages in terms of structure, function and stage of development (Lindsay, *et al.,* 2000). Some of this difference stems from the age and therefore the size of the individuals. As assemblages mature, individuals have time to grow and develop often, resulting in higher proportions of adult organisms than younger assemblages. Adults often perform either different functional roles to juveniles, or perform the same functional role to a much greater extent. This is well illustrated by tunicates which dominate much of the available benthic substratum in Hartlepool marina.

Most larval tunicates rely on the re-absorption of body tissues for nutrients and so do not feed externally (McHenry, 2005). Once recruited, most juvenile tunicates perform a similar functional role as adults by filter feeding and removing nutrients and particulates from suspension in the water column. However their filtering capacity is directly proportional to their wet weight, at approximately 80 ml per hour per gram wet weight for *Ciona intestinalis* (Carlisle, 1966). This example demonstrates that the functional influence of an individual *C. intestinalis'* on its assemblage and the adjacent environment varies with its size, which is inextricably linked to its maturity.

It is not just the functional role of an assemblage that changes with maturity. Previous studies have produced clear examples where diversity is strongly linked with assemblage age. The historical view is that younger assemblages are dominated by low numbers of opportunistic species, whereas older assemblages are still often dominated by small number of species, so support a wider range of relatively rare species which increases the overall diversity (Stehli & Wells, 1971; Lindsay, *et al.,* 2006). The direction of this relationship is not always consistent and appears to depend on the particular characteristics of dominant species within the assemblage (Lindsay, *et al.,* 2006). The relationship between assemblage age and diversity is complex, however, most evidence suggests there is a link between diversity and age which reinforces the need to understand how the advance of OA will affect assemblages of all ages and different successional stages.

Succession is the term that describes the process by which living assemblages change in terms of composition and structure and develop over time (Cowles, 1911; Connell & Slatyer, 1977; Fairfull & Harriott, 1999) This flux is attributable to many circumstances including the loss if individuals from predation or old age, creation of new space, competitive exclusion,

seasonal recruitment, periodic disturbance and evolutionally adaptation (Cowles, 1911; Connell & Slatyer, 1977; Fairfull & Harriott, 1999).

Vacant substratum can be theoretically considered as a platform of empty ecological niches which is then subject to the process of recruitment followed by succession. Each niche represents the potential of space and other resources required by an individual or species to grow and reproduce. Individuals of the same species generally demand similar niche characteristics stimulating intra-specific competition. As each new species recruits and establishes, a different niche becomes filled. The niche requirements of different species vary, but they inevitably overlap with respect to basic requirements like food and space, so the recruitment of more than one species often draws recruits into inter-specific competition.

These intricate processes of inter and intra-specific competition are inherent drivers of succession. The process is further complicated by the interaction of extrinsic factors such as environmental conditions and supply-side larvae, i.e. larvae which do not originate from the assemblage itself. The influence of OA on this multi-factorial process is as yet, largely unknown, primarily a result of the considerable technological challenges involved in experimentally investigating this subject, as described in Chapter 1.

The potential for OA to disrupt or alter multiple components of succession influence is vast. In the simplest example, heterogeneous tolerance to low pH conditions would lead to the death of individuals and species, with the immediate creation of vacant niches in otherwise well-established assemblages. As different species possess different functional roles in

assemblages, the loss of one or multiple species creates the potential to considerably alter assemblage structure and function.

Empty niches in established assemblages are often swiftly occupied due to the close proximity of other species, many of whom are capable of rapid lateral vegetative reproduction, as in the case of the tunicate *D. listerianum.* (Vance, *et al.,* 2008). Increased dominance by opportunistic fast growing species occupying niches vacated by low pH intolerant species provides another example of how OA has the potential to shift marine assemblage dynamics.

OA is a change to the chemical composition of sea water, and so the potential effects of this phenomenon are not just species specific or limited to one environment type. It has the potential to affect every organism in the oceans. This means that OA may also affect other more subtle processes which also play a crucial role in driving ecological succession such as the supply and diversity of larvae, and the abundance and diversity of predators.

As discussed in Chapter 1, even basic requirements of established assemblage members may be altered by the advance of OA such as the speciation and subsequent bioavailability of basic nutrients such as nitrogen and phosphorous. The intrinsic and extrinsic factors that drive succession and the potential influence of OA are highly interwoven. The main processes involved and the potential influences of OA are simplified and visualised in Figure 28.

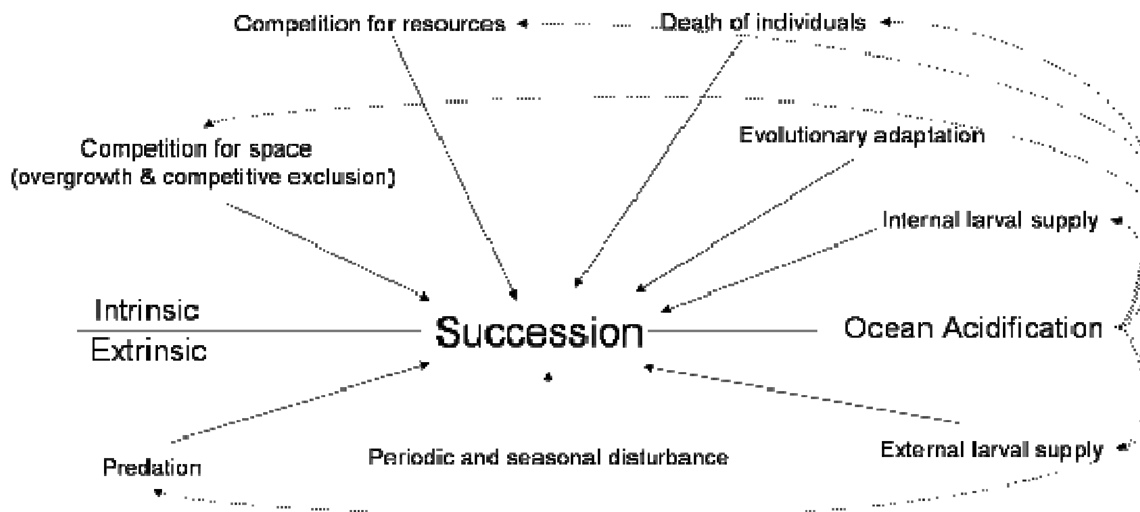


Figure 28. *Schematic diagram to show relationship between intrinsic and extrinsic factors that drive successional change in marine assemblages and the potential influence of marine acidification. Internal larval supply referrers to larvae produced by individuals within the assemblage.*

The links between OA and successional processes could lead to widescale changes in assemblage performance. Understanding and quantifying the extent of this potential is a vital and so far under explored area in marine biology. Without data collected from established assemblages undergoing succession that are also being exposed to low pH conditions that closely recreate OA, it is impossible to accurately predict how ecosystem performance may change in the future.

5.1 - Aims of this experiment:

The study reported in this Chapter aimed to use the apparatus described in Chapter 1 to measure established assemblage succession under the influence of simulate OA conditions. In order to achieve this objective, established assemblages were exposed to low pH conditions in the field and the following response variables were measured: percentage cover of individuals, shell weight, somatic tissue weight, somatic tissue weight to shell weight ratio

(mussels and barnacles) gonad weight, and gonad weight to tissue weight ratio (tunicates). These data were analysed in order to provide evidence to answer the following hypothesis:

1: Low pH conditions alter the structure of established fouling assemblages in Hartlepool marina

2: Exposure to low pH conditions induces physiological changes in exposed individuals.

5.2 - Methods

In January 2006, thirty 15 x 15 cm^2 PVC settlement panels were deployed vertically in Hartlepool Marina. The panels were attached along their top edge with cable ties to a rope that was horizontally suspended 1m deep. The rope was attached to a floating pontoon and ensured that all panels were kept at a uniform depth regardless of tide or lock action. Ensuring the panels were kept at a uniform depth was considered important to ensure that environmental factors such as light were kept as uniform as possible across all panels.

Prior to immersion, all panels were evenly roughened for 1 minute with coarse sand paper (grit size 60) on an electric orbital sander, in order to ensure homogeneous rugosity between panels. The panels were left in this orientation to mature for 26 months until March 2008. After the maturation period the panels closely resembled other non-manipulated artificial hard substrata in the marina and were dominated by established assemblages of *M. edulis*, *B. crenatus*, *C. intestinalis* and *A. aspersa* (Sugden, 2007).

On the second of April 2008, nine panels were at chosen at random and removed from the rope. A pull saw was used to trim the panels down to 12 x 12 cm^2 to enable them to fit on the experimental apparatus described in Chapter 2. Trimming was required as the panels were originally deployed for another experiment, but 12 x 12 cm^2 is the optimum size for the apparatus used in this study. After trimming, the panels were re-attached to the rope and left for one month to recover until the third of May when they were removed from the rope once more and fixed to the base plate of the experimental apparatus with cable ties. The apparatus, complete with established assemblages, was deployed under the same pontoon where the panels had matured, to ensure identical ambient conditions. Deployment was in exactly the same manner as described in Chapter 3 but with the addition of the established assemblages. When in position, the nine panels were randomly assigned one of three conditions; pH 100 years (7.74), air control (pH 7.95), and no gas control (pH 7.94). Each treatment or control chamber was replicated three times, n =3.

Gas supplies were controlled and maintained as described in Chapter 2 in order to sustain the desired environmental conditions around each panel. Water sampling was conducted every two days throughout the duration of the experiment according to the method described in Chapter 2, in order to measure the environmental profiles being created by the apparatus.

After the first two week recovery period, the gas supply to the apparatus was momentarily halted and the whole apparatus was recovered to the pontoon. The top half of the frame was removed and high quality digital images were taken of each panel. Photography and image analysis was conducted as described in the Methods section of Chapter 4. During these few minutes, time was taken to gently probe the assemblages to aid later identification of the

residing species from the photographs and ensure that the assemblage was fairly represented by a percentage coverage data set.

After sampling in this fashion, the apparatus was swiftly reassembled and repositioned under the pontoon and the gas supply re-connected. During each sampling event the total time where the assemblage were not in contact with the gas supply was no more than 20 minutes and the panels were removed from the water for no more than ten minutes, once per month. This process was repeated once a month from May until the end of the experiment in September 2008.

At the end of the experiment in September 2008, the panels were harvested and taken back to the laboratory for end point analysis in exactly the same way as described in Chapter 4. In addition to the analysis of mussels and tunicates, all individuals of the barnacle *B. crenatus* were also extracted from the established assemblages. Care was taken to remove all visible animals in entirety, with the exception of the basal plate of *B. crenatus* which frequently disintegrated into unmanageable fragments. In order to reduce the introduction of error, all individuals leaving basal plates were excluded from further analysis.

The somatic tissue of the barnacle was extracted from the test and both the test and tissues were dried in accordance to the method described in Chapter 4. After drying, measurements were made of the dry test weight, dry tissue weight and the ratio of dry tissue to test using the same micro balance as described in Chapter 4. The internal volume of the barnacle tests was also measured using the same micrometres previously described. Measurements were made of height, basal width and opercula diametres, all at the longest dimension, and used to

calculate the internal volume. The formula used assumed the barnacle were cone shaped: Volume = $1/3\pi r^{2*}$height

5.2.1 - Statistical Analysis – end point responses

MANOVA conducted in SPSS version 15 was used to test for differences between the following end point response variables; *M. edulis* shell thickness in the centre of the shell and the bottom of the dorsal scar, *M. edulis* shell weight, *M. edulis* somatic tissue weight, *M. edulis* shell to somatic tissue ratio, *C. intestinalis* somatic tissue weight, *C. intestinalis* gonad weight, *C. intestinalis* gonad weight to somatic tissue weight ratio, *B. crenatus* tissue weight, test weight and ratio of test weight to tissue weight and ratio of internal volume to tissue weight (data in Appendix 9.3). All data were tested for normality (Anderson darling, Minitab statistical software version 15) and transformed accordingly. Ratio data was arc-sin transformed (Table 17).

Table 17*. End point response variables, transformation type and test of normality.*

Response Variable	Transformation	Anderson Darling Test of Normality p =
C. intestinalis tissue weight	None	0.31
C. intestinalis gonad weight	Square root	0.05
C. intestinalis tissue weight to gonad weight ratio	Arc - sin	0.10
M. edulis tissue weight	Log^{10}	0.1
M. edulis shell weight	None	0.1
M. edulis tissue weight to shell weight ratio	Arc - sin	0.4
M. edulis shell thickness, center	None	0.10
M. edulis shell thickness, bottom of scar	none	0.01
B. crenatus test weight	Log^{10}	0.50
B. crenatus test volume	Square root	0.21
B. crenatus tissue weight to test weight ratio	Arc - sin	0.05
B. crenatus tissue weight	None	0.07

5.2.2 - Statistical Analysis – treatment conditions

pH data obtained from water sampling conducted during the five month experimental run time were analysed using a repeated measures ANOVA and a *Ryan-Einot-Gabriel-Welsch F post hoc* test, both conducted in SPSS version 15. All pH data were statistically normal producing $p = 0.05$ in all cases.

5.2.3 - Statistical Analysis – assemblage responses

In order to test for differences between the species composition in the established assemblages, the percentage cover data obtained at each month from May until September were analysed using Primer Software version 6.13. Bray Curtis similarity matrixes were calculated representing the percentage cover of all species on all settlement panels at each month. These matrixes were analysed using ANOSIM to test for similarity between assemblage composition between panels exposed to reduced pH and the two controls.

Additional exploration of the multivariate percentage cover data was conducted with Principal Response Curve (PRC) analysis to test for the effect of the introduction of CO_2 on percentage cover of the resident species over time. Significance of the analysis was verified by a Monte Carlo test. Both tests were performed using R statistical software (R, 2008).

5.3 - Results

5.3.1 - pH control

Statistical analysis of the water sample data confirmed that the apparatus and technique used in this experiment produced an experimental pH profile that was significantly different from both the controls (p = 0.001, Table 18). Post hoc testing revealed the introduction of CO^2 produced environmental conditions around the settlement panels which were approximately 0.3 – 0.36 of a pH unit lower that the controls, which also represented ambient pH in Hartlepool marina (Table 19). A pH decrease of this magnitude is predicted to occur by the year 2100 according to the models produced by Caldeira & Wickett, (2003). The two controls differed only by a $\bar{x}$ average of 0.1 of pH unit over the five month duration of the experiment.

A running $\bar{x}$ value of pH 7.90 was calculated from the ambient pH data collected by the data logger (data in Appendix 9.1).This value deviates from the $\bar{x}$ control pH values by 0.01 - 0.05 and deviates from the $\bar{x}$ low pH treatment value by 0.31 of a unit.

Table 18*. Repeated measures ANOVA table showing test for difference between the experimentally reduced pH conditions produced by the introduction of CO2 and an air control and no gas control.*

Source	Type III Sum of Squares	Df	$\bar{x}$ Square	F	*p.*
Intercept	18271.46	1	18271.46	107507.310	<.01
Treatment	7.72	2	3.86	22.74	.02
Error	1.02	6	.17		

Table 19. *Post hoc analysis of experimentally reduced pH conditions produced by the introduction of* CO_2 *compared to an air control and no gas control. n = 3 in all cases.*

	Treatment	N	Subset	
		1	2	1
Ryan-Einot-Gabriel-Welsch F(a)	Low pH	3	7.59	
	Air control	3		7.89
	No gas control	3		7.95
	Sig.		1.00	.33
Ryan-Einot-Gabriel-Welsch Range(a)	Low pH	3	7.59	
	Air control	3		7.89
	No gas control	3		7.95
	Sig.		1.00	.33

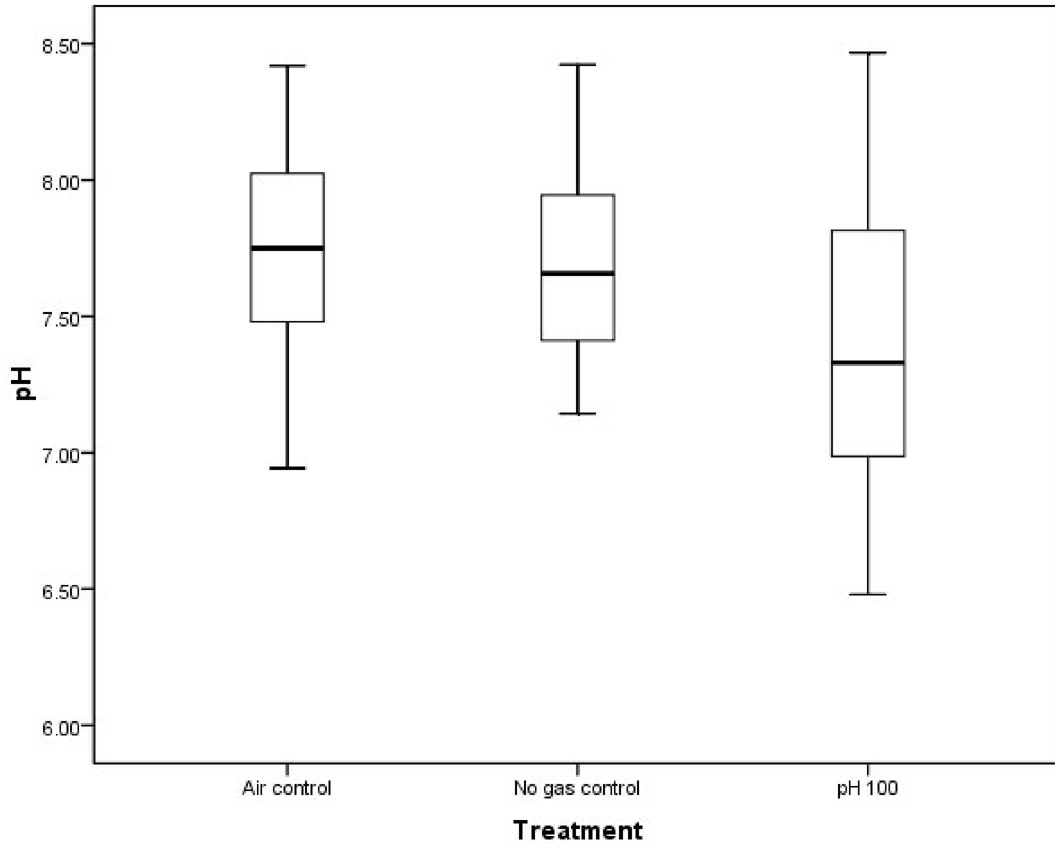


Figure 29. *Box plot of water sample pH taken from experimental chambers. Horizontal lines indicate the median and bars indicate 95 % confidence intervals.*

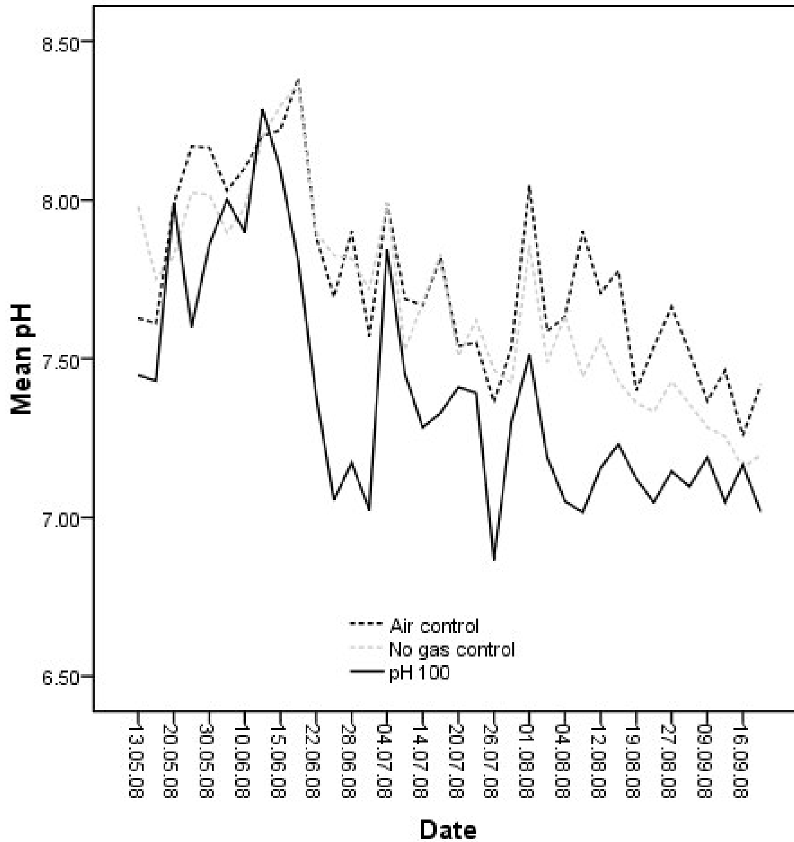


***Figure 30**. $\bar{x}$ pH of water from experimental chambers. Error bars are removed for clarity. The variance around the $\bar{x}$ values is shown in Figure 29.*

The time series pH data shows the controls and data logger reporting variable but comparable pH values until the 4th of July. From this point onwards a clear drift can be seen between the data logger and the treatments and controls. This drift is attributed to a measurement error and is addressed in the final discussion, Chapter 8. The water samples taken before the drift occurred were analysed with a separate repeated measures ANOVA and again the treatment effect was significant ($p = 0.05$), Table 20-21. Data after this point (post 4th of July) were not analysed separately as the extent of the drift is not possible to separate from the background data. Although variable, the average pH values in Figures 29 & 30 show differences did occur between treatments and controls.

Table 20. *Repeated measures ANOVA table showing test for difference between the experimentally reduced pH conditions produced by the introduction of CO_2 and an air control and no gas control data from before drift occurred at 4th of July only.*

Tests of Between-Subjects Effects

Source	Type III Sum of Squares	df	$\bar{x}$ Square	F	P
Intercept	8355.318	1	8355.318	136577.883	<.001
Treatment	2.914	2	1.457	23.816	<.01
Error	.367	6	.061		

Table 21. *Post hoc analysis of experimentally reduced pH conditions produced by the introduction of CO_2 compared to an air control and no gas control.*

	Treatment	N	Subset	
			1	2
Ryan-Einot-Gabriel-Welsch F[a]	pH 100 years	3	7.66	
	No gas control	3		7.95
	Air control	3		7.98
	Sig.		1.00	.59
Ryan-Einot-Gabriel-Welsch Range[a]	T2	3	7.66	
	NGC	3		7.95
	Air	3		7.98
	Sig.		1.00	.59

5.3.2 - Assemblage Composition.

The established assemblages measured for this experiment consisted of the same thirteen species as the younger assemblages in Chapter 4, yet the relative proportions of these species were different. In general, these assemblages consisted of thirteen macro-fouling species and biofilm.

Table 22. *Species list from all panels excluding biofilm*

Mytilus edulis	Bivalve mollusc
Balanus crenatus	Arthropod crustacean
Ectocarpus siliculosus	Filamentous brown algae
Punctaria plantaginea	Brown algae
Pomatoceros triqueter	Tube dwelling polychaete worm
Obelia dichotoma	Erect bryozoan
Bugula flabellata	Erect bryozoan
Membranipora membranacea	Encrusting bryozoan
Ciona intestinalis	Solitary tunicate
Clavelina lepadiformis	Solitary tunicate
Ascidiella aspersa	Solitary tunicate
Botrylloides leachii	Colonial tunicate
Diplosoma listerianum	Colonial tunicate
Botryllus schlosseri	Colonial tunicate

The negative R value outputs generated by the ANOSIM assemblage composition analysis are considered to support the null hypothesis; the low pH treatment had no significant effect on the percentage cover of different species on the settlement panels compared to assemblages exposed to control and ambient conditions. This view is taken as a negative R value suggests that the variance between replicate samples is equal to or greater than the variance between treatments.

Table 23. *ANOSIM results used to test for differences between percentage coverage of species on panels exposed to either pH 7.95 (no gas control) 7.89 (air only control) and 7.59 (introduction of CO_2) at each sampling point.*

Date	**Global R**	**Significance *p* =**
08.05.08	-0.25	0.89
20.05.08	-0.16	0.79
19.06.08	-0.16	0.81
23.07.08	-0.14	0.72
15.08.08	-0.17	0.75
19.09.08	-0.13	0.78

The nMDS (Figure 31) plot clearly shows how the fouling assemblages on all settlement panels underwent temporal change during the experiment, regardless of treatment. There is no clear pattern of one treatment type being distinct from the others over time, a conclusion supported by lack of significant results from the ANOSIM test.

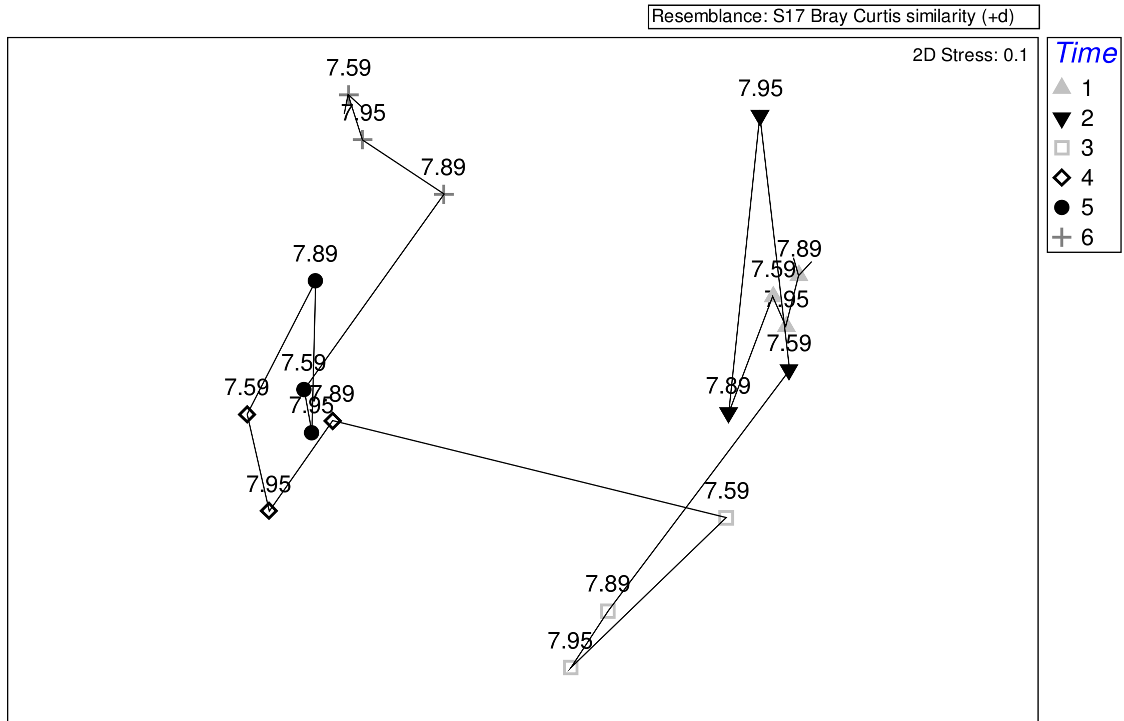


Figure 31. *Non metric multi-dimensional scaling showing the relationship between fouling assemblages exposed to different pH exposures over time. 7.89 = Air control, 7.95 = No gas control, 7.59 =* CO_2 *introduced, pH 100 years. Each point represents the* $\bar{x}$ *average assemblage composition derived from three replicate settlement panels exposed to the same treatment or control.*

Figure 31 shows how the temporal change in all assemblages regardless of treatment was attributed to the decrease in percentage cover of *E. siliculosus* and the increase in percentage cover of *C. intestinalis* during the growth season. This pattern is consistent with normal annual assemblage development in Hartlepool (Prendergast, 2007).

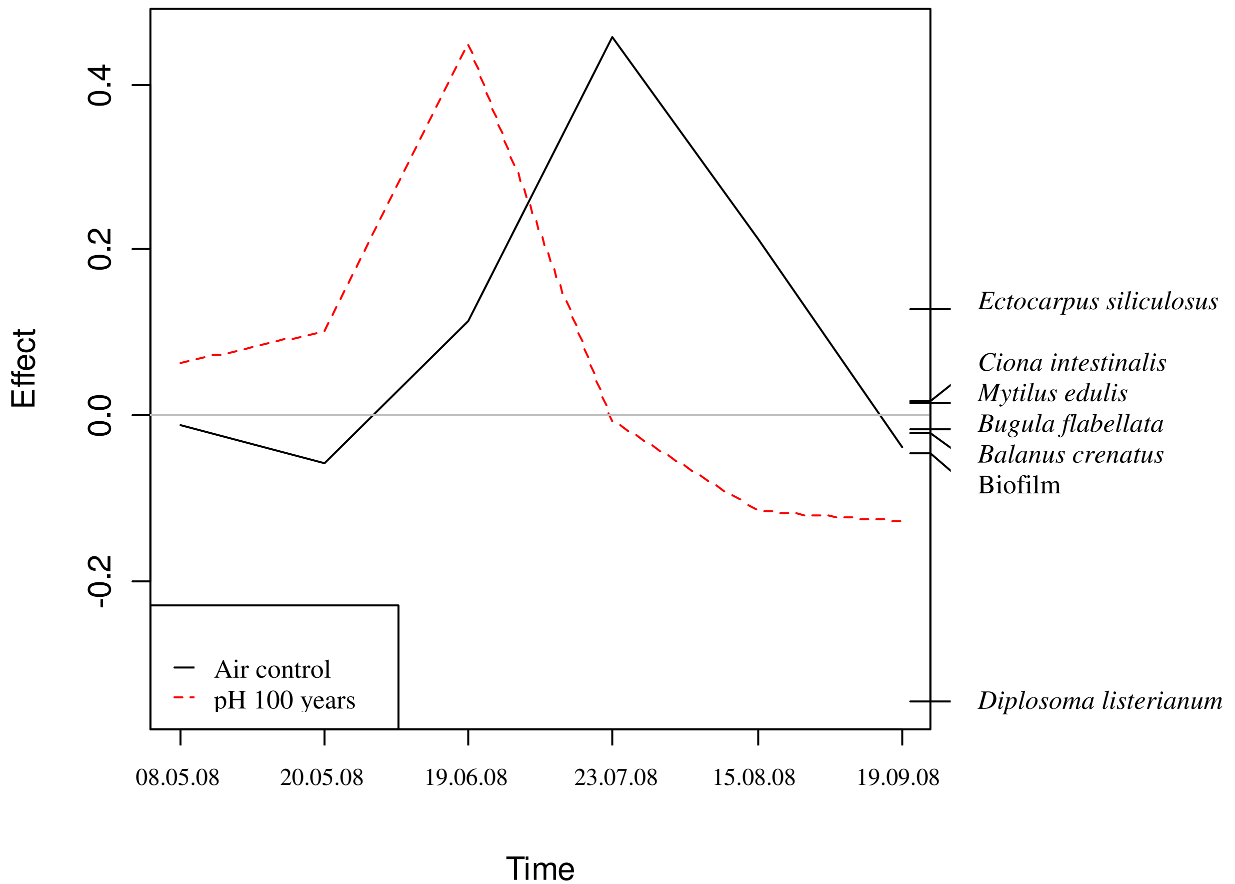


Figure 32. *Principal Response Curve Analysis (PRC) showing effect of introduction of CO_2 (treatment pH 100 years) on the percentage cover of species on settlement panels over time. Species weights for species contributing more that 10% coverage are shown on the right of the plot.*

Table 24 *Model: PRC (response = spp, treatment = pH, time = Time)*

	Df	**Variance**	**F**	**N.Permutations**	**P**
Redundancy Analysis	1	0.013596	1.9527	199	1
Residual	36	0.250659			

PRC analysis shows a very similar multivariate response from control and treatment assemblages. Monte Carlo testing of the PRC analysis produces a non-significant (p=1) result confirming the lack of response of the established assemblages to the low pH, high CO_2 treatment. The negative species weighting of *Diplosoma listerianum* suggests that this species did not follow the main response pattern.

5.3.3 - End point response variables

In all cases, no significant difference (p= >0.05) was detected between the characteristics in Table 17, measured from individuals that had been growing under pH 7.95 (no gas control) pH 7.89 (air control) and pH 7.59 (introduction of CO_2).

5.4 - Discussion

Results from this experiment suggest that the mature assemblages in Hartlepool marina are tolerant of a pH decrease of 0.3 of a unit for at least five months in duration without displaying any measurable variation in assemblage structure or physiological characteristics such as somatic tissue, gonad or shell weight. This finding leads to the acceptance of null hypothesis 1: low pH conditions *do not* alter the structure of established fouling assemblages in Hartlepool Marina and null hypothesis 2: exposure to low pH conditions *do not* induce physiological changes in exposed individuals.

Initially this is surprising given that a pH decrease of 0.3 of a unit represents an increase in the concentration of H^+ ions by as much as ~80%. These results also differ in comparison to the newly recruited assemblages measured for the previous Chapter, where the assemblage composition differed significantly between low pH and control panels, two months after recruitment.

This lack of response of mature assemblage succession after exposure to different pH treatments measured for this Chapter may be attributable in part to the increased age of the residing individuals. The maximum age of individuals in this experiment is 24 months compared to five months in the experiment in Chapter 4 where responses to low pH treatments were detected. Age of individuals may affect the resilience of the community to pH change.

Analysis of the results of the previous Chapter suggested that species that were most responsible for contributing to the difference between low pH and control panels were *D. listerianum*, *Ectocarpus siliculosus* and *Clavelina lepadiformis,* and the multi-species microbial biofilm assemblage. However, these organisms, only constituted a minor percentage of the total coverage in the mature assemblages measured in this Chapter, with each species generally representing less that 15% of the total cover. The only exception was *E. siliculosus,* which was highly represented in the developing assemblages and also contributed as much as much as approximately 55% of the cover on the mature assemblage panels during May.

In both experiments *E. siliculosus* decreased in percentage cover during the season as it was overgrown by tunicates displaying rapid growth. However, it was the rapid overgrowth by *C. lepadiformis* in particular in which lead to a marked decrease in *E. siliculosus* in the previous experiment. *C. lepadiformis* was far less abundant in the mature assemblages, and no significant overgrowth of *E. siliculosus* occurred.

The lack of response to treatments in this experiment may be explained by the relatively low abundance of *C. lepadiformis*, the species which showed a positive response to low pH

exposure during the experiment described in Chapter 4. The low abundance of this species in the mature assemblages at time zero strengthens the argument that low pH responses are more likely at early successional stages, simply because younger assemblages support more pH responsive species. This concept requires more work to understand the direction of the pH response. As stated in the previous Chapter, at present it is not clear weather *C. lepadiformis* responds positively to low pH and overgrows *E. siliculosus* or the capacity of *E. siliculosus* to resist overgrowth is reduced (a negative response) or a combination of the two.

Both the ANOSIM and PRC assemblage analysis techniques produced a common lack of response from the low pH treatment. This common result gives added confidence that a true impression of the data was produced. The PRC analysis clearly shows a pattern of assemblage change over the experiment, and this pattern is common to both treatment and control assemblages. The species weighting shows that *E. siliculosus* contributes most to the pattern shown in the PRC plot. This is most likely to be overgrowth of *E. siliculosus* by other dominant species such as *C. lepadiformis* resulting in competitive exclusion of *E. siliculosus.*

The lack of significant difference between the low pH assemblages and the control assemblage in this study may simply be attributable to an inherent capability of these fouling species to deal with flux in environmental pH. It is possible that this tolerance is particularly acute in an environment such as Hartlepool marina, where considerable variation in background pH occurs in comparison to the open ocean as result of the relatively small body of water and the relatively large influence of water ingress and egress via the sluice gate.

The environmental conditions in Hartlepool marina are subject to more variability compared with the open ocean in general. This being the case, it is possible that that the species and

individuals measured for this study may simply display high tolerance to low pH conditions. The same study conducted in a fully open system may produce measurable differences in the response variables that were not detectable in this investigation.

An environment with these characteristics may serve to accelerate genetic adaptation for traits such as pH tolerance by selecting only those individuals which are able to survive the environmental fluctuation. This situation has the potential to rapidly produce highly tolerant sub populations. This tolerance, if it exists, may be mechanistically produced by genetic variation in efficiency of membrane ion pumps or other acid base homeostatic processes. A genetic assessment of species living inside and just outside Hartlepool may produce evidence to validate this assumption. An investigation of this nature would be of considerable interest, as if such rapid genetic adaptation was possible, the prospects of benthic marine invertebrates may be more certain for the future.

This study used a range of end point physiological characteristics of benthic invertebrates together with percentage cover of different species to test for differences between communities exposed to low pH conditions and assemblage exposed to ambient pH. The end point traits such as shell thickness were chosen as they relate directly to the process of calcification, and also the active dissolution of calcareous tests, which have both been demonstrated as being affected by OA simulation (Gattuso & Buddemeier, 2000; Riebesell, *et al.,* 2000; Andersson, *et al.,* 2008; Gutowska, *et al.,* 2008; Lough, 2008; Moy, *et al.,* 2009).

The fact that no significant difference was measured between these response variables from low pH and control organisms does not necessarily mean that differences did not occur, as they may have been associated with other attributes. For example, this experiment used

gonad weight in *C. intestinalis* as a proxy to represent metabolic resource allocation under pH stress. As the viability of the gonad was not measured directly, it may have been affected by the low pH conditions produced during this experiment, but this effect remained undetected.

An alternative approach that could be adopted by similar investigations would be to investigate the functional performance of test species such as filtration rates in tunicates or cirri beating in barnacles. Responses such as these could easily be measured *in situ* using a submersible video camera providing a more holistic approach to organism response to low pH conditions rather than crude end point characteristics. Alternative direct variables may be more responsive to pH stress and therefore more appropriate to measure when attempting to detect differences between assemblages on relatively short timescales. When attempting to interpret the results of this investigation it is also important to acknowledge the time scale of the experiment. Although it is a significant advance on many experiments, an observation period of five months actually represents a relatively short period of time in relation to the succession of a multi species assemblage.

Several constraints combined to limit the observation period of this study, but it must be considered that extended monitoring could bring to light alternative responses to the ones measured here. The latter is especially true given that this experiment spanned the recruitment peak in Hartlepool marina. High levels of larval supply and recruitment pressure could have served to dampen down any sucession related assemblage structure related responses to low pH conditions that may have occurred at alternative times in the growth season.

In summary, this experiment produced no evidence to suggest that established fouling assemblages, twenty four months old responded to five months of exposure to seawater approximately 0.3 pH units lower than ambient, in terms of percentage cover of resident species and a range of responses related to tissue, organ and shell characteristics. These findings contrast to observations in younger assemblages comprising of different ratios of species together with younger, smaller individuals, therefore suggesting low pH tolerance may be heterogeneous between species and also dependant on individual age or size within species. Further development of this method should include greater replication of experimental units to increase the representative size of the test assemblages. In addition, increased observational periods ideally spanning a whole growth season would assist in separating any influence of unusually high recruitment pressure during natural seasonal peaks from low pH responses.

Chapter 6

Ocean Acidification and Biofilms II. Diversity at a Low pH Vent Site

6.0 - Introduction

Thus far this thesis has concentrated on the development and implementation of methods to allow experimental *in situ* pH manipulation to mimic future OA conditions. While this approach has successfully modified the pH of seawater in the field, is believed to be novel and addresses significant gaps in the literature, the value of this method is limited by the duration of the experiments. The considerable maintenance associated with the method makes it best suited to experiments of relatively short duration. This Chapter uses a volcanic vent site to measure the chronic effects of low pH exposure on biofilms.

Chronic exposure to low pH environments is likely to induce increased metabolic effort to be directed towards homeostasis rather than other metabolically demanding life process such as calcification (Borowitzka & Larkum, 1976; Feely, *et al.,* 1988; Gattuso & Buddemeier, 2000; Riebesell, *et al.,* 2000; Berry, *et al.,* 2002; Yates & Halley, 2006; Andersson *et al.,* 2007; Andersson, *et al.,* 2007; Balch & Fabry, 2008; Gutowska, *et al.,* 2008; Hall-Spencer, *et al.,* 2008; Hofmann, *et al.,* 2008; Kuffner, *et al.,* 2008; Lough, 2008; Manzello, *et al.,* 2008; Riebesell, *et al.,* 2008; Wood, *et al.,* 2008). Chronic shifts in energy allocation resulting from low pH exposure may not become evident after short term exposures to low pH which may explain some of the results described in this thesis so far.

Other fundamental factors that influence the long term persistence of a species such as fecundity are difficult to measure in short term experiments. Gamete viability and ultimately reproductive success are areas which are likely to be affected by OA, yet to date only a limited number of species have been investigated in relation to this aspect and even these examples are limited to short term laboratory experiments with one generation of organisms (Kurihara, *et al.,* 2004; Kikkawa, *et al.,* 2004; Kurihara, *et al.,* 2007; Kurihara, 2008; Naik & Naidu, 2008; Clark, *et al.,* 2009).

Marine scientists are a long way from understanding how organisms in their current evolutionary state will respond to future environmental conditions such as OA. Predicting future ecological functioning is made even more challenging because organisms are constantly evolving in response to, amongst other factors, environmental conditions. It is therefore imperative when attempting to predict ecosystem properties of the future to understand the evolutionary potential of present day organisms in relation to known environmental phenomena such as OA. The impact of OA on evolution is currently poorly represented in the literature (Collins & Bell, 2004), largely as a consequence of the difficulty in maintaining long term experiments that involve reductions of pH on the scale expected to occur with the onset of OA. Understanding the potential for marine organisms to evolve increased tolerance to low pH conditions resulting from OA, as well as increased thermal tolerance in response to global warming, is one of the least understood but most pressing issues in current marine biology.

Attempts have been made to fill these information gaps with laboratory and mesocosm experiments under manipulated pCO_2 conditions. Such studies have identified species and processes that appear vulnerable to OA: see Chapter 1. However, even the most rigorous of

this style of experiment is inherently constrained by attempting to measure complex natural processes, in simplified artificial environments. These approaches are not capable of accurately recreating the complex assemblage interactions and nutrient fluxes that exist in ocean ecosystems.

The constraints of laboratory based experimentation are particularly pertinent when attempting to measure responses of microbial communities. Laboratory based microbial experiments are frequently conducted at unnatural cell densities and are unable to accurately recreate viral populations which control bacterial population diversity and abundance in natural systems (Martinez-Martinez, 2006). Without taking into account the factors which control microbial populations, laboratory experiments are unlikely to produce results that can be extrapolated into real systems and, until now, no suitable field sites were available in which to conduct experiments to address this knowledge gap.

Many of the constraints of laboratory based experimentation have recently been overcome by Hall-Spencer, *et al.,* (2008) who have used cold CO_2 vent sites to serve as natural laboratories in which to study the responses of marine organisms to low pH environmental conditions *in situ*. Seepage of cold volcanic CO_2 through the sub-tidal bedrock around the island of Ischia, off the coast of Naples, creates zones where the carbonate saturation state of the seawater is similar to predicted future OA conditions. This localised phenomenon provides a unique opportunity to measure ecological interactions and evolutionary organism responses to chronic high pCO_2 in the field, where these conditions are thought to have persisted for over one hundred years and possibly thousands of years (Tedesco, 1996). The vent site provided a unique opportunity to grow marine biofilms under conditions that mimic OA scenarios *in*

situ, and measure the associated microbial diversity. In addition, this site allows the potential to investigate microbial adaptation to long term low pH conditions.

The structure, formation and influence of marine biofilms were discussed in detail in Chapter three. As with all living assemblages, even mature biofilms are thought to be temporally and spatially plastic in terms of diversity and relative abundance of species, and the extent of this plasticity varies between environments (Yannarell & Triplett, 2005; Anderson-Glenna, *et al.,* 2008). The advance of OA is predicted to lower the environmental pH in shallow ocean waters by approximately 0.7 pH units, possibly for thousands of years (Caldeira & Wickett, 2003). pH variation of less than 0.6 of a unit has been reported as being either the primary or secondary factor in determining biofilm species composition in fresh water systems (Yannarell & Triplett, 2005). As pH change is known to affect biofilm composition, there is potential for OA to alter current assemblage structuring processes such as recruitment, as well as nutrient cycling. Currently, the likelyhood or magnitude of this alteration is unknown.

6.1 - Aims of this Chapter

The Chapter aims to collect data to provide information to test the following hypothesis:

1: Microbial diversity within established biofilms on sea grass leaves varies with pH

2: Microbial diversity within young biofilms growing on glass microscope slides at 1 m depth varies with pH

3: Microbial diversity within young biofilms growing on glass microscope slides at 4 m depth varies with pH.

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6.2 – Methods

To test these hypotheses, three experiments were performed at a low pH volcanic vent site (Ischia, Gulf of Naples) described by Hall-Spencer, *et al.,* (2008). The effect of different pH environments on biofilm formation were tested on biofilms growing on different substrata and at different depths. The leaves of sea grasses support an easily harvestable microbial film which had developed naturally at the low pH vent site. The leaf itself provides a relatively standardised substratum in terms of micro-texture and leaves were present at each of the different pH zones allowing the effect of environmental pH on biofilm diversity to be measured. However, some sea grasses are known to produce anti-microbial compounds which may influence the response to reduced environmental pH (Jensen, 1998; Mayavu, *et al.,* 2009) so biofilm formation on artificial microscope slides was also measured to test for an influence of environmental pH on a different substratum to address hypothesis two. Hypothesis two was tested by measuring biofilms that were grown on the benthos which may have influenced their early development as the vent gas is released from the benthos, possibly affecting biofilm formation in terms of small scale hydrodynamics (Vieira, *et al.,* 1993). To separate these influences, a separate experiment was conducted to test hypothesis three where the effect of pH on young biofilms grown on microscope slides at 1 m depth was measured.

Hall-Spencer, *et al.,* (2008) identified areas around the volcanic vent site (40° 043.84' N; 13° 57.08' E) where the escaping volcanic gas, mainly carbon dioxide, created zones where the seawater pH was consistently lower than ambient, see Hall-Spencer, *et al.,* (2008) for breakdown of different gas fractions. These zones were to the North of the island: N1, pH 8.14, N2, pH 7.87, N3, pH 7.09 and to the South of the island: S1, pH 8.14, S2, 7.83, S3 pH 6.57. (Figure 33).

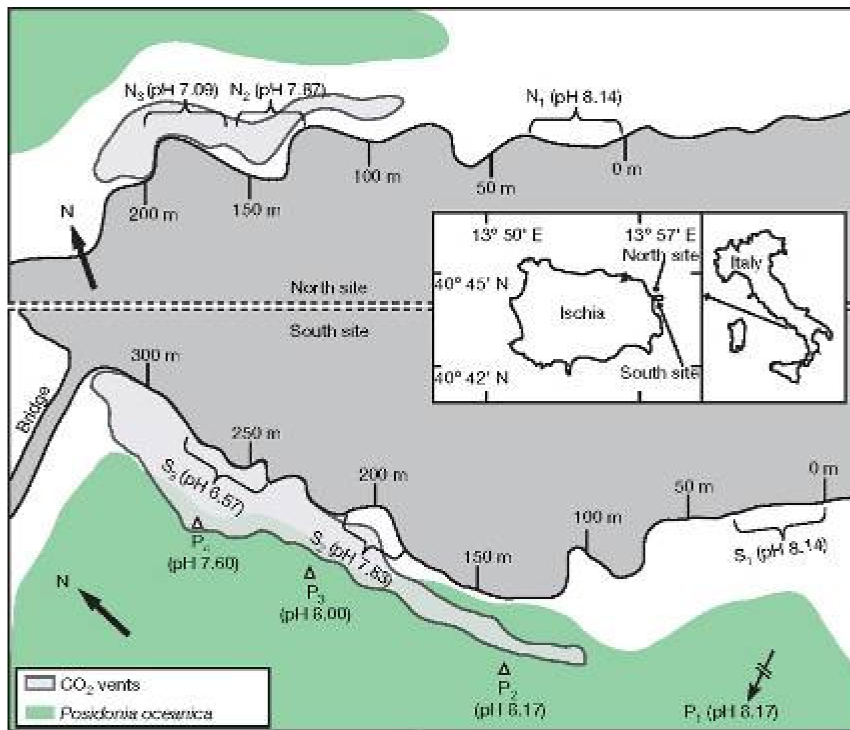

Figure 33. *Map of vent site and different pH areas.* $\bar{x}$ *surface pH is shown at 35-m-wide rocky-shore stations N1–N3 and S1–S3.* $\bar{x}$ *sub-tidal pH is shown at stations P1–P4, together with the distributions of* CO_2 *vents and P. oceanica sea-grass meadows. Reference station P1 was at a 3-m depth, 400 m from the arrow shown. Taken from Hall-Spencer et al., (2008).*

To address hypothesis one, biofilms were harvested from leaves of the sea grass *Posidonia oceanica* growing at different pH zones. In addition, biofilms were grown on autoclaved microscope slides (ground edges, plain glass 76mm x 26mm 0.8mm to 1.0mm thick, Fisher brand) that were deployed in both shallow (1 m) and deep (4 m) water at each of these zones. Shallow water slides were slotted and glued into slits cut in plastic floats, with five slides per float.

The floats were attached to ropes that were anchored to the sea bed (see Figure 35) so the slides were suspended near the surface at a constant depth of 1 m. Deep water slides were slotted into slits made in artificial sponges that were fixed to the seabed at a depth of 4 m (Figure 34).

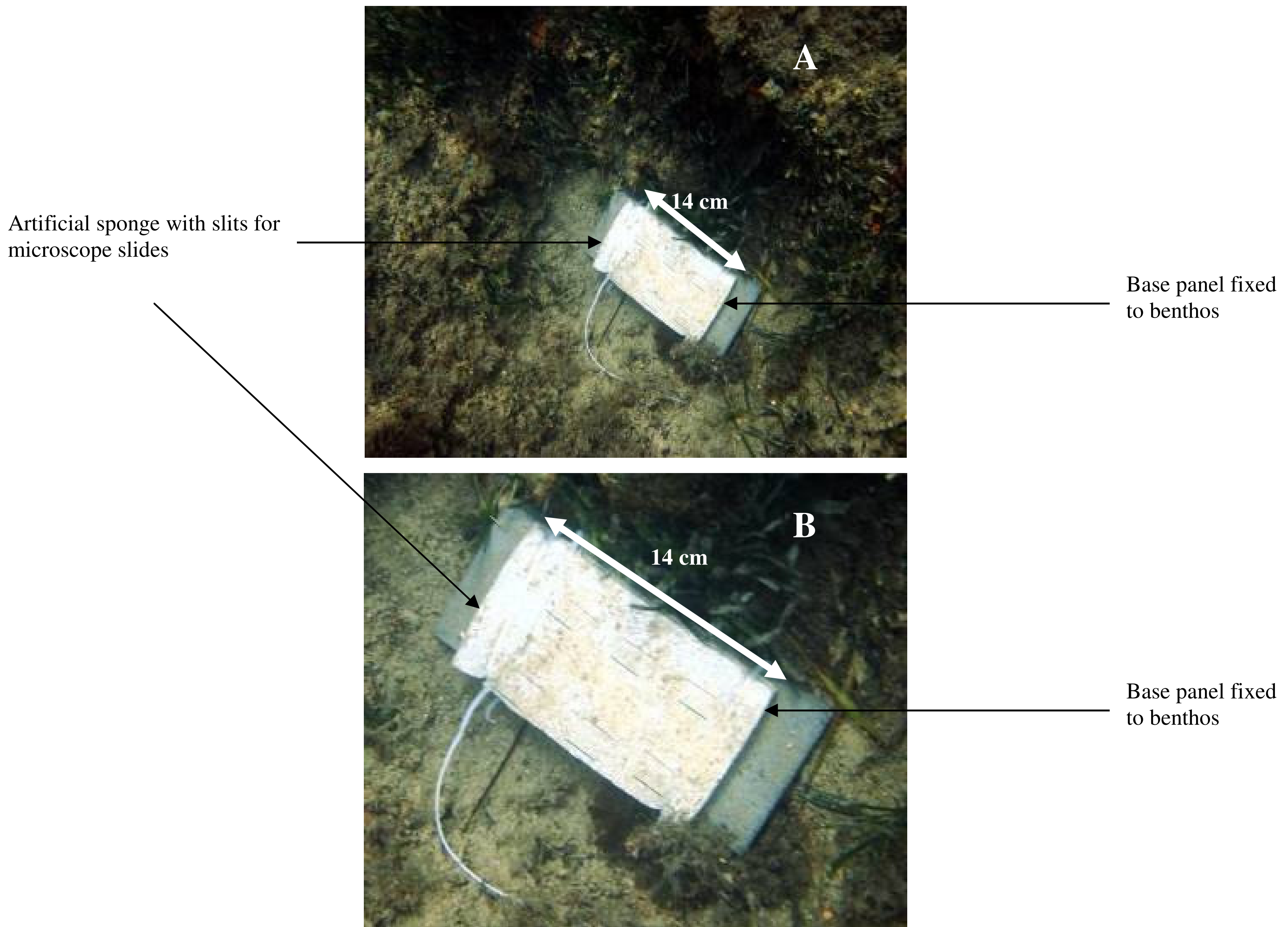

Figure 34, *Image A shows base plate and artificial sponge fixed to the benthos. Image B is enlarged from the previous image to show slits in the sponge into which microscope slides were slotted to grow biofilms in 4 m deep water. The white sponge is 14 cm long to give scale. Photographs courtesy of Riccardo Rodolfo-Metalpa.*

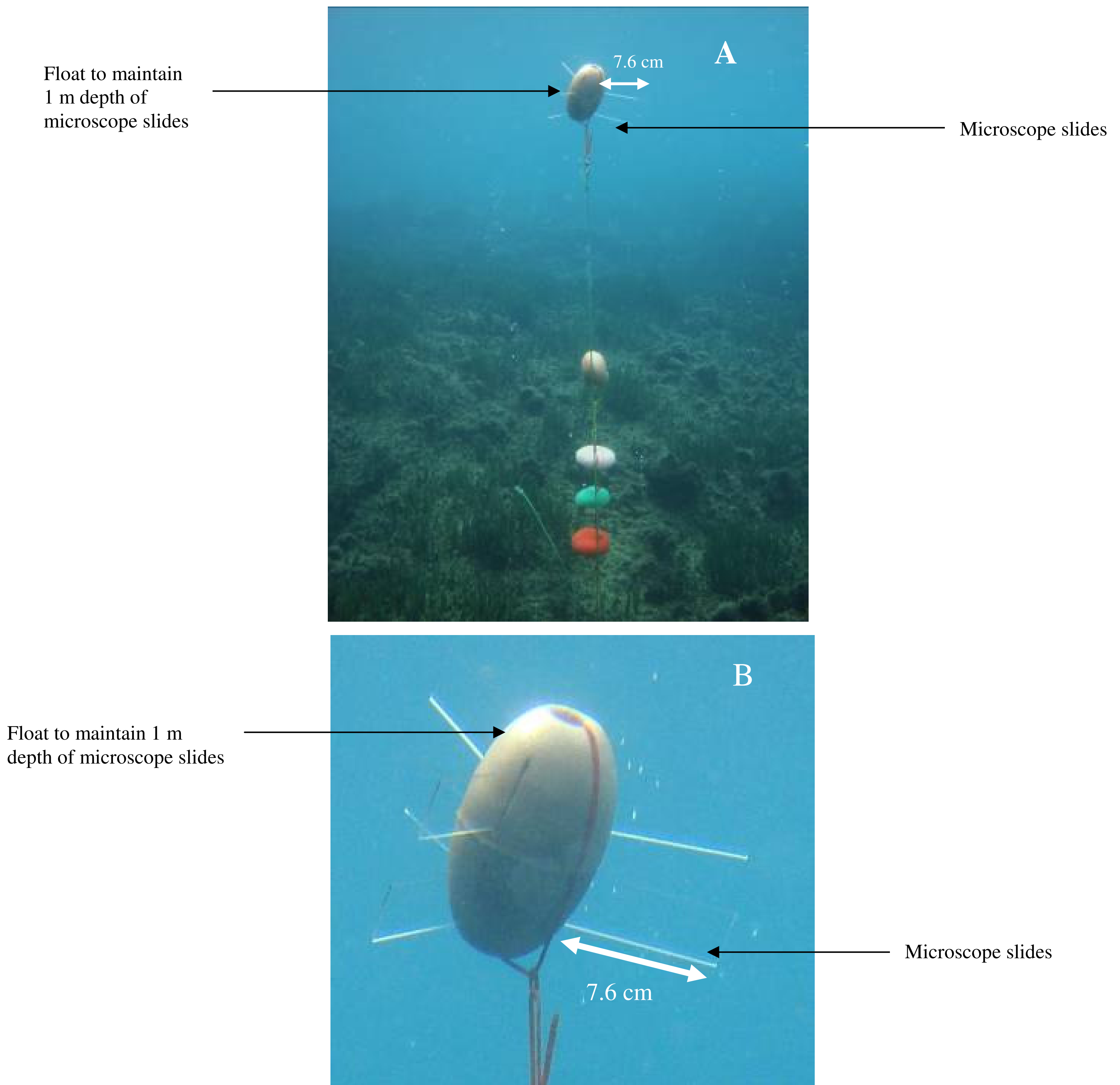

Figure 35, *Image A shows the float that was anchored to the benthos with rope at a depth of 1 m. The white green and red objects attached lower down the rope are related to a different experiment. Image B is enlarged from the previous image to show the slits in the float into which microscope slides were slotted to grow biofilms in 1 m deep water. Photographs courtesy of Riccardo Rodolfo-Metalpa.*

All slides were deployed on the 8th of April 2008 and left un-manipulated until collection on the 12th of May 2008. After the deployment, slides were recovered using gloves and aseptic technique, and the surface of each slide was immediately swabbed with an autoclaved cotton bud. The cotton bud tip was snipped with sterile scissors into an autoclaved 1.5 ml Eppendorf tube containing 99.9% ethanol. Leaves from the sea grass *P. oceanica* were plucked from the sea grass meadow from within the different pH sites and recovered to the surface. The biofilm from the top of the leaf was harvested using the same cotton bud method as the slides. All Eppendorfs were stored at 4 °C and shipped back to the U.K., where upon arrival they were stored at – 80 °C until analysis.

6.2.1 -Molecular Analysis overview

DNA was extracted from the samples and amplified using PCR. The amplified DNA was run on DGGE gels to provide a coarse measure of diversity within and between samples. Bands that were both unique and common to samples were extracted from the gels and sequenced in an attempt to establish the functional roles of the isolated microbes.

6.2.2 - DNA Extraction and 16s rRNA Gene Amplification

Biofilm samples were removed from the -80 °C freezer and the lid of each Eppendorf was punctured three times using a sterile needle. The samples were then freeze-dried for 24 hours to remove the ethanol. After removal of the ethanol, the extraction buffer from the DNAeasy DNA extraction kit for blood and tissue (see Chapter 3 for details) was added to the each Eppendorf. The cotton bud tip was manipulated in the extraction buffer using sterile tweezers to remove as much visible material as possible from the tip before discarding. PCR and DGGE were conducted according to the same method described in Chapter 3. DGGE gels

were run with each sample repeated three times to ensure consistency between gels. Once suitable quality gels were obtained, individual gels were placed between two acetate sheets, carefully wrapped in cling film and stored at -20°C until immediately prior to the picking of bands for sequencing

6.2.3 - Sequencing

Immediately prior to the picking of bands, the gels were removed from the freezer, carefully unwrapped and generously bathed in 1 x TAE (Tris – acetate) buffer solution to prevent fragmentation during thawing. Gels were placed on a UV light box and the appropriate band of interest was identified using a printout of the digital image of the gel. The band was picked using a 200 µl pipette tip, and the resulting plug of gel was extracted and placed in 100 µl of clean water and stored overnight. 1 µl of this product was then used as the target DNA in a PCR reaction conducted with the same method as previously described.

Samples were prepared for sequencing by purifying 30µ of PCR product (DNA concentration of approximately 12 µg per µl) with a Zymo Research DNA + Concentrator -5. Samples were eluted in 10µl of clean water. Following purification, 40µl clean water was added to each sample and DNA concentration in all samples varied between 10 and 18 µg per µl. Samples were sent to Geneius Labs, Newcastle, U.K. for sequencing and were supplied as 1 µl of purified sample mixed with 1µl of 357 forward Primer without the GC clamp. Sequences were matched with the Blast programme, using the Microbes option on the National Centre for Biotechnology Information (NCBI) webpage (NCBI, 2010).

6.2.4 -Statistical Analysis of DGGE data.

Bionumerics software version 5.0 was used to analyse the DGGE gel images and to generate data representing the presence or absence of each band identified in each gel. The presence or absence of bands on the DGGE gels generated data that was considered a proxy for microbial diversity, with each band representing a particular species or sub species (Muyzer, 1993).

The DGGE band data were analysed in the multivariate statistics program, Primer Software version 6.13, where Bray-Curtis similarity matrixes were calculated representing the presence or absence of bands on different gels. These matrixes were analysed using Non Metric Multidimensional Scaling (nMDS) and one way Analysis of Similarity (ANOSIM) to test for the effect of the factor pH (6.57 – 8.17) on microbial diversity within biofilms growing on microscope slides at 1 and 4 m deep and on *P. oceanica* leaves on microbial assemblage composition. Initially, all data were analysed for the influence of pH separately. If no effect of pH was measured on multiple factors they were pooled and analysed together for an effect of pH.

Different sites around the vent site are subjected to different pH waters as described by Figure 33, with some of the sites being exposed to the same pH. pH was not directly measured by this study, instead environmental characterisation of the sites was based on data supplied by Hall-Spencer, *et al.,* (2008). Because pH was not directly measured for this study, the factor site was excluded from the analysis as it correlated 100% with pH because pH data was based on site data supplied by Hall-Spencer, *et al.,* (2008).

6.3 - Results

Originally, six replicate microscope slides were deployed at each pH zone, and at each depth. Several samples were lost as a combined result of adverse weather conditions and/or lack of DNA after extraction and PCR, resulting in n = 3 for all microscope slide samples and n = 3 (pH 8.14) and n= 2 (pH 7.83) for the *P. oceanica* samples. The surviving samples produced between 10 – 26 bands on the DGGE gels, many of these being very faint (Figure 36). The majority of these bands were between 1000 and 500 base pairs long.

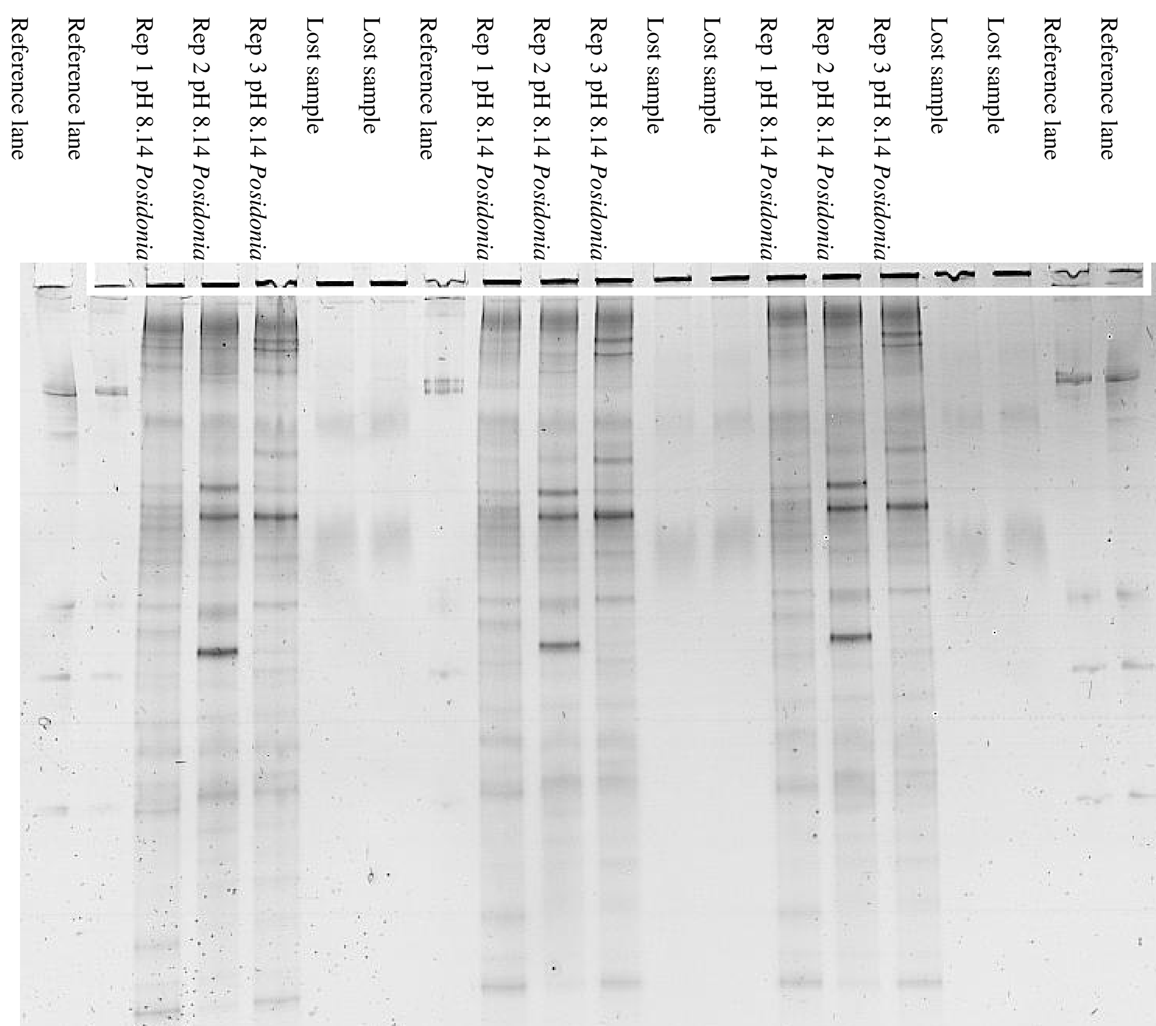

Figure 36: *Example DGGE gel showing 16s RNA extracted from biofilm samples obtained from Posidonia leaves in the 8.14 pH zone. Reference lanes contain bands of known base pair length for comparison to samples. Each replicate is run on three separate lanes to ensure consistency. Lost samples did not produce sufficient DNA during the extraction phase to produce a signal in the gel.*

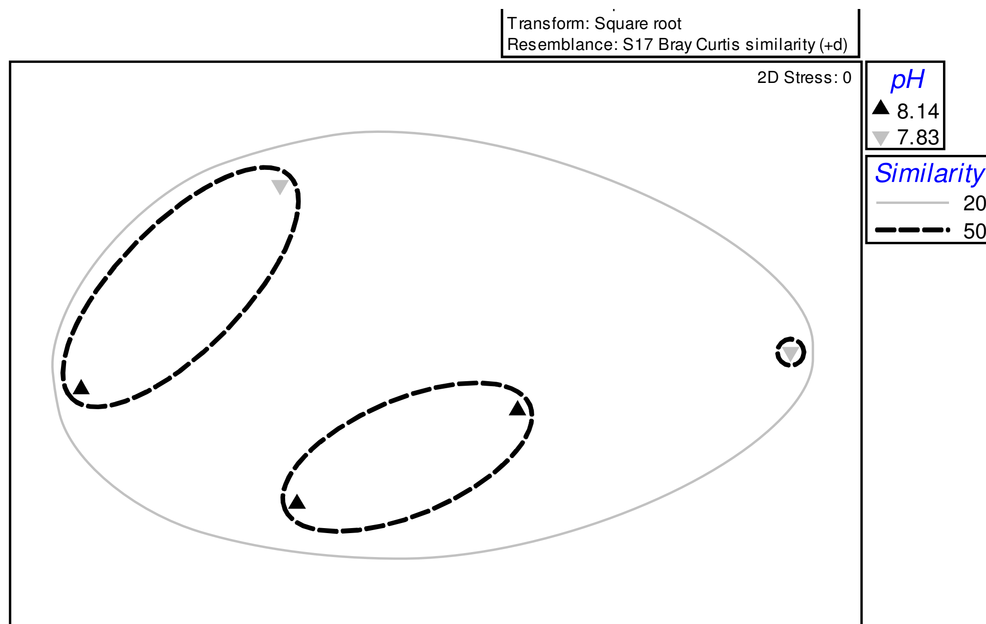

Figure 37: *nMDS plot representing microbial diversity in biofilms grown on P. oceanica leaves in pH zones 8.14, n=3, and 7.83, n=2. Data have been square root transformed, similarity shown as a percentage.*

nMDS analysis of biofilm microbial diversity data from *P. oceanica* leaves does not show any obvious pattern relating microbial diversity to pH. Unfortunately, only five replicate *P. oceanica* biofilm samples produced a sufficiently clear signal in the DGGE gels to allow meaningful analysis which limits the power of this analysis to detect an effect of pH. ANOSIM analysis of the *P. oceanica* biofilm data confirms this lack of effect (pH 8.14 and 7.83, R = -0.018, p = 0.61) but this analysis is also compromised in terms of power as a result of low numbers of samples.

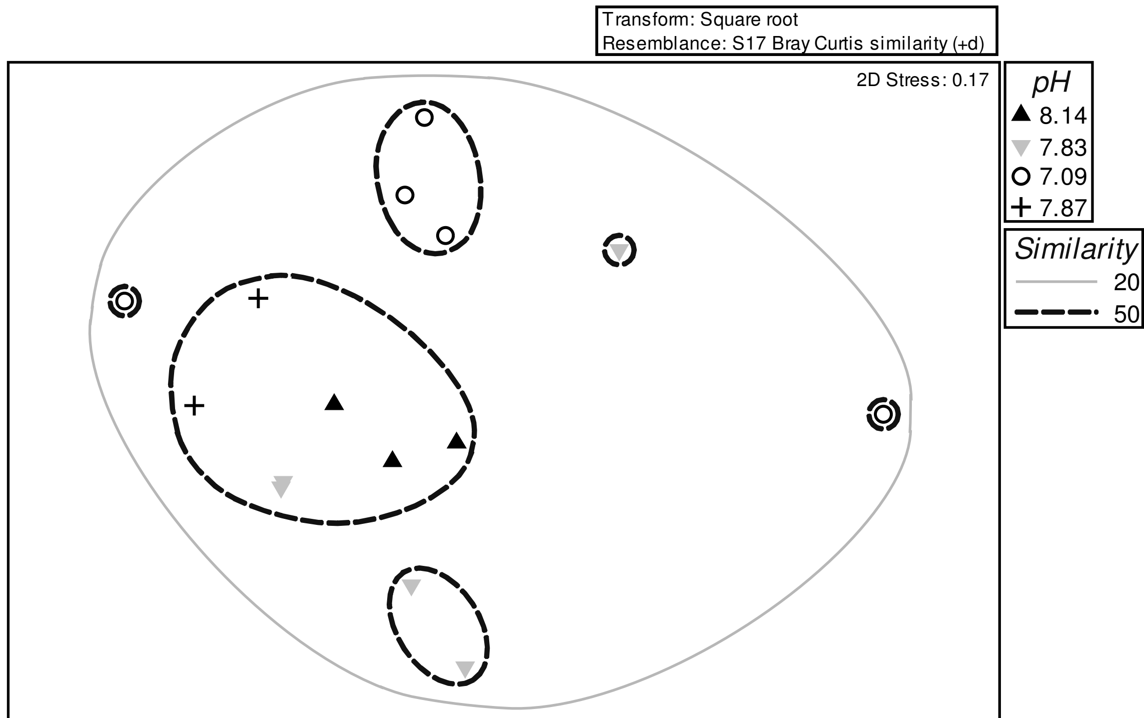

Figure 38*: nMDS plot representing microbial diversity in biofilms grown on 1 m deep microscope slides in pH zones 8.14, n=3, 7.83, n=4, 7.09, n=4, and 7.87, n=2. Data have been square root transformed, similarity shown as a percentage.*

nMDS analysis of biofilm microbial diversity data from microscope slides at 1 m deep does not show any obvious pattern relating microbial diversity to pH. This analysis is also limited in power by poor replication. Groups of samples from the same pH zone are present, particularly for samples from the pH 7.09 zone. However, other samples from within the same pH zone are separated indicating high within site heterogeneity with respect to biofilm diversity. ANOSIM analysis to compliment the nMDS plots supports this lack of relationship (microscope slides only, all pH zones, 1 metre deep, R = 0.14, p = 0.11.

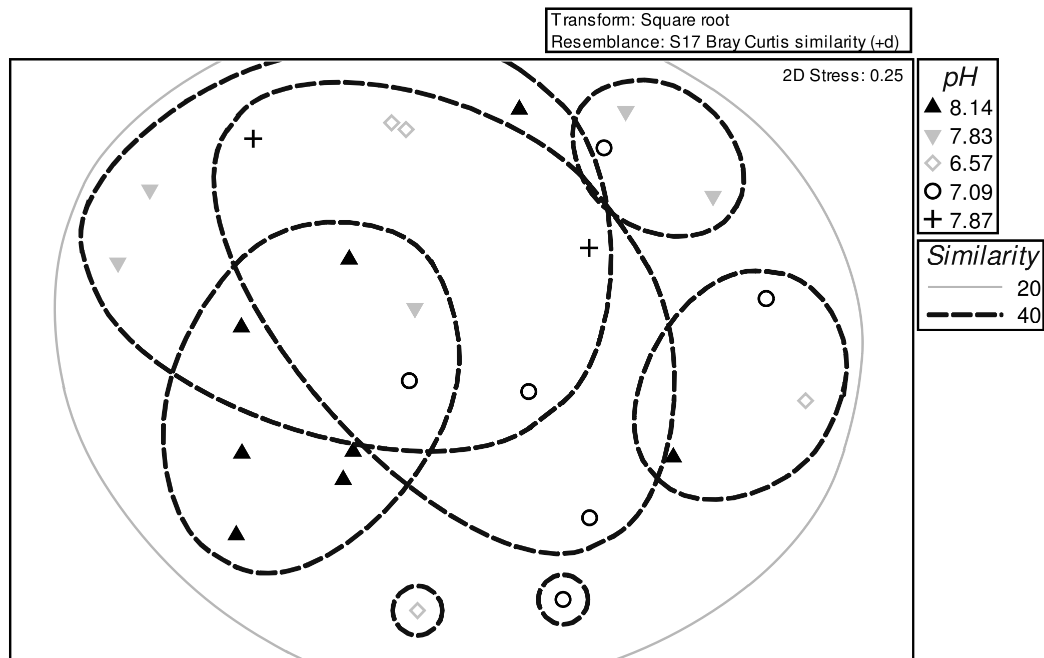

Figure 39*. nMDS plot representing microbial diversity in biofilms grown on 4 m deep microscope slides in pH zones 8.14, n=8, 7.83, n=5, 6.57, n=3, 7.09, n=7, and 7.87, n=2. Data have been square root transformed, similarity shown as a percentage.*

nMDS analysis of biofilm microbial diversity data from microscope slides at 4m deep does not show any obvious pattern relating microbial diversity to pH. Groups of samples from the same pH zone, particularly from the 8.14 zone, show up to 40% similarity. However, in a similar way as for the 1 m microscope biofilms, several samples from the same pH zone show considerable dissimilarity, again indicating heterogeneity of biofilm diversity with each pH site. This is lack of relationship between pH and microbial diversity is also reflected in the ANSIOM analysis of the 1 m microscope biofilm data, R = 0.14, p = 0.05.

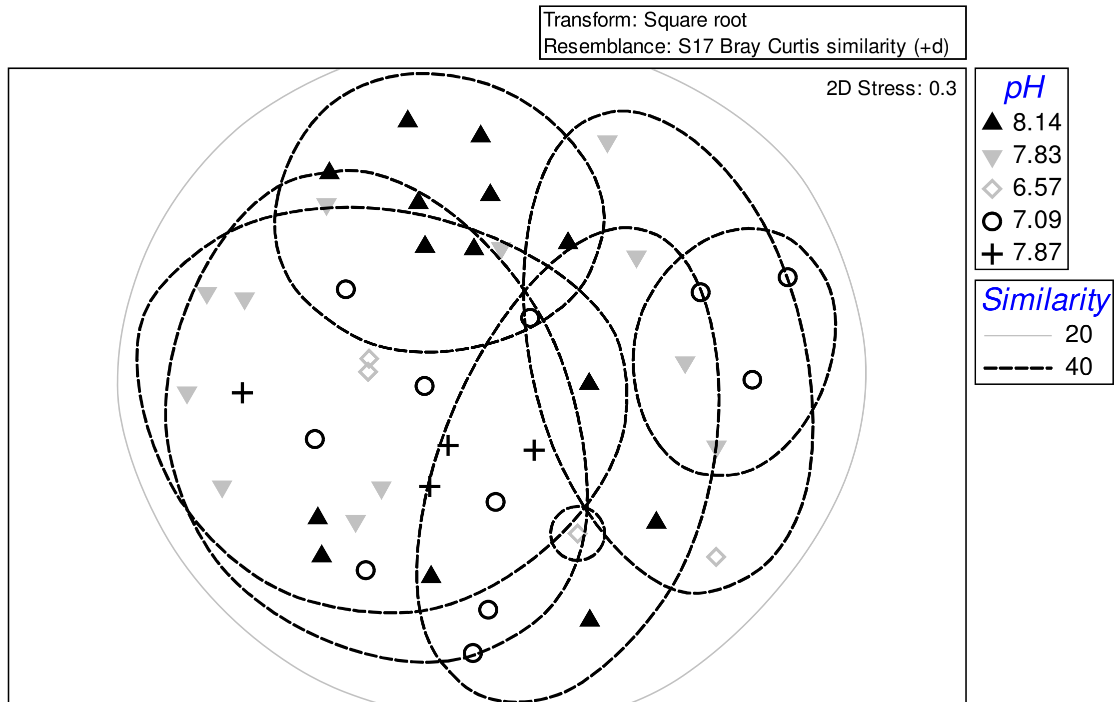

Figure 40. *nMDS plot representing microbial diversity in biofilms from pooled P. oceanica and microscope slides. Data are pooled by the factors depth and pH and substratum. Data have been square root transformed. P. oceanica samples n = 5, microscope slide samples n = 40, similarity shown as a percentage.*

As all analysis thus far has failed to produce a measurable effect of pH on biofilm microbial diversity, data were pooled by substratum type and depth and re-analysed. This pooled analysis has the added advantage of increasing the number of samples from each pH area, and therefore increasing the power available to detect any response of microbial diversity to pH. Even with this added power, no obvious patterns are shown by the nMDS plot, and no significant effect of pH was shown by the ANOSIM analysis, R = 0.158, p = 0.04).

***Table 25**. ANOSIM table showing R values to represent the similarity between microbial communities isolated from biofilms grown under different pH vent sites (pH 7.09, 7.83, 7.87 and 8.14) different substrata (microscope slides and P. oceanica) and different depths (1 and 4 m). Data represent the presence or absence of a particular band produced on a DGGE gel, a proxy for microbial diversity.*

Test for:	**Factors**	**Global R**	**Significance *p* =**
Effect of pH on diversity (microscope slides only)	all pH zones, 1 m	0.14	0.11
Effect of pH on diversity (microscope slides only)	all pH zones, 4 m	0.14	0.07
Effect of pH on diversity (*P. oceanica* leaves only)	pH 8.14, 7.83, 4 m	-0.018	0.61
Effect of pH on diversity (data pooled by pH and depth)	all pH zones, all depths, all substrates	0.15	0.04

6.3.1 - DGGE band Sequencing

Close matches between sequences obtained from the biofilm samples and the NCBI microbial database suggest that the DGGE method successfully separated 16s RNA strands of different lengths (Table 26). This close matching and differentiation between bands indicates the DGGE method was successful in that different bands did actually represent different species or sub species of microbes with the samples. Sequences were obtained from the shallow water biofilms, however they lacked sufficient length and cleanliness to allow meaningful matches on the NCBI database.

Table 26: *Sequencing results. DNA extracted from selected bands on the DGGE gel were sequenced and blasted on the NCBI microbes database. None of the bands sequenced from 1 m deep microscope slides produced good quality sequences.*

DGGE band #	Sample origin	pH	NCBI access #	Closest relative/ NCBI Ref	Similarity	Phylogenetic group
1	*Posidonia*	8.17	ZNSWC6YY016	*Parvibaculum lavamentivorans* NC_009719	99%	Proteobacteria
2	*Posidonia*	8.17	ZNTND99X016	*Maricaulis maris* NC_008347	96%	Proteobacteria
3	*Posidonia*	7.83	ZNV0KXPE014	*Methylobacterium Chloromethanicu* NC_011757	95%	Proteobacteria
4	*Posidonia*	7.14	ZNVZDEH9016	*Synechocystis* sp NC_000911	91%	Cyanobacteria
5	*Posidonia*	7.14	ZNWCTY56012	*Nodularia spumigena* NZ_AAVW01000053	92%	Cyanobacteria
6	4 metre deep slides	7.09	ZNWPUR17016	*Escherichia coli* NZ_AAJT02000128	92%	Proteobacteria
7	4 metre deep slides	7.87	ZNXBM7PT012	*Cyanothece* sp. NZ_AAXW01000002	90%	Cyanobacteria
8	4 metre deep slides	8.14	ZNXT0HVP014	*Crocosphaera watsonii.* NZ_AADV02000003	91%	Cyanobacteria
9	4 metre deep slides	8.14	ZNYUZ1T7012	Roseobacter sp. AzwK-3b NZ_ABCR01000010	94%	Cyanobacteria

6.4 - Discussion

Microbial diversity within marine biofilms growing at a low pH natural vent site was investigated and described in this Chapter. The effect of pH on microbial diversity within the biofilms was investigated with biofilms that had grown on natural *P. oceanica* leaves and also on microscope slides growing at 1m and 4m. No distinction was measured between the microbial diversity in biofilms growing in different pH sites. This lack of pH effect was consistent across biofilms grown on *P. oceanica* leaves and on microscope slides at 1m and 4m depth.

The sequenced genomic coding obtained from DGGE bands showed close matches with different microbes on the NCBI microbial database. This provides confidence that the DGGE method successfully separated 16s RNA strands of different lengths, ensuring that different bands did actually represent different species or sub species of microbes with the samples (Muyzer, 1993).

The general lack of effect on microbial diversity attributed to pH at the vent site is surprising. Analysable biofilm samples were obtained from pH 7.09 zones which represent a 1.11 pH unit deviation compared to the ambient pH of seawater, 8.2. A pH decrease of this magnitude equates to a theoretical ~ 330% increase in the concentration of H^+ ions in the environment. A 330% increase in any environmental parametres including pH can be considered a considerable stress. Higher organisms respond to similar stress levels by diverting metabolic energy to acid / base homeostasis (Seibel & Walsh, 2002). This response represents an energetically unsustainable shift in energy resource allocation, generating potential alteration to assemblage composition. Results from this Chapter suggest that marine biofilm associated microbes do not respond in the same way to pH decreases in the range that are expected to occur as a result of ocean acidification.

Two of the microbes sequenced from *P. oceanica* biofilm samples, *Maricaulis maris* and *Methylobacterium chloromethanicu,* are known to be associated with carbon scavenging, especially from carbon rich sources such as methane (NCBI, 2010). Methane was present in the vent gases, albeit at low concentrations (Hall-Spencer, *et al.,* 2008). It is likely therefore, that the microbial communities present within biofilms at this vent site are atypical of the wider environment, and are specialised to optimise growth under the chemical enrichment

provided by the vent gases. It is possible that microbes from other environments not exposed to gases other than normal atmospheric gases at normal partial pressures would respond differently to pH change of the same magnitude. Although not definitive, the sequence matches suggest that three out of five microbes isolated from *P. oceanica* leaves biofilms are known to be involved in important environmental processes such as the degradation of hydrocarbons in the case of *Parvibaculum lavamentivorans*, organic nutrient transfer by *M. maris* and nitrate and nitrite assimilation by *Synechocystis* sp (NCBI, 2010). This gives support that even in atypical environments marine biofilms are implicated in nutrient cycling.

M. maris is also described as being tolerant to a range of environmental conditions such as temperature (NCBI, 2010). Tolerance of atypical environments enables these organisms to survive in nutrient rich niches such as hydrothermal vent sites which are frequently associated with extreme environmental conditions, (Crespo-Medina, *et al.,* 2009). Adaptation to harsh environmental gradients may account for in part, the lack of difference between diversity in *P. oceanica* biofilms isolated from different pH vent sites. It is likely that as a response to the conditions they encounter utilising nutrient rich niches, many of these species have evolved tolerance to environmental stresses such as pH. Deeper water carbon rich vents are known to experience extreme environmental pH values in the range of 3.8 units, well outside ambient conditions (Gamoa, 2001).

Biofilm samples taken from control sites provided a measure of how unique microbial communities were within the vent site compared to comparable biofilms outside the site. No difference in microbial diversity was detected within biofilms from low pH and ambient pH sites in this study. It should be considered that the environmental parametres pH was not directly measured during this study, instead the different pH zones were defined by

conditions described by Hall-Spencer, *et al.,* (2008), which could have been subject to temporal change.

Although their initial formation is fast, biofilms do exhibit clear stages of succession and development (Acuna, *et al.,* 2006). The *P. oceanica* leaves sampled for this study were collected from adult plants which although not directly aged, are likely to be at least one year old (Ott, 1980; Guidetti, 2000b) These biofilms are likely to represent a more advanced successional state compared to the microscope slide films which were deployed for just over four weeks.

The *P. oceanica* samples may be considered to be of greater ecological value as they represent a natural substratum type in the marine environment, capable of facilitating natural biofilm formation. Biofilm formation and development is known to be largely dependant on the physical and chemical properties of a substratum, particularly the presence or absence of organic particles (Characklis, 1981; Cooksey 1995; Maki, 1999; Marshall & Bowden, 2000; O'Toole, 2000; Aguilera, *et al.,* 2007; Qian *et al.,* 2007). It is reasonable to assume the latter is going to differ considerably between autoclaved glass microscope slides and natural *P. oceanica* leaves.

Sea grasses are known to contain anti-microbial compounds (Jensen, *et al.,* 1998; Mayavu, *et al.,* 2009). Microscope slides represent unnatural surfaces for biofilm colonisation and sea grass leaves contain anti-microbial substances. It may be possible that biofilms present on other natural surfaces such as benthic rocks and sediments which are both natural and non-antimicrobial, would respond differently to pH change. Investigating the pH response of

biofilms growing on other natural surfaces at the vent site is considered an important addition to this work.

Explanations for the lack of pH response measured in this study include the possibility that other diversity influencing parametress existed at the site that exerted a greater influence on diversity than environmental pH. Parametress such as salinity and temperature could have been more important in terms of affecting diversity (see Rodolfo-Metalpa, *et al*., 2010). pH however, can be ruled out as having a dramatic effect on microbial diversity at the vent site, according to data obtained in this study. However this conclusion must be taken in context with limitations previously described.

The unbalanced design with fewer replicates of *P. oceanica* biofilms compared to microscope slides may preclude underlying trends of pH response that only repeated sampling on a wider scale would highlight. It is also possible that the DGGE method used here was simply not refined enough to pick up slight differences in microbial assemblage structure resulting from the different substratum types. Although beyond the scope of this study, the area of molecular techniques is advancing rapidly and a more sensitive method of analysis, such as the use of clone libraries and Florescence *In Situ* Hybridization (FISH) may detect substratum specificity and other pH or depth influences not detected here.

Biofilm microbial diversity was not seen to respond to changes in environmental pH at either 1m or 4m. This general lack of response is also unexpected as several other parametress such as light and temperature are commonly related to depth. In addition, the volcanic vents at this site release gas through the benthos into the water column. It would be expected therefore that any diversity affecting capacity of the gas may exert a greater influence closer to the

point source of release, compared to shallower areas where the potential for buffering of any pH alteration is increased. Enhanced dissolution of CO_2 facilitated by the greater pressure may also occur at deeper zones, creating the potential for a vertical pH gradient effect, which in turn can alter microbial diversity.

These results are unexpected as biological communities exposed to environmental stress are frequently characterised by low diversity with a minority of stress tolerant species out competing the others (Menge & Sutherland, 1987; Mackey & Currie, 2001; Witman, *et al.,* 2008; Gray, 2009). One explanation may be that the vent environments fluctuate in terms of pH with variation in rates of venting gas and prevailing weather conditions. This fluctuation may facilitate the existence of opportunistic populations of microbes which proliferate in micro-climates created by patchy dissolution of vent gas throughout a particular site.

It is my feeling that caution should be exerted when drawing conclusions from these data. Medium scale spatial and temporal variation in species composition of biofilms inhabiting the vent site are to be expected, as described in other aqueous environments (Yannarell & Triplett, 2005). Inherent patchiness is very difficult to experimentally account for at this site, given that constant flux in the rate of vent gas dissolution could easily lead to the false identification of false trends and relationships between factors. It is also entirely possible that other environmental gradients not measured in this study, such as temperature, salinity and small scale hydrodynamics also occur within the area, contributing to the lack of influence of pH on microbial diversity measured between zones.

The vent site at Ischia represents a unique opportunity to study the chronic effects of low pH environments on marine organisms of all sizes in the field. This approach is especially

valuable for OA research as it helps to distinguish between acute, toxic effects of high pCO_2 treatments often used in laboratory based OA experiments, and real effects of chronic low pH exposure of the sort that are likely to occur as a result of progressive OA. However, despite the ecological and experimental value of the site, we are unable draw firm conclusions from this investigation due to the poor replication of samples caused by either the loss of samples due to storms, lack of extractable DNA, or insufficient amplified DNA in the PCR product.

In order to fully understand how these biofilm microbes will respond to OA driven pH change, future investigations should continue to measure microbial diversity at sites like Ischia. Particular emphasis should be directed towards extensive temporally and spatially replicated sampling regimes which allow for the inherent patchiness of marine microbial populations to be accounted for, and separated from microbial diversity shifts triggered by the low pH vents. Alternative quantitative and easily replicable molecular techniques other than DGGE would assist with the identification of potentially subtle shifts in microbial assemblage composition.

No effect of low environmental pH on microbial diversity within biofilms was measured on natural substratum, or artificial substratum at two different depths. Low sample sizes may have masked pH effects, yet pooled data with higher statistical power still failed to show an effect of pH decreases on a similar scale to those expected as a result of OA. Further work at this vent site on biofilms from other substrate types and with more replication is recommended. In addition, the measurement of other variables such as salinity, hydrodynamics and temperature which could influence microbial diversity is suggested to help separate the role of environmental pH decrease on biofilm diversity from other factors.

Chapter 7

Industrial Applications

7.0 - Introduction

The conceptual phase of this thesis led to a spin-out concept that was further developed and successfully patented as a novel non-toxic antifouling treatment, patent number GB2459854, (Appendix 9.4.). This Chapter describes the background industrial requirement for this low pH technology and the testing and development procedure.

7.1 - Biofouling

Marine biofouling refers to an accumulation of undesirable marine growth on an artificial surface immersed in marine water. The fouling process starts with the attraction of organic and inorganic particals onto un-colonised surfaces via Brownian motion, Van der Waals forces and hydrostatic charges (Chambers *et al.,* 2006). This primary layer, also known as the conditioning layer (Callow & Callow, 2002) is often the precursor to biofilm development, and is described in Chapter 3. Biofilms and cues from biofilm associated microbes subsequently interact both physically and chemically with higher fouling organisms (Pawlik, 1992; Wieczorek, 1996; Hadfield, 1998; Olivier, 2000; Zhao, 2002; Hung, *et al.,* 2005a; Lam, *et al.,* 2005b; Lau, *et al.,* 2005; Bao, 2007; Qian, *et al.,* 2007), also see Chapter 4. The result is a multi-species fouling assemblage typically consisting of micro-organisms such as marine bacteria, diatoms, fungi, unicellular algae, protozoa and macro-organisms such as barnacles, molluscs, sponges, tunicates, bryozoans and tube forming polychaete worms (Callow & Callow, 2002).

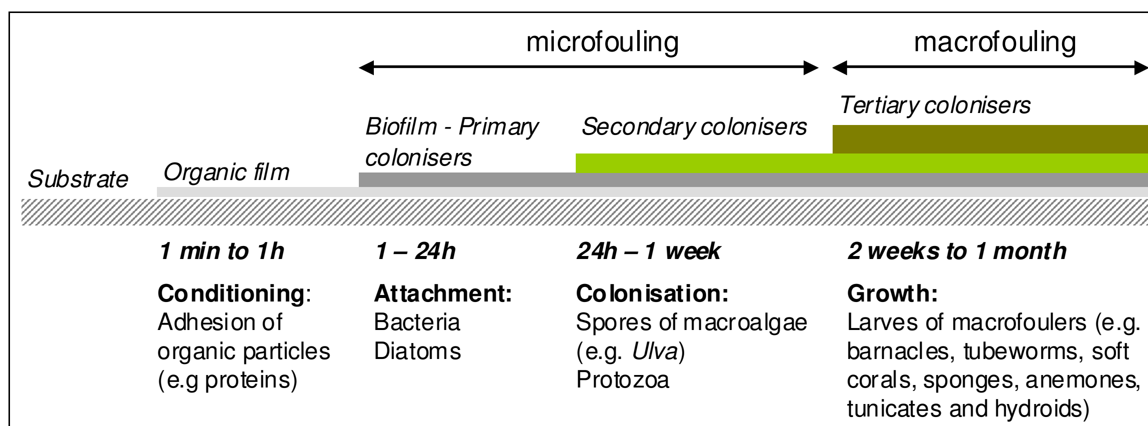

Figure 41. *Biofouling development adapted from Yebra et al., (2003) and Chambers et al.,(2006) by Karine Laffont and Annie Linley*

The process of biofouling is often described as a linear process, with one stage followed by the next as shown in Figure 41. However, under certain conditions marine larvae settle in the absence of biofilms (personal observations) and biofilms often undergo a process of sloughing which effectively reverts the film back to an earlier stage of colonisation (Lewandowski, 2000). This sloughing means that biofilm formation and development is a circular process and the different stages do not necessarily follow each other sequentially.

Biofouling causes severe problems in many industries where artificial surfaces are in contact with marine water, with typical examples being the fouling of ship hulls (WHOI, 1952), power station cooling water pipes (Bott, 2001), ballast water tanks (Hewitt, *et al*., 2009), marine sensors (Delauney & Compère, 2010), aquaculture structures (Lane & Willemsen, 2004; Dürr & Watson, 2010) and off-shore platforms (Hardy, 1981;Yan & Yan, 2003;Yan & Yan *et al*., 2004). A well developed fouling assemblage adds dramatically to the complexity of a surface causing increased drag and hydrodynamic load exerted on man made structures (Schultz, 2004, 2007). Hauling and moving heavily fouled equipment can prove problematic

as once out of the water, the pay load of a fouled surface is considerably higher than an un-fouled surface due to the high water content of the fouling organisms. This can lead to increased structural stress and load on joints and welds (Braithwaite & McEvoy, 2005)

Increased weight, drag and load on ship hulls causes reduced hull efficiency and increased fuel consumption (Schultz, 2004, 2007). The impact of biofouling on ship hull performance varies depending on hull design, but heavy fouling can decrease ship speed by up to ten per cent (Schultz *et al.*, 2003; Yebra *et al.,* 2004). The increased resistance in the water caused by fouling can add forty per cent to fuel consumption in severe cases, which contributes to an estimated twenty million tonnes of CO_2 produced globally each year as a result of fouled ship hulls, according to the United States Office of Naval Naval Research (ONR).

Biofouling causes further problems in marine industries by hindering maintenance work and preventing early detection of corrosion. Not only can biofouling conceal early stages of corrosion by covering the affected areas, it can increase the rate of ferric metal corrosion as a result of microbially influenced corrosion (MIC) (Beech, 2002; Videla & Characklis, 2002). As a result of MIC, corrosion rates are accelerated due to the oxidising nature of metabolic products from organisms such as sulphate reducing bacteria (SRB) residing within biofilms and microbial mats (Geesy & Beech *et al.,* 2000; Hamiliton, 2003). Dense fouling on aquaculture cages can prevent sufficient water exchange between the enclosure and the environment, creating adverse conditions for the inhabitants, as well as expensive cleaning and maintenance issues (Paul & Davies, 1986; Brake & Parsons, 1999).

The biological implications of biofouling are also considerable. Adult fouling organisms adhered to mobile artificial surfaces such as ships hulls represent a viable supply of gametes

into potentially foreign environments (Faubel & Gollasch, 1996; Gollasch, 2002; Vaate *et al.,* 2002; Sylvester *et al*., 2010) The threat posed by invasive or potentially invasive species transported in this manner (and through other vectors such as ballast water) constitutes the second most important threat to marine biodiversity after habitat destruction (Bax; 2003; Molnar *et al*., 2008).

Estimates of how much biofouling costs the affected industries in total are scarce. The annual end user market for the commercial ships and leisure market in 2001 was estimated at nearly seven hundred million pounds sterling excluding VAT, indicating the scale of the problem (Keystones Inc, 2001). Notably this figure only relates to shipping and also excludes other market sectors such as aquaculture, power station cooling and off-shore oil and gas industries. Problems arise when trying to put a cost on the impacts of biofouling as it is very difficult to fully account for the long term cost of habitat alteration resulting from biological invasions (Lewis & Coutts, 2010). Consequently any figures which claim to represent the full cost of biofouling should be considered conservative.

Fouling prevention is not a new industry. Early antifouling treatments involving tars, copper sheeting, animal skins and fats are all described in the literature (Plutarch, 1870; WHOI, 1952; Bertram, 2000; Anderson, 2004; Almeida *et al.,* 2007). Later technologies involved coatings which self polished or leached toxic compounds such as copper to poison and deter marine life from settling. Many of these treatments were partially effective in deterring fouling but had significant disadvantages such as lack of specificity for target species, limited longevity and most notably in the case of tributyltin (TBT), considerable and persistent negative environmental repercussions, see Bryan & Gibbs (1991) and deMora (1996) for a full review.

The environmental consequences of toxic compounds in antifouling coatings stimulated legislative convention International Maritime Organisation (IMO) banning the inclusion of TBT on coatings in 2008 stating that “vessels shall not bear such compounds on their hulls or external parts or surfaces or shall bear a coating that forms a barrier to such compounds from the underlying non-compliant anti-fouling systems” (IMO, 2001). The resolution called for a global prohibition on the application of organotin compounds which act as biocides in antifouling systems on ships by 1st January 2003, and a complete prohibition by 1st January 2008 (IMO, 2001).

Despite the environmental fate of the active constituents, TBT based coatings are effective antifoulants, so much so that in 1999 the IMO estimated that 70% of the world’s commercial fleet were using TBT based antifouling systems (IMO, 2001). The antifouling protection offered by TBT was highly effective leading to vast economic benefits to the shipping industry (Evans, 1999; Abott & Abel, 2000; Thomason, 2010). As a result, the prohibition of many of the TBT based products from the market has created a considerable gap.

This gap has been partially filled by several different types of coating, but with much emphasis on copper based biocidal coatings, which currently make up the largest proportion of global antifouling coating sales (pers comm with sales representatives from Jotun Ltd). Copper based biocidal coatings have been engineered to release biocides in several ways such as self polishing, controlled depletion (Bertram, 2000; Anderson, 2004; Almeida *et al.,* 2007) and with the addition of booster biocides (Readman, 2006).

All of these technologies provide antifouling performance but fundamentally rely on the toxic effect of copper on marine organisms and also show significant loss in performance over time resulting in the need for frequent maintenance and re-application (Bertram, 2000; Anderson, 2004; Almeida *et al.,* 2007).

Recent shifts towards non-toxic coatings have resulted in the production of foul release technologies which rely on very low surface energies to prevent fouling organisms from firmly adhering to a treated surface (Almeida *et al.,* 2007, Finne & Williams, 2010). These coatings are generally either organosilicones, often in the form of ploychlorodimethysilohexane (PDMS), or fluropolymers (Finne & Williams, 2010). Once sufficient hydrodynamic shear force is applied across the surface by a moving vessel, the loosely attached organisms are dislodged. This concept is suitable for fast moving vessels that are frequently in use, but problems arise with fouling species becoming firmly adhered after prolonged periods of inactivity, especially during lay-up (Finne & Williams, 2010), and the apparent disregard to low surface energies shown by some organisms when settling (Chaudhury, *et al.,*1995). It is generally accepted across the industry that what is needed to address these issues is a coating or technology which is environmentally benign, capable of deterring fouling in static water conditions, and also exhibits a foul release capability (Finne & Williams, 2010).

7.1.2 - Organisms response to low pH

Thus far, this thesis has described the response of marine organisms to pH change in the range that is expected to occur as a result of ocean acidification. These data show a general lack of response to pH decreases of up to 0.7 of a unit. However, the biological response data in the literature suggest that organisms exhibit varied and diverse responses to environmental

pH change (Hendriks *et al*., 2010). Generally speaking, the lower the pH, the more severe the biological response (Hendriks *et al*., 2010). This trend led to the idea of using small amounts of CO_2 to create low pH surfaces that deter the settlement of fouling species. The attraction of this concept is considerable as ships already produce CO_2 to use as a fire retardant to be pumped into holds containing combustible cargos, and all inputs, products and by-products involved in this system occur naturally in seawater. What was lacking was a suitable delivery system with which to administer to CO_2 into the environment.

7.1.3 - Conception and Prototype

In order to address the main concepts in this thesis, there was a requirement to develop a system that allows the user to experimentally lower the pH of sea water *in situ* and sustain this manipulation to gauge the response of marine organisms to pH change similar to OA. This concept requires the sustained supply of CO_2 into the environment via some form of delivery medium. The delivery medium must also be coupled with a control mechanism if this system is to be used experimentally, as natural environmental variability exits with respect to pH, and other factors that influence pH such as temperature and hydrodynamic fluctuation. The introduction of a feedback loop provided the required pH control for the experiments described thus far in the thesis.

During the development of the system described and applied in this thesis, other CO_2 delivery options were considered. One of these options involved introducing CO_2 through permeable membranes. Gas permeable membranes have been used by the oil and gas industry for decades as a means of fractioning off a particular gas from a blend of different gases, for a review see Bernado *et al.,* (2009). Modern silicone polymer membranes were particularly attractive for this application as they characteristically display a high degree of flexibility

between the silicon and oxygen chain that forms their chemical structure (Stern *et al.* 2003). This flexibility effectively creates channels through which relatively large molecules, including CO_2 can pass (Vieth, 1991). As a result of this inherent flexibility, silicone polymer membranes exhibit one of the highest degrees of permeability to CO_2 of the major commercially available polymers (Stern *et al.* 2003). In addition, silicone polymers are relatively cheap, resistant to harsh environmental gradients and immersion in seawater, as well as being chemically inert.

A concept was developed that involved supplying CO_2 behind a gas permeable silicone membrane that was fixed in a gas tight gasket. The whole unit could then be immersed and used to create a low pH zone *in situ*. At the conceptual stage, it was anticipated that this low pH zone would be swiftly buffered by the carbonate in the surrounding seawater, so the low pH zone would effectively be a boundary layer immediately adjacent to the membrane. The dimensions of such a boundary layer would make it difficult to use this technology to expose well established assemblages of large fouling organisms to measure their response to OA, but it may provide a successful approach to deter fouling organisms.

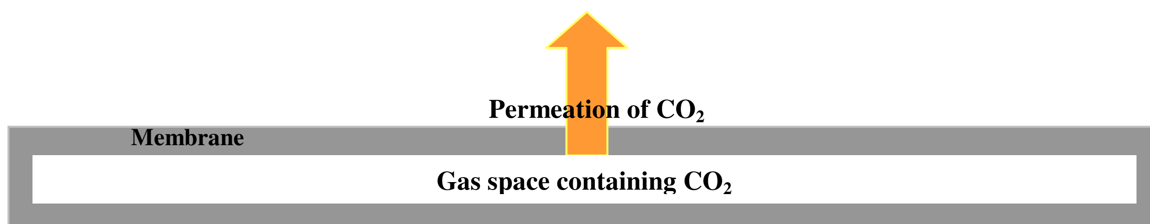

Figure 42: *Conceptual diagram of membrane gas permeation unit*

The next steps involved taking the concept and testing it in the laboratory as a proof of concept trial to determine if a low pH boundary layer was achievable using this principal. The

second stage of the development was a field trial to determine if the low pH boundary layer had any influence on fouling *in situ.*

7.1.4 - Proof of concept

In order to test this concept of low pH boundary layers induced by CO_2 permeating through silicone membrane, a basic pilot study was conducted in the laboratory with the aim of discovering:

- is low pressure CO_2 able to pass through a commercially available silicone membrane?

  and if so:
- what was the approximate pH created in the boundary layer?
- what were the approximate dimensions of the boundary layer?

7.2 – Methods

Seawater was collected from Sunderland Marina on 08.03.2007 and taken back to the laboratory where it was filtered through a 11µ Whatman cellulose paper filter to remove any large particles. 500 ml of filtered seawater was then poured into a 500 ml beaker which contained 20 drops from the universal indicator solution bottle supplied in a Hanna Instruments chemical pH test kit (product code HI -3881). A membrane test gasket was constructed from a 75ml Nalgene container by removing the lid and cutting a 1.5 cm radius hole in the top. The test membrane (clear silicone polymer sheet, 1.5 ml thickness supplied by Silex ltd, Hampshire, UK) was then placed between the lid and the container and gently screwed back on until firm and gas tight.

The membrane test gasket was secured within the beaker of seawater with metal bench clamps so that the gasket was vertically orientated and 10 cm below the surface of the seawater. Once secured in position, CO_2 (99.0 % industrial grade supplied by British Oxygen Company, BOC) was introduced into the Nalgene container from a 5,000,000 Pa cylinder via a Polyvinylchloride (PVC) tube (0.5 cm diametres). The internal pressure within the membrane test gasket was not measured. The surface of the membrane in the gasket was filmed before the introduction of gas and during the introduction using a Canon G4, 4 $x10^6$ pixels camera on its video setting (Figure 43).

7.3 - Results

The pH indicator solution was green when initially added to the seawater prior to the introduction of CO_2 into the membrane test gasket. This colour corresponds to approximately pH 8.0 according to the pH indicator chart supplied with the Hanna instruments pH test kit. After the CO_2 was introduced into the into the membrane test gasket, a distinct colour change in the boundary layer above the membrane was visible, changing from green to bright orange (Figure 43). The bright orange colour produced in the boundary layer corresponds to approximately pH 5.5 according to the pH indicator chart supplied with the Hanna instruments pH test kit. This qualitative test confirms that the boundary layer pH had changed as a result of the introduction of CO_2 into the membrane test gasket.

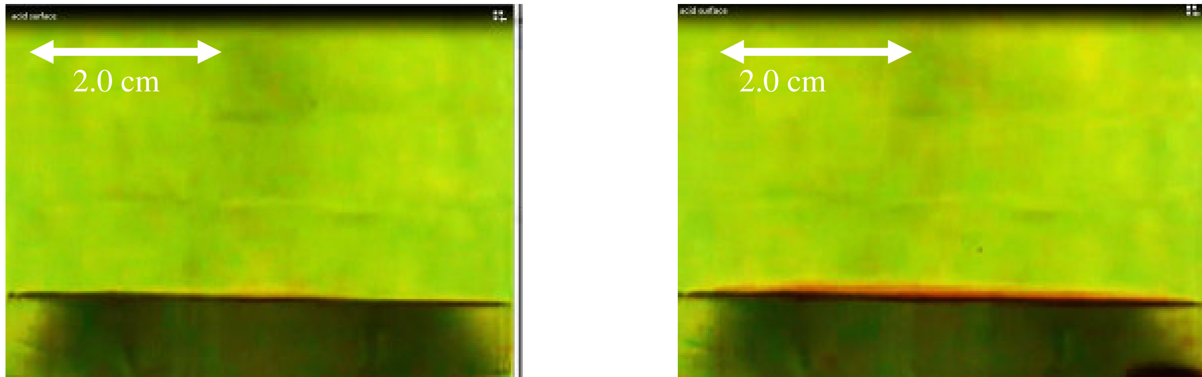

Figure 43. *Side view image of the membrane test gasket before (left image) and after (right image) the introduction of CO_2 under the membrane for a 15 second time period.*

Although the video analysis only produced qualitative information, it served as a proof of concept that actively permeating CO_2 through a silicone membrane produces carbonic acid of approximately pH 5.5 in ambient seawater. If normal seawater is approximately pH 8.2, a pH drop of 2.7 units to pH 5.5 represents a significant modification of boundary layer chemistry. The potential of this boundary layer to disrupt the fouling process described in the introduction of this Chapter remained un-known, as did the capacity of technique to maintain the boundary layer modification whilst being subjected to hydrodynamic flux and in the field. In order to measure the antifouling capacity of this technique, a field trial was conducted as phase two of the development process.

7.4 - Field Trial

A field trial of the membrane antifouling system was conducted to address the following questions:

- Does the addition of CO_2 make the silicone membrane less likely to foul than an untreated membrane?
- How long does the antifouling capacity last?
- Is the antifouling capacity generalised or specific to certain fouling species?

7.5 - Methods

A static water field trial of the membrane antifouling technique was conducted at Hartlepool Marina, UK. For a full site description see Chapter 2. Three of the panels were 10 x 10 cm square, 3 mm thick and made from grey PVC supplied by Bay Plastics of North Shields, Tyne and Wear, U.K. These three panels were evenly roughened for 1 minute with coarse sand paper (grit size 60) on an electric orbital sander, in order to ensure homogeneous rugosity between panels. The next three panels were made of the same PVC base, but with the addition of a layer of silicone polymer membrane (clear silicone polymer sheet, 1.5 ml thickness supplied by Silex ltd, Hampshire, UK) that was adhered to the surface of the panel with a non-toxic silicone based adhesive (Silver label silicone aquarium sealer supplied by Seapets ltd, UK). These two types of panel acted as controls for the main treatment type where CO_2 was introduced to create the low pH boundary layer effect.

The remaining three panels were again made from the same PVC base but with the addition of three 8 cm long, 1 cm wide PVC ribs fixed to the front surface. The same silicone membrane was then glued to these supporting ribs and fixed in place with a 3 mm thick, 1 cm wide, grey PVC gasket to seal the unit, (Figure 43). The gasket was fixed in place by nylon cheese head slotted screws (Radio Spares ltd part number, 527-993) that passed through holes drilled through the panel and were secured in place by nuts on the reverse of the panel. CO_2 (99.0 % industrial grade supplied by British Oxygen Company, BOC) was introduced into the space between the membrane and the PVC back plate from a 5,000,000 Pa cylinder via 0.5 ID PVC tube where the pressure was regulated at approximately 13,000 Pa.

The gas supply tube was connected to the panel via push fittings (Algard brand supplied by Seapets ltd, UK) that were glued into the back of the panel. Any minor gas leaks in the panels were sealed with the same silicone adhesive previously described. All panels were fixed with cable ties in a random order to a frame made from 3.6 cm ID white PVC pipe. The frame was suspended from a floating pontoon in the marina so that the panels were maintained vertically at a depth of 1 m. The array of nine test panels was deployed in this way from September 2007 until March 2008.

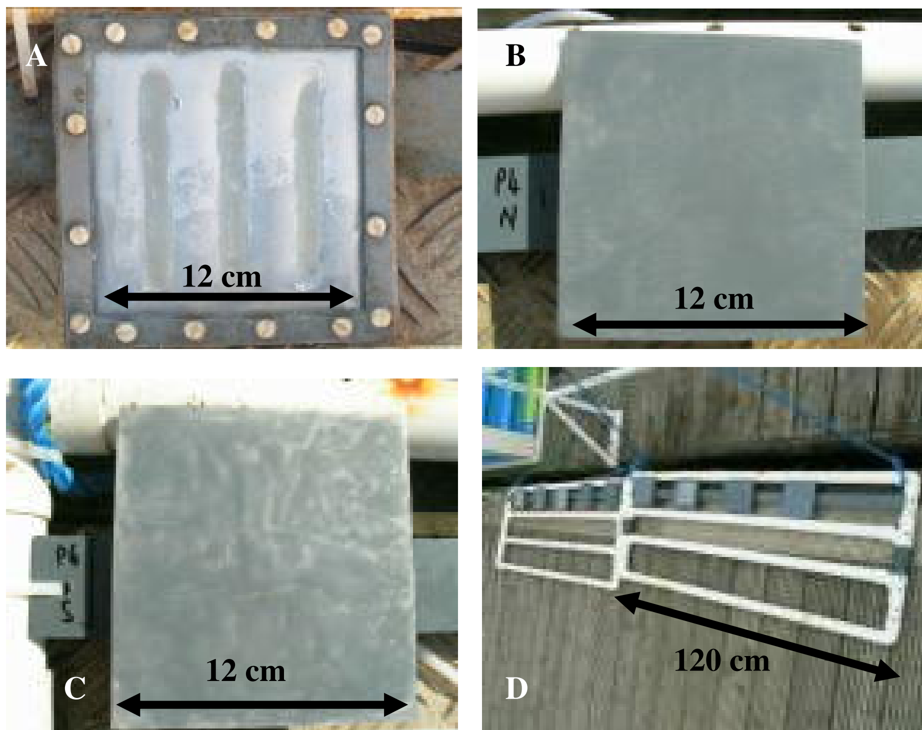

Figure 43. *Top left: Silicone membrane and CO_2 panel, top right: PVC panel, bottom left: a silicone membrane panel and bottom left: the frames that supported the panels.*

During the six month deployment, the panels were checked every two weeks for gas leaks and debris and the CO_2 supply was changed as required. After six months the panels were removed and photographed (Canon G4, 4 x10^6 pixels, RAW file format) and returned to their original position until the end of the experiment in March 2008. The digital photographs were cropped to 11 x 11 cm using Photoshop CS software, which served both to standardise the size of the image and remove a 1 cm wide perimetres from each image to reduce the influence of any edge effects in further analysis (Underwood, 1997). This one centimetre perimetres exclusion also neatly excluded the PVC gaskets which were neither roughened nor permeable to the CO_2. The images were then analysed using ImageJ image analysis software (ImageJ, 2007) by creating a grid arrangement of 100 dots as a layer on each image. The particular species under each dot was identified and used to represent 1 % cover, see Sugden, (2008). Gaps and open space were classed as biofilm.

7.6 - Analysis

Percentage cover data of the fouling species on all panels were arcsine transformed and tested for an effect of treatment type in SPSS version 17 using a general linear model, one way analysis of variance (ANOVA). Ryan-Einot-Gabriel-Welsch F (REGWF) post hoc tests also performed in SPSS version 17 were used to attribute significance of treatment effect. Bray Curtis similarity matrixes were calculated using Primer Software version 6, representing the percentage cover of all species on all settlement panels at six months. These matrixes were analysed using Non Metric Multidimensional Scaling (NMDS) and Analysis of Similarity (ANOSIM) to test for similarity in assemblage composition between panel type. If significant differences were detected by the ANOSIM test, further analysis was conducted with Similarity Percentages (SIMPER) to determine the rank percentage contribution of the different species to the total dissimilarity. In all cases n = 3 for the treatment and control.

7.8 - Results

After six months deployment, clear differences between panel types were present. Multi species fouling assemblages had grown on the PVC and silicone only control panels. A list of all species present on panels of all types is shown in Table 27. Growth was present on the silicone with CO_2 panels, although this was considerably reduced compared to the controls when viewed with the naked eye. Considerable fouling had also grown on the frames supporting the panels (Figure 44).

Table 27. *Species list from all panels excluding biofilm*

Balanus crenatus	Arthropod crustacean
Ectocarpus siliculosus	Filamentous brown algae
Pomatoceros triqueter	Tube dwelling polychaete worm
Bugula flabellata	Erect bryozoan
Membranipora membranacea	Encrusting bryozoan
Ciona intestinalis	Solitary tunicate
Clavelina lepadiformis	Solitary tunicate
Ascidiella aspersa	Solitary tunicate
Botrylloides leachii	Colonial tunicate
Botryllus schlosseri	Colonial tunicate

.

Figure 44. *Panels after 6 month static water immersion trial in Hartlepool Marina. From left to right: Silicone membrane and* CO_2 *panels, PVC panels, and silicone membrane panels. All panels are 10 x 10 cm square.*

Uni-variate analysis of the fouling on the panels showed there was a clear and highly statistically significant (p = 0.002) effect of panel type on the dependant variable; total fouling percentage cover (Table 28). Post hoc testing attributed this panel type effect to the membrane with CO_2 panels (Table 29).

***Table 28**. One way general liner model ANOVA showing the effect of panel type on the total fouling percentage cover after six months static water immersion. All data are arcsine transformed, n=3.*

Source	Type III Sum of Squares	df	$\bar{x}$ Square	F	*P*
Corrected Model	1.13[a]	2	.56	22.57	<0.01
Intercept	12.97	1	12.97	516.92	<0.01
Treatment: panel type	1.13	2	.56	22.57	<0.01
Error	.15	6	.02		
Total	14.26	9			
Corrected Total	1.28	8			

***Table 29**. Post hoc test for the ANOVA above showing the significantly different sub set of variance attributed to the membrane with CO_2 panels.*

	Treatment: panel type	N	Subset	
			1	2
Ryan-Einot-Gabriel-Welsch F[b]	Membrane with_ CO_2	3	0.70	
	Membrane	3		1.39
	PVC_control	3		1.50
	Sig.		1.00	0.43
Ryan-Einot-Gabriel-Welsch Range[b]	Membrane with_ CO_2	3	0.70	
	Membrane	3		1.39
	PVC_control	3		1.50
	Sig.		1.00	.43

Multivariate analysis in the form of MDS plots and ANOSIM tests offer a more refined analytical approach compared to the omnibus; total fouling percentage cover response variable used in the ANOVA. Panel types were analysed by comparing the similarity between

percentage coverage of all the different species present. These tests again show how the fouling assemblages growing on the different panel types are clearly different (Figure 45).

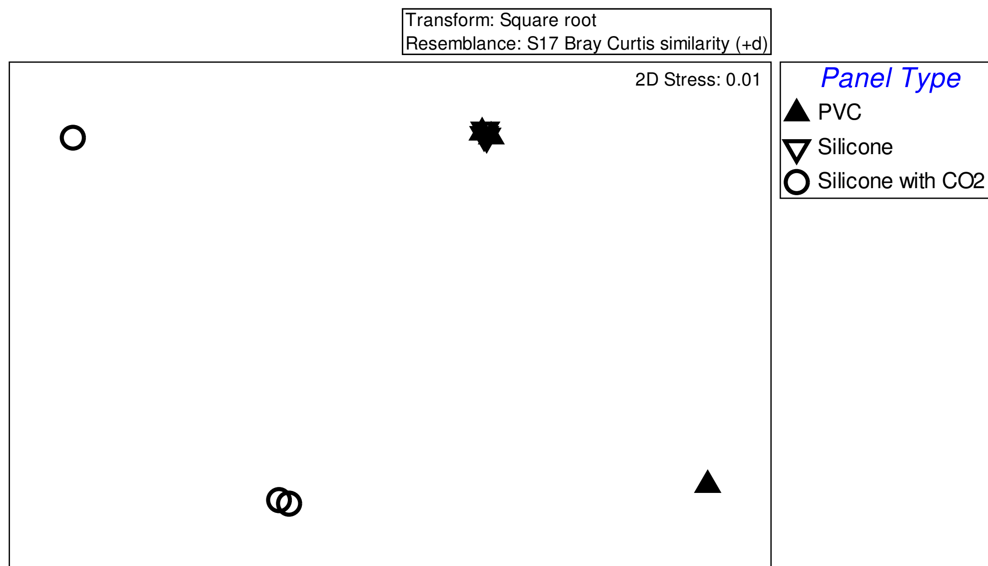

Figure 45. *MDS plot showing similarity between fouling assemblages on all panels after 6 month static water immersion trial in Hartlepool Marina. Data have been arcsine transformed .*

With the exception of one outlier, the PVC and silicone panels support a very similar fouling assemblage structure, while the silicone with CO_2 panels are distinctly different. These findings mirror the total fouling percentage cover response variable measured in the ANOVA. ANOSIM tests of the similarity between assemblages on different panel types confirms that this finding is significantly more likely to occur as a result of the difference between the fouling assemblages on the different panel types than as a result of chance (global R = 0.44, p= 0.04).

SIMPER analysis provides more detailed information relating to the fouling type contributing to the similarity *within* panel types (Table 30) and contributing to the dissimilarity *between* panel types (Table 31).

Table 30. *SIMPER analysis showing the fouling types and their relative contribution to the similarity within each panel group. Av.Abund = Average abundance, Av.Sim = Average similarity, Sim/SD = Similarity standard deviation, Contrib% = Contribution to dissimilarity, Cum.% = Cumulative dissimilarity.*

Panel type: PVC: Average similarity: 75.61

	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Membranipora membranacea	6.14	22.11	16.39	29.24	29.24
Ciona intestinalis	4.41	14.57	3.63	19.27	48.51
Bugula flabellata	3.73	13.93	4.80	18.42	66.94
Biofilm	3.41	11.19	2.56	14.79	81.73
Balanus crenatus	2.10	7.24	3.49	9.58	91.31

Panel type: Silicone: Average similarity: 84.28

	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Membranipora membranacea	5.00	19.68	14.38	23.35	23.35
Ciona intestinalis	4.39	17.30	17.00	20.53	43.88
Bugula flabellata	4.37	16.09	10.15	19.09	62.97
Biofilm	3.05	11.97	25.53	14.20	77.17
Ascidiella aspersa	3.60	10.70	4.58	12.70	89.87
Clavelina lepadiformis	1.52	5.77	17.00	6.84	96.71

Panel type: Silicone with CO_2: Average similarity: 61.79

	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Bugula flabellata	3.37	23.90	3.24	38.68	38.68
Ciona intestinalis	4.02	23.25	7.99	37.63	76.30
Biofilm	1.99	14.64	8.30	23.70	100.00

Table 31. *SIMPER analysis showing the fouling types and their relative contribution to the dis-similarity between each panel type*

Panel types: PVC and Silicone, Average dissimilarity = 26.06

	Contribution to dissimilarity %
Balanus crenatus	16.88
Ascidiella aspersa	16.58
Clavelina lepadiformis	12.15
Botrylloides leachii	11.03
Membranipora membranacea	9.85
Ciona intestinalis	8.80
Biofilm	8.52
Bugula flabellata	6.84

Panel types: PVC & Silicone with CO_2, Average dissimilarity = 42.84

	Contribution to dissimilarity %
Membranipora membranacea	35.24
Balanus crenatus	12.16
Ciona intestinalis	10.67
Ascidiella aspersa	10.07
Biofilm	9.54
Botrylloides leachii	5.94
Pomatoceros triqueter	5.45
Bugula flabellata	4.88

Panel types: Silicone & Silicone with CO_2, Average dissimilarity = 39.76

	Contribution to dissimilarity %
Membranipora membranacea	28.86
Ascidiella aspersa	19.36
Ciona intestinalis	10.88
Botrylloides leachii	9.17
Biofilm	7.28
Bugula flabellata	7.08
Clavelina lepadiformis	6.93
Botryllus schlosseri	5.31

SIMPER analysis in Tables 30 and 31 show that the fouling species that contributes most to the dissimilarity between the two control panel types (PVC and Silicone only) and the silicone membrane with CO_2 was *Membranipora membranecea.* The percentage cover of *Ciona intestinalis* is a consistently high contributor to similarity *within* panel types, and makes a consistent contribution to dissimilarity between panel types at around 10%.

7.9 - Discussion

This study describes the conception and development of a novel, non-toxic antifouling technology. The technology works by exploiting the natural production of carbonic acid that takes place when CO_2 dissolves in seawater, the same reaction that drives ocean acidification. CO_2 can be introduced behind silicone membranes, which due to their polymer structure, are highly permeable to certain gases such as CO_2. As the CO_2 mixes and dissolve with the seawater in the boundary layer above the membrane, the carbonic acid that is formed can reduce the boundary layer pH to around 5.5, as described in the laboratory trial in this Chapter.

The results of the field study demonstrated that the combination of CO_2 and silicone membrane remarkably outperformed either PVC or silicone membrane without the addition of gas, with respect to antifouling performance during a 6 month static water immersion trial. These results look very promising and this technology has many attractive attributes compared to conventional antifouling practices.

Firstly, as mentioned in the introduction, all of the inputs, outputs and by-products of this technique are natural compounds which are nontoxic and occur in the environment, albeit at reduced concentrations. Secondly, large ships such as Very Large Crude Carriers (VLCC) already have onboard inert gas generators to provide different mixes of gasses to add to cargo holds to reduce explosion risks associated with highly volatile cargos. These gas generators combust fossil fuels and could be used together with the existing infrastructure to produce a suitable CO_2 supply.

However, the original conception of this technology involved using scrubbed exhaust emissions from ships main engines and auxiliary generators, thus illuminating the need for additional energy use. Thirdly, although the exact chemical make-up of the silicone polymer membranes used in this study was not determined, many foul release coatings currently on the market are based on variations of organo silicone polymers, often in the form of ploychlorodimethysilohexane (PDMS) (Almeida *et al*., 2007). It is reasonable to assume that there are physical characteristics common to silicone polymers, perhaps with gas permeability being one of them. If this was the case, it would make the large scale production of this novel technology appealing as it does not constitute a vast departure from existing coatings.

While these preliminary trials appear very exciting there are several important factors to consider when evaluating this approach. Firstly, although the silicone membranes with the addition of gas considerably outperformed the other panel types tested here, the other panel types were not designed to have any antifouling capabilities, but were merely acting as controls for the enhanced silicone membranes. This being the case, the performance of the silicone membranes should be evaluated in trials with other coatings specifically designed with antifouling capabilities.

Another important consideration is that these trials were designed to be the preliminary stage to a wider technology development plan, and so were conducted with a "does it work?" attitude, as opposed to a "how does it work?" approach. The result is that the actual pH that the enhanced panels were creating in their boundary layers was not measured in the field. While the majority of the antifouling capability of the CO_2 enhanced panels is attributed to the formation of carbonic acid in the boundary layer, further manipulative experimentation is

required to quantify the boundary layer pH in the field and to test for the influence of uncontrolled factors.

One of these potential un-controlled factors that may have indirectly contributed to the lack of fouling on the CO_2 enhanced panels was the flexing of the membrane. The panels were fixed to a frame, which was in turn attached to a floating pontoon. Any wind movement or movement caused by people walking on the pontoons caused the panels to move up and down in the water column. All panels were fixed at the same height, so any depth variation would have been applied evenly to all panels. However, the effect of slight fluctuations in depth was far more important in the CO_2 enhanced panels. As the gas enhanced panels moved down in the water, the outside pressure increased and the gas was compressed, causing the surface of the membrane to become slightly concave. Once the depth variation has ceased, the membranes would return to their original state.

The influence of this fluctuation in surface morphology is likely to have influenced the antifouling performance of the gas enhanced panels, as this principal of flexible surfaces is similar to that which makes cetacean skins such effective antifouling surfaces (Chambers *et al*, 2006). This surface fluctuation is not considered to be a potential problem with this technique, and it still a result of the introduction of gas beneath a permeable membrane, albeit an indirect one. Further work is needed to separate out the relative influences of the low pH boundary layer and the antifouling effect of flexible membranes. It is also important to state that there are several inherent problems with using membrane based technology as antifouling coatings. These issues centre on low mechanical and abrasion resistance together with technical problems of supplying and maintaining a gas supply to a coating that will be exposed to variable external depth related pressures.

From an ecological perspective, it is interesting that certain species such as *Membranipora membranecea* were notable in terms of it's consistently low abundance on the gas enhanced panels. This is to be expected, given that the species possesses a thin calcium carbonate super-structure, and spreads itself in thin inflexible layers. In this respect, the species could be suspected as being highly susceptible to both the low pH boundary layers created by this technique, as well as the flexible surface effect. From an antifouling coatings point of view, this is interesting as encrusting bryozoans such as *M. membranecea* are classed as problem species and are resistant to many traditional antifouling techniques.

The ability to modify boundary layer pH also has many other exciting ecological applications. The boundary layers created in the laboratory trial for this study were approximately pH 5.5. A boundary layer at this pH is too low to be meaningfully used in relation to ocean acidification studies, however it is well within the range of the pH conditions that are expected to occur in the event of failure, leakage or seepage from CO_2 sequestration sites. In this capacity, it may be possible to use a similar principle to the one developed in this Chapter to understand how recruitment and growth of benthic species may be affected by low pH environments. The membrane itself could be modified by changing its rugosity to make its surface energies and other characteristics more similar to natural substratum, while maintaining its gas permeability. This could be achieved by adhering milled and size graded natural substratum to the membranes surface, or by incorporating sized and powdered substratum in the curing process during the silicone polymer production.

In relation to ocean acidification studies, meaningfully manipulated pH boundary layers could be produced by reducing the diffusion gradient of gas through the membrane. This can

be carried out by reducing the gas pressure or increasing the thickness of the membrane itself or by using a lower pCO_2 gas mix. All of these modifications would result in control over the pH of the boundary layer which would make the technique suitable for ocean acidification experiments. It would be particularly suitable for examining the potential for macro invertebrates to recruit and grow in reduced pH conditions. Perhaps the most exciting application of this technique in an ecological context is the ability to examine the effect of microbial adaptation to low pH environments in the field. So far, the ability of organisms to adapt to OA change is one of the big information gaps that needs to be addressed before the severity of the pH change in the oceans can be evaluated.

This novel method of modifying boundary layer pH *in situ* described in this Chapter has many exciting applications both as a potential non-toxic antifouling strategy and as an ecological tool to asses marine organisms responses to low pH environments. The antifouling capacity is under investigation and this technique has been successfully patented (Appendix 9.4) and steps for further development are underway. The ecological potential of this technique is yet to be fully exploited. This concept is a good example of how there is significant industrial and academic potential to be gained from simple, low tech adaptations of existing technology for specific applications.

Chapter 8

Discussion

8.0 -Methods and Approach

A review of OA literature presented in the first Chapter of this thesis highlighted considerable gaps in what is a comparatively small body of information compared to other global themes in marine biology. This general lack of knowledge stems partly from the fact that OA is a relatively recently discovered phenomenon and partly because conducting experiments to investigate OA is technically challenging in the laboratory, and widely regarded as impractical for a field based approach. As a result, a large percentage of the literature alluding to the potential future impacts of OA is theoretical or conceptual and not based on manipulative experimental data. The inability to collect, analyse and interpret empirical data relating to OA greatly retards our ability to understand the potential impacts of this phenomena using a hypothesis testing, scientific approach.

Specifically, notable gaps in the literature appeared concerning ecological responses of marine organisms to the advance of OA such as recruitment, development and succession. Although crucial to life in the seas, the technology did not exist to investigate these processes in the field, under the influence of lowered environmental pH conditions. The few studies that had attempted to examine these processes were confounded by the many artefacts associated with mesocosms, inappropriate acid treatments or acute exposure periods.

This thesis aimed to make headway in filling the knowledge gaps by conceiving and designing an experimental apparatus that enabled researchers to investigate the ecological responses of OA on marine organisms in the field. The main objective was to use the apparatus designed for this thesis to investigate the *in situ* responses of microbial biofilms, recruiting and established macro invertebrates and algae to low pH conditions that simulate OA, in the field. In addition, the findings of these manipulative experiments would be compared to the only well described field site where marine organisms are naturally exposed to low pH conditions produced by high pCO_2 in seawater, Ischia in Italy (Hall-Spencer *et al*., 2008).

This thesis has been successful at achieving the primary objective. In general, this work has proved that it is possible to partially contain a body of water in a field environment, and manipulate the pH of that water to recreate environmental conditions similar to those which are predicted to occur as a result of OA. This can be accomplished while maintaining equal water exchange and larval supply between treatments and controls, allowing ecological process to be examined whilst the pH is experimentally manipulated.

The settlement panel and hood design of the apparatus, as described in Chapter 2, restricts water movement sufficiently to allow a controlled introduction of CO_2 to be used to change the pH to the desired level. This works in conjunction with a feedback system which maintains the pH of the seawater surrounding the settlement panel. This system is believed to novel at the time of publishing and presents many advantages to the marine scientist investigating the effects of OA.

One major advantage of this method is that the pH controller that manages the feedback system can be adjusted to control the pH anywhere between ambient pH and approximately 5.5. This provides great scope for measuring the effects of OA at pH values that represent a whole range of future scenarios. It must be noted however, that the closer the pre-set value is to the ambient pH, the less stable the pH becomes over long term run times. This is probably attributable to the pre-set functions of the microprocessor in the pH controller which could be developed further to better suit this application of the technology.

During the two week long biofilm experiment described in Chapter three, the controller was able to maintain two discrete pH treatment levels. However, when two treatment levels were attempted for the longer five month experiments described in Chapters 4 and 5, analysis revealed that the treatment level closest to the ambient pH was not significantly different from the ambient pH. The panels exposed to this failed treatment were excluded from further analysis. Another advantage of this method is that a whole range of marine organisms can be investigated to assess their responses to OA. In theory, any organism that can be secured to the settlement panel can be tested. This applies to microbes, macro invertebrates and algae. It may even be possible to use cages containing mobile invertebrates or fishes on scaled up versions of this apparatus.

The morphology of a test organism is an important consideration here however, as development of the technique demonstrated that a steep pH gradient occurs within the hood. Therefore any organisms with a substantial vertical architecture may experience a different pH exposure at the top of its form compared to the bottom.

The concept of partially containing of a body of water around a settlement panel also offers the unique prospect of investigating two or more synergistic effects of climate change on one test assemblage. This same technique could be applied to introduce experimentally manipulated temperature, sediment and nutrient loads. This unique multi-faceted approach provides the prospect of gaining a holistic idea of how marine assemblages will respond to the consequences of living in a high CO_2 world.

As with any experiment, especially one involving novel methods, there are some notable disadvantages or scope for improvement associated with this technique. Firstly, the maintenance involved with this technique is considerable, including the changing of gas cylinders, retrieval of heavily fouled frames onto marinas and water sampling every other day. There is also an ethical issue concerning the injection of CO_2 directly into the sea, although the importance of the results of these experiments is deemed by this author, to be worth the environmental costs.

Another disadvantage of this method is the variability of the pH treatment caused by the constant cycle of water entering the hoods, being treated with the influx of CO_2, and then mixing with the ambient sea water causing the pH to increase once more. This cycle is unavoidable in this design, but with increased understanding of the local hydrodynamics around the panel and hood, the effect of this variation could be minimised.

The use of baffles on the edge of the hood may further restrict water movement resulting in less mixing which in-turn would require less gas to create the desired low pH effect. This could be one option that might smooth the internal pH profile around the settlement panel. Early prototype chambers incorporating baffles were trialled but initially rejected as the

reduced flow appeared to induce flocculation of sediments on the settlement panel, introducing an un-controlled variable into the experimental design. Further experimentation with different designs of baffles could produce a balance between pH stability and flocculation.

The differences between low pH and control panels have been attributed directly to the low pH effect generated by the novel experimental apparatus. Although the apparatus successfully fulfilled its design brief by creating and maintaining low pH micro-climates in the field, it is possible that other environmental gradients that were not measured by this study may have influenced the outcomes described in this Chapter. Light, temperature, nutrient flux and larval density were not directly measured, but they were also not intentionally manipulated. As the experiment was conducted in a randomised block design with all materials and distances kept homogeneous between treatment and control panels, the effect of any natural heterogeneous variability in these factors would have been minimised.

The cost, time and man power required to operate and maintain the system used in the study is not trivial. As a result n = 3 with respect to treatment and control settlement panels. With such limited statistical power, any significant results can be considered as strong effects. The downside is there is a considerable likelihood that other subtle, yet important results may have been cast aside as type II errors. It must be also considered that all conclusions based on the low pH panels are drawn from a combined mere 432 cm^2 of settlement panel. It would be unwise to extrapolate ecosystem wide responses from such limited spatial analyses. It is hoped however that these findings can form the basis of future work which will further enhance the technique developed here, and explore the ecological relationships resulting from low pH exposure detected in this investigation.

In defence of the method applied in this thesis, it was designed to simulate OA, a process in which atmospheric CO_2 dissolves in surface seawaters. The rate of this dissolution is also variable and is affected by factors such as wind speed, wave height and temperature. A degree of variation in pH treatment is therefore acceptable, although the range and rate of fluctuation permissible is open to debate.

Although not a direct fault in the method, this technique relies on the long-term deployment of pH probes in the field environment. This is problematic due to the potential for pH probes to drift between calibrations, and also as a result of fouling on the probe surface. Although calibration and cleaning of the probes was conducted every two days during this study, the potential for drift in the readings to alter the outcome of these experiments is high, as input of CO_2 into the chambers is controlled by the readings produced by the pH probes.

Drift in pH readings occurred in the experiments described by Chapters 4 and 5. This drift is attributed to a problem with the pH probe used for analysing the water samples. Both the pH controller probes and data logger did not show the same drift and provided consistent readings with regular calibration. The pH probe used for analysing the water samples was also calibrated at the same frequency (every two days before use) but showed a gradual drift after the 4th of July. This malfunction was not picked up during the experiment as pH data from the water samples were not fully analysed until the end of the experiment. The discretion between treatments after the 4th of July is subject to uncertainty, so separate analysis of pH data prior to this point was conducted to provide confidence in the response variables. The problems associated with long-term deployment of pH probes also made the use of *in situ* pH logging in each chamber prohibitively expensive in this instance.

Another potential source of error was introduced into this design because all three low pH hood and panel pairs were controlled by one feedback loop. The loop was controlled by a single reference pH probe in one of the three hoods. It is very likely that the local hydrodynamics surrounding each hood and panel were not totally homogeneous meaning that the introduction of CO_2 into the chambers may have been required at slightly different times in order to achieve the smoothest possible internal pH profile. Ideally, each low pH hood and panel pair would have been controlled by its own independent feedback loop, producing tighter control of the internal pH. Although independence of control would assist in reducing the variability in the pH treatments, it would also introduce considerably more complexity and cost to the method.

This area of marine science is expanding rapidly and several major steps forward have occurred since starting this thesis which has helped to steer and consolidate the research effort investigating OA. One of the most important is the book produced by EPOCA (Guide for Best Practices in Ocean Acidification Research and Data Reporting. EPOCA, France) which presents a set of guidelines and instructions aimed to assist researchers to perform correct practice when measuring aspects of carbonate chemistry relating to OA experiments. This guidance is very helpful in increasing transferability of results in a rapidly developing field, but unfortunately was not available at the outset of this project. Although not specifically dedicated to field pH manipulative research, this document would have benefited this project by presenting a clear procedure for analysis of water samples with respect to carbonate chemistry parametress, ensuring standard practice was adhered to.

8.1 - Biological Response: Microbial

The overwhelming impression generated when reviewing the results of this work is that the task of understanding how marine organisms will to response OA is far from complete. Considerable further investigation is needed to verify the conclusions arrived at in this thesis. The latter is not a failing of this work, but merely a reflection of the complexity of the issues being addressed here, together with the lack of similar studies with which to compare and contrast the results. No strong microbial response to low pH conditions was detected during the short term biofilm study conducted at Hartlepool (Chapter 3). This lack of response was echoed by the microbial diversity patterns at the vent site, Ischia, Italy. These results are surprising but consistent across different spatial and temporal scales.

In Hartlepool, although the ambient pH is prone to fluctuation, the deployment of the experimental apparatus immediately created what were effectively micro climates of low pH water that existed for two weeks. In contrast, the vent site at Ischia has been subjected to low pH conditions for at least 100 years (Tedesco, 1996). A rational behind the results observed in this study is that the micro flora that established during the Hartlepool experiment was early generalist, opportunist colonisers, inherently tolerant of variable environmental gradients. In contrast, the biofilm assemblage detected in the low pH Ischia samples came from surrounding populations where low pH tolerant species had been established for many years and had become adapted to life in high CO_2 conditions.

Although this is a speculative suggestion, it is creditable, as long term exposure to low pH conditions is likely to induce genetic adaptation in microbes due to their considerable and rapid adaptive potential. Complete and thorough sequencing of the microbes from high and

low pH sites at Ischia would provide evidence to test this speculation, as would conducting longer term trials of the same type at Hartlepool.

Other significant differences exist between the Hartlepool and Ischia studies which makes comparison between the two difficult. The duration (four weeks Ischia, two weeks Hartlepool) depth (1-4 m Ischia, 30 cm Hartlepool) and the difference between the treatment pH and ambient pH (up to 1.57, Ischia, 0.2, Hartlepool) all varied between sites.

Because of the importance of microbial activity in biogeochemical cycles, it is vital this area of work be advanced to understand how OA will affect these global life sustaining process. It is the recommendation of this study that similar methods are used to the one described in this thesis, but with experiments that are conducted in fully marine sites, not in marinas. Performing similar experiments in sites where the control pH is more stable will make the controls more stable compared to the treatments, which will increase the chance of detecting differences between treatments and controls.

8.2 - Biological Response: Macro

The response of established macro invertebrate and algal assemblages to low pH conditions differed considerably from that of recruiting assemblages. Twenty-four month old assemblages appeared largely unaffected by five months exposure to a reduction in environmental pH of 0.3 of a unit, whereas assemblage structure in recruiting assemblages was significantly altered during month two by a reduction in environmental pH of 0.2 of a unit. This variation of susceptibility to low pH conditions may be simply due to increased age and size of individuals in the established assemblages. Adulthood may present organisms

with more metabolic leeway to deal with environmental stresses as a result of reduced metabolic activity dedicated to growth when compared with juveniles.

These findings suggest that established assemblages in temperate marinas are unlikely to be affected by low levels of OA. However, un-colonised substrata created by disturbance, sea level rise or death of established individuals, may exhibit different assemblage structure under early stages of development as a result of OA. The wider ecological implications of a change in developing assemblage structure remain unknown. Importantly, these conclusions are based on results of five month trials. The longer term exposure that will occur with the progression of OA may induce very different results.

It is surprising that recruitment success, which is known to be largely a chemically mediated process, did not appear affected by the considerable change in chemistry produced by 0.2 of a unit decreases in pH. There were, however, other factors that influenced recruitment in this study which may have masked the effect of pH alone.

8.3 - Final Appraisal

This study has made three significant contributions in the effort to understand the effects of OA on marine assemblages. Firstly, the possibility now exists to experimentally manipulate the pH of a partially contained body of water in the field, and measure biological responses of organisms residing within that body of water. Secondly, this work has demonstrated that changes in assemblage structure are likely to occur as a result of OA of 0.2 units, although these changes may be limited to early stages of succession. Thirdly, the technology described in Chapter 7 presents an example of alternative methods that can be applied to help address major knowledge gaps associated with the response of marine organisms to OA.

It is hoped that the conceptual, methodological and experimental advances reported in this thesis will provide marine scientists with fresh expectations and scope to advance this new area of field based OA experimentation.

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Appendix 9.1

Table 32. Raw data from *in situ* logger monitoring ambient water parametress in Hartlepool marina 30 cm from settlement panels

Salinity ppt	DO mg/l	Temp ^{0}C	pH
34.99	9.84	11.33	8.21
35.00	9.86	11.33	8.7
35.00	9.71	11.33	8.32
35.00	9.68	11.33	8.35
35.00	9.63	11.32	8.46
34.99	9.68	11.32	8.5
34.99	9.55	11.32	8.6
34.99	9.44	11.32	8.47
34.99	9.48	11.32	7.81
34.98	9.75	11.32	8.07
34.99	9.76	11.32	8.53
35.00	9.71	11.32	8.43
34.98	9.57	11.32	8.26
34.99	9.54	11.32	8.2
34.99	9.46	11.32	8.3
34.99	9.67	11.32	8.28
34.99	9.66	11.32	8.37
34.99	9.50	11.32	7.85
34.98	9.53	11.31	8.94
34.98	9.49	11.32	8.23
34.99	9.54	11.31	8.64
34.99	9.67	11.31	8.11
34.99	9.64	11.32	8.56
34.99	9.91	11.32	8.54
34.99	9.89	11.32	8.3
34.99	9.82	11.32	8.36
34.99	9.81	11.32	8.34
34.98	10.27	11.32	8.36
34.99	10.21	11.32	8.44
34.99	9.94	11.32	8.43
34.99	10.05	11.32	8.37
34.99	10.15	11.32	8.39
34.99	9.91	11.31	8.38
34.98	9.89	11.32	8.42
34.99	9.90	11.31	8.42
34.99	9.94	11.30	8.42
34.99	9.98	11.30	8.39
34.99	10.32	11.31	8.39
34.99	10.23	11.31	8.39
34.99	10.64	11.31	8.37
34.99	10.75	11.31	8.36
34.99	10.48	11.31	8.41
34.99	10.36	11.31	8.42
34.99	10.46	11.31	8.37
34.99	10.38	11.32	8.38
34.99	10.30	11.31	8.35
34.99	10.42	11.31	8.33
34.99	10.41	11.31	8.36
34.99	10.85	11.31	8.35
34.99	10.67	11.31	8.37
34.99	10.63	11.31	8.37

Salinity ppt	DO mg/l	Temp ^{0}C	pH
34.99	10.43	11.31	8.34
34.99	10.27	11.31	8.31
34.99	10.40	11.31	8.4
34.99	10.61	11.31	8.42
34.99	10.37	11.31	8.51
34.98	10.26	11.31	8.22
34.99	10.39	11.31	8.21
34.99	10.47	11.31	8.4
34.99	10.42	11.31	8.33
34.99	10.57	11.31	8.15
34.99	10.45	11.31	8.19
35.00	10.37	11.31	8.53
34.99	10.43	11.31	8.39
34.99	10.52	11.31	7.83
34.99	10.40	11.31	8.78
34.99	10.29	11.31	7.9
34.99	10.51	11.31	8.04
34.99	10.45	11.31	8.46
34.99	10.31	11.31	8.53
35.01	10.37	11.31	8.41
34.99	10.36	11.31	7.75
34.99	10.28	11.31	8.01
34.99	10.20	11.31	8.48
35.00	10.25	11.31	8.71
34.99	10.20	11.31	8.29
35.00	10.18	11.31	8.21
35.00	10.09	11.31	8.1
35.00	10.27	11.31	8.12
34.99	10.05	11.31	8.03
35.00	9.94	11.31	7.98
35.00	10.12	11.32	8.26
34.99	9.90	11.31	7.93
35.00	9.64	11.31	8.29
35.00	9.85	11.31	8.24
35.00	9.81	11.32	8.23
34.99	9.89	11.31	8.41
35.00	9.93	11.31	8.44
35.00	9.90	11.31	8.22
34.99	9.90	11.31	8.17
35.00	10.03	11.31	7.96
34.99	10.21	11.31	8
34.99	9.98	11.31	8.39
34.99	9.90	11.31	8.06
35.00	9.88	11.30	8
34.99	9.71	11.29	8.27
34.99	9.66	11.30	8.07
34.99	9.68	11.30	8.13
34.98	9.97	11.30	7.87
34.99	10.01	11.30	8.44
34.99	10.01	11.30	8.6
34.99	9.91	11.30	8.52
34.99	9.86	11.30	7.79
35.00	9.84	11.31	8.35
34.99	9.77	11.31	8.15
34.99	9.69	11.31	7.98
34.99	9.81	11.31	8.26
35.00	9.65	11.32	8.56

Salinity ppt	DO mg/l	Temp ^{0}C	pH
34.99	9.68	11.31	8.1
34.99	9.57	11.31	8.24
35.00	9.51	11.31	8.08
35.00	9.46	11.31	8.27
34.99	9.40	11.31	7.72
34.99	9.33	11.32	8.26
35.00	9.20	11.31	8.56
34.99	9.19	11.31	8.32
35.00	9.25	11.31	8.6
35.00	9.96	11.31	8.37
34.99	9.94	11.31	8.33
35.00	10.27	11.32	8.34
35.00	10.13	11.31	8.29
35.00	10.04	11.32	8.02
35.00	10.07	11.32	8.07
35.00	9.65	11.31	8.03
35.00	9.71	11.31	8.24
35.00	9.66	11.32	8.1
35.00	9.71	11.32	7.98
35.00	9.93	11.31	7.97
35.00	9.96	11.31	7.84
34.99	10.00	11.31	7.64
35.00	9.93	11.31	8.56
35.00	9.85	11.30	7.54
34.99	9.66	11.30	7.64
35.00	9.45	11.31	8.37
35.00	9.33	11.30	8.29
35.00	9.34	11.30	8.3
35.00	9.25	11.29	7.93
35.00	9.32	11.30	8.09
35.00	9.13	11.30	8.18
35.00	9.32	11.29	7.98
35.00	9.41	11.30	7.91
34.99	9.29	11.29	8.08
34.99	9.39	11.29	8
35.00	9.35	11.30	7.99
35.00	9.40	11.30	8.01
34.99	9.66	11.30	8.04
34.99	9.79	11.30	7.98
34.99	9.82	11.30	8.08
34.99	9.87	11.30	8.18
34.99	9.78	11.30	7.91
34.99	9.91	11.30	8.26
34.99	9.85	11.30	7.62
34.99	9.65	11.30	7.95
34.99	10.03	11.44	8.26
35.00	10.03	11.45	8.15
35.00	9.93	11.44	8.06
34.99	9.91	11.45	8.09
34.99	10.14	11.45	8.02
35.00	10.05	11.45	8.05
34.99	9.88	11.45	7.68
35.00	9.83	11.45	7.68
34.99	9.87	11.46	7.73
34.99	9.88	11.46	7.66
34.99	9.91	11.46	7.59
34.99	9.78	11.46	7.6

Salinity ppt	DO mg/l	Temp ^{0}C	pH
34.99	9.76	11.46	7.98
35.00	9.82	11.46	8.02
34.99	10.08	11.46	8.13
34.99	10.03	11.46	8.04
34.99	9.95	11.46	8.1
34.99	10.00	11.46	8.07
34.99	9.84	11.45	8.14
34.99	9.65	11.45	8.1
34.99	9.95	11.45	7.86
35.00	9.98	11.45	8.24
35.00	9.97	11.46	8.22
34.99	10.23	11.46	8.05
34.99	10.01	11.46	7.62
34.94	9.94	11.45	7.8
34.79	9.85	11.45	8.07
34.65	9.95	11.45	8.19
34.67	9.80	11.45	8.16
34.65	9.79	11.45	8
34.73	9.91	11.46	8.14
34.64	9.92	11.46	8.01
34.86	9.77	11.46	8.15
34.64	9.63	11.46	8.17
34.68	9.81	11.46	7.98
34.63	9.77	11.46	8.08
34.88	9.71	11.46	8.07
34.69	9.81	11.46	7.99
34.98	9.69	11.46	8.01
34.99	9.81	11.46	8.09
34.99	9.71	11.46	8.11
34.99	9.56	11.46	8.12
34.99	9.92	11.46	8
34.99	9.69	11.46	8.17
34.99	9.63	11.46	8.16
34.99	9.58	11.46	8.04
34.99	9.54	11.46	8.16
34.99	9.73	11.46	8.15
34.99	9.71	11.46	8.15
34.99	9.71	11.46	7.96
34.99	9.56	11.46	7.95
34.99	9.47	11.46	8.14
34.98	9.44	11.46	7.98
34.98	9.35	11.46	8.05
34.98	9.48	11.46	8.07
34.98	9.49	11.46	8.18
34.98	9.30	11.46	7.77
34.98	9.47	11.46	7.82
34.98	9.53	11.46	8.31
34.98	9.42	11.46	7.74
34.98	9.32	11.46	8.16
34.98	9.29	11.46	8.1
34.98	9.58	11.46	7.93
34.98	9.60	11.47	8.04
34.98	9.81	11.47	7.99
34.98	9.88	11.47	8.03
34.98	9.68	11.47	7.97
34.98	9.58	11.47	8.06
34.98	9.57	11.47	8.05

Salinity ppt	DO mg/l	Temp ^{0}C	pH
34.98	9.66	11.48	8
34.98	9.64	11.48	8.08
34.98	9.85	11.48	8.03
34.99	9.77	11.49	8.09
34.98	9.91	11.49	8.04
34.99	9.80	11.49	8
34.98	9.85	11.49	7.96
34.99	9.89	11.50	7.99
34.99	9.67	11.50	8.09
34.98	9.53	11.50	7.98
34.98	9.68	11.50	7.97
34.98	9.91	11.50	7.98
34.98	9.91	11.50	7.99
34.98	9.88	11.51	8.05
34.99	9.98	11.51	7.92
34.99	10.09	11.51	7.96
34.98	9.98	11.51	7.8
34.98	10.02	11.51	8.05
34.98	9.90	11.51	7.93
34.98	9.84	11.52	8.17
34.98	9.93	11.52	8.02
34.98	10.03	11.52	8.02
34.98	9.90	11.52	8.15
34.98	9.87	11.52	8.19
34.98	9.77	11.52	8.16
34.98	9.72	11.52	8.08
34.98	9.82	11.52	8.1
34.98	9.86	11.51	7.9
34.98	9.89	11.52	7.89
34.98	9.80	11.52	7.89
34.98	9.56	11.52	7.8
34.98	9.38	11.52	8.11
34.98	9.52	11.52	8.28
34.99	9.46	11.52	8.3
34.98	9.30	11.53	7.91
34.98	9.36	11.52	7.93
34.98	9.39	11.52	8.06
34.98	9.62	11.52	7.96
34.98	9.78	11.52	8.06
34.98	9.59	11.52	8.07
34.98	9.54	11.52	7.98
34.98	9.66	11.52	8.08
34.98	9.67	11.52	8.06
34.98	9.58	11.52	8.03
34.98	9.55	11.52	8.05
34.98	9.45	11.52	7.99
34.98	9.23	11.52	7.89
34.99	9.06	11.52	8.05
34.99	9.34	11.52	7.85
34.99	9.19	11.52	8.15
34.99	9.16	11.52	8.17
34.98	9.14	11.52	8.14
34.98	9.17	11.52	7.93
34.98	9.38	11.52	8.06
34.98	9.59	11.52	7.89
34.98	9.50	11.52	7.76
34.99	9.50	11.52	8.05

Salinity ppt	DO mg/l	Temp ^{0}C	pH
34.99	9.63	11.52	8.06
34.98	9.48	11.52	7.86
34.98	9.24	11.52	8.05
34.99	9.34	11.52	7.88
34.99	9.49	11.53	7.97
34.99	9.28	11.43	8.01
34.99	9.11	11.45	7.97
34.99	9.08	11.45	7.99
34.99	9.48	11.47	8.05
34.99	9.66	11.48	8.04
34.99	9.53	11.47	8.01
34.99	9.53	11.46	8.05
34.99	9.41	11.46	7.98
34.99	9.59	11.46	7.94
34.99	9.45	11.46	8.04
34.99	9.43	11.46	8.02
34.99	9.39	11.47	7.93
34.99	9.41	11.47	8.02
34.99	9.44	11.48	7.96
34.99	9.61	11.48	8.01
34.99	9.48	11.51	8.03
34.99	9.76	11.52	8.02
34.99	9.87	11.52	8.02
34.99	9.64	11.52	8.03
34.99	9.91	11.52	8.03
34.99	9.97	11.52	7.97
34.99	9.94	11.52	7.97
34.99	9.99	11.52	7.97
34.99	9.89	12.22	8
34.99	9.66	12.22	8.08
34.99	9.59	12.22	8.07
34.98	9.92	12.22	8.23
34.99	9.77	12.22	8.36
34.99	9.74	12.22	8.07
34.99	9.79	12.22	8.22
34.99	9.62	12.22	8.17
34.99	9.52	12.22	8.35
34.99	9.89	12.22	8.07
34.98	10.29	12.22	8.25
34.98	9.97	12.22	8.15
34.98	9.96	12.22	8.15
34.97	10.05	12.22	8.09
34.98	10.08	12.22	8.14
34.97	10.10	12.21	8.15
34.97	10.01	12.22	8.18
34.98	9.99	12.22	8.18
34.98	10.67	12.21	8.12
34.98	10.46	12.22	8.13
34.98	10.35	12.22	8.14
34.98	10.32	12.22	8.19
34.98	9.96	12.22	8.05
34.98	9.91	12.22	8.07
34.98	10.26	12.22	8.2
34.98	9.91	12.21	8.32
34.97	9.96	12.21	8.03
34.97	9.69	12.21	8.15
34.98	9.56	12.21	8.16

Salinity ppt	DO mg/l	Temp ^{0}C	pH
34.98	9.37	12.21	8.16
34.97	9.53	12.21	8.35
34.97	9.58	12.22	7.98
34.97	9.76	12.21	7.95
34.98	9.62	12.21	8.35
34.97	9.35	12.21	7.9
34.97	9.25	12.20	8.08
34.98	9.19	12.20	8.42
34.98	9.20	12.21	8.2
34.98	9.54	12.21	8.21
34.98	9.66	12.21	7.93
34.98	9.92	12.21	7.87
34.98	10.07	12.21	8.27
34.98	10.18	12.20	8.2
34.98	10.00	12.20	8.23
34.98	10.01	12.20	7.92
34.98	10.05	12.20	7.99
34.98	9.95	12.20	8.1
34.98	10.33	12.20	8.14
34.98	10.16	12.20	8.14
34.98	10.21	12.19	7.94
34.98	10.31	12.20	8.03
34.98	10.18	12.19	8.02
34.98	10.01	12.20	8.3
34.98	10.00	12.19	8.14
34.98	10.09	12.20	7.99
34.98	9.88	12.19	8.25
34.97	9.94	12.19	8.22
34.98	10.21	12.19	8.36
34.97	10.65	12.18	8.22
34.97	10.26	12.18	8.07
34.97	10.20	12.18	8.4
34.97	10.01	12.17	7.71
34.97	10.34	12.18	7.68
34.98	10.22	12.18	8.23
34.97	9.85	12.17	8.28
34.98	10.08	12.18	8.1
34.97	10.27	12.18	8.07
34.97	10.97	12.18	7.78
34.98	10.38	12.17	8.28
34.98	10.44	12.18	8.16
34.99	10.10	12.17	8.13
34.99	10.20	12.18	7.83
34.98	10.91	12.17	8.32
34.98	10.42	12.17	7.92
34.99	10.05	12.17	8.33
34.98	9.86	12.17	7.91
34.98	9.99	12.17	8.03
34.98	10.21	12.17	8.07
34.99	10.11	12.16	7.98
34.99	10.00	12.16	7.82
34.99	10.15	12.16	7.96
34.98	10.52	12.16	7.83
34.99	10.52	12.16	7.81
34.98	10.76	12.16	7.81
34.98	10.42	12.16	7.86
34.98	10.10	12.15	7.84

Salinity ppt	DO mg/l	Temp ^{0}C	pH
34.98	10.19	12.15	7.88
34.99	10.46	12.14	7.86
34.98	10.38	12.14	7.83
34.98	10.13	12.14	7.78
34.98	10.13	12.13	7.77
34.98	10.11	12.13	7.71
34.98	10.08	12.13	7.55
34.98	10.10	12.13	7.82
34.98	10.25	12.12	7.98
34.98	10.22	12.12	7.83
34.98	10.08	12.12	7.95
34.98	10.04	12.12	7.95
34.97	10.10	12.12	7.89
34.97	10.47	12.12	8.02
34.97	10.30	12.12	8.01
34.98	10.26	12.12	8.11
34.98	10.17	12.12	7.8
34.98	10.50	12.12	7.97
34.98	10.53	12.11	7.99
34.98	10.44	12.11	7.98
34.98	10.17	12.11	7.98
34.98	10.14	12.11	7.9
34.98	10.29	12.10	7.96
34.98	10.24	12.10	8.01
34.98	10.58	12.10	8.01
34.97	10.16	12.10	7.8
34.98	10.09	12.10	7.83
34.98	10.22	12.10	7.89
34.98	9.75	12.10	8.02
34.98	9.69	12.09	7.92
34.98	9.83	12.10	7.97
34.98	9.74	12.10	7.84
34.97	9.58	12.10	8.06
34.98	9.64	12.09	8.15
34.98	9.47	12.09	7.79
34.98	9.47	12.09	8.24
34.98	9.51	12.09	8.21
34.97	10.06	12.09	7.76
34.98	10.41	12.09	7.82
34.99	10.11	12.08	8.06
34.98	9.72	12.08	8.03
34.99	9.60	12.08	7.67
34.99	9.53	12.08	8.05
34.99	9.56	12.08	7.8
34.99	9.66	12.08	7.98
34.99	10.07	12.07	7.87
34.99	10.11	12.07	7.85
34.99	10.16	12.07	7.89
34.99	10.18	12.07	7.8
34.99	10.29	12.07	8.05
34.99	10.10	12.07	8.04
34.99	9.99	12.07	7.98
34.99	9.73	12.06	8.07
34.99	9.64	12.06	7.88
34.99	9.62	12.06	8.15
34.99	9.67	12.06	7.95
34.99	9.68	12.05	7.66

Salinity ppt	DO mg/l	Temp ^{0}C	pH
35.00	10.15	12.05	7.76
35.00	10.39	12.05	8.04
34.99	10.34	12.05	7.92
34.99	9.93	12.05	7.76
34.99	9.52	12.05	8.15
34.99	9.45	12.05	7.79
34.99	9.66	12.05	7.8
35.00	9.82	12.05	7.93
35.00	10.71	12.05	7.97
34.99	10.50	12.05	8.03
35.00	10.77	14.60	7.93
35.00	10.72	14.60	7.74
35.00	10.21	14.60	7.76
35.01	10.45	14.60	8.27
35.01	9.86	14.60	8.22
35.01	9.72	14.60	7.93
35.02	9.69	14.60	8.18
35.01	9.42	14.60	8.07
35.02	9.21	14.60	7.92
35.02	8.99	14.60	8.2
35.01	8.95	14.60	8.11
35.01	8.92	14.59	7.81
35.00	9.23	14.59	7.9
35.01	9.05	14.59	8.04
35.01	8.89	14.58	7.98
35.00	8.80	14.58	7.98
35.01	8.77	14.58	7.88
35.00	9.24	14.58	7.89
35.01	9.63	14.58	8.02
35.00	9.68	14.58	8.11
35.00	9.54	14.58	7.97
35.01	9.29	14.58	7.91
35.01	9.33	14.57	7.99
35.01	9.89	14.57	7.95
35.01	9.92	14.58	7.9
35.01	10.17	14.58	8
34.96	9.82	14.58	7.88
34.90	9.87	14.58	7.73
34.88	9.83	14.58	7.82
34.89	9.94	14.57	7.86
34.89	9.63	14.58	7.87
34.92	9.65	14.58	7.91
34.92	10.21	14.58	7.99
34.92	9.76	14.58	8.37
34.95	9.42	14.57	8.09
34.96	9.23	14.57	7.96
34.96	9.74	14.57	7.92
34.96	9.85	14.58	7.91
34.97	10.29	14.57	8.04
34.98	10.00	14.57	7.9
34.99	10.12	14.57	7.93
34.99	9.85	14.57	7.93
34.99	10.06	14.57	7.92
34.99	9.64	14.57	7.94
34.99	9.39	14.56	8.04
34.99	9.32	14.56	7.89
35.00	9.16	14.56	8.13

Salinity ppt	DO mg/l	Temp ^{0}C	pH
35.00	9.91	14.57	7.66
35.00	9.56	14.56	7.66
35.00	9.40	14.55	8.29
35.00	9.28	14.54	8.11
34.99	9.23	14.54	8.13
34.99	9.22	14.53	7.7
35.00	9.32	14.53	7.83
35.00	9.31	14.52	8.2
35.00	9.49	14.52	8.05
35.00	9.53	14.52	7.82
34.99	9.32	14.52	7.86
34.99	10.12	14.52	8
35.00	9.67	14.52	7.85
35.00	9.74	14.52	7.86
35.00	9.67	14.52	8.07
34.99	9.31	14.52	7.81
35.00	9.35	14.52	7.74
35.00	9.31	14.52	7.78
34.99	9.51	14.52	7.75
35.00	9.25	14.51	7.85
35.00	8.99	14.52	7.57
35.00	8.76	14.51	7.88
35.00	8.91	14.52	8.19
35.00	9.83	14.52	8.1
34.99	9.91	14.52	7.85
34.99	9.74	14.52	7.86
35.00	9.56	14.52	7.72
35.00	9.85	14.51	8.08
34.99	9.59	14.51	7.85
35.00	9.66	14.51	7.98
34.99	9.61	14.51	7.99
35.00	9.57	14.51	7.78
35.00	9.77	14.51	7.96
34.99	9.54	14.51	7.92
34.99	9.24	14.51	7.89
35.00	9.35	14.51	8.01
34.99	9.84	14.52	7.91
34.99	9.93	14.52	7.92
34.99	9.88	14.52	7.97
34.99	9.70	14.53	7.98
34.99	9.33	14.53	7.9
34.99	9.47	14.53	8.06
34.99	9.33	14.53	8
34.99	9.41	14.52	7.88
34.99	9.26	14.52	8.04
34.99	9.51	14.53	8.02
34.99	9.71	14.53	8
34.99	9.67	14.54	7.89
34.99	9.64	14.53	8.01
34.99	9.60	14.52	7.95
35.00	9.50	14.52	7.95
34.99	9.29	14.53	7.99
35.00	9.00	14.51	8.34
34.99	8.86	14.52	8.28
35.00	8.75	14.52	7.85
34.99	8.78	14.53	7.91
34.99	8.92	14.54	7.7

Salinity ppt	DO mg/l	Temp ^{0}C	pH
34.99	9.09	14.52	7.86
34.99	8.86	14.54	7.78
35.00	9.10	14.53	8.11
34.99	9.04	14.51	7.76
34.99	9.18	14.52	7.85
34.99	8.97	14.52	7.88
34.99	9.00	14.52	7.86
34.99	9.19	14.53	7.98
34.99	9.56	14.52	7.89
34.95	9.72	14.51	7.89
34.95	9.48	14.52	7.87
34.94	9.60	14.53	8.03
34.94	9.49	14.52	7.94
34.95	9.27	14.52	8.04
34.96	9.18	14.52	7.91
34.97	9.31	14.52	8
34.97	9.28	14.52	7.92
34.95	9.61	14.52	7.94
34.95	9.53	14.51	8.06
34.96	9.67	14.53	7.91
34.96	9.65	14.52	7.99
34.95	9.89	14.53	8.31
34.94	9.84	14.52	8.09
34.92	9.82	14.54	8.1
34.93	9.84	14.54	7.84
34.93	9.85	14.53	7.82
34.94	9.89	14.54	7.97
34.97	9.91	14.54	7.88
34.92	10.07	14.53	8.07
34.97	10.07	14.54	8.14
34.95	9.99	14.54	8.13
34.97	10.16	14.54	7.87
34.98	10.79	14.54	7.81
34.98	10.14	14.53	7.81
34.98	9.83	14.55	7.76
34.98	10.55	14.55	8.01
35.04	11.23	14.54	7.91
35.06	10.74	14.54	8.11
35.07	10.32	14.55	8.22
35.05	10.32	14.54	8.08
35.04	10.45	14.54	8.11
35.01	10.17	14.53	7.76
35.02	10.00	14.54	7.63
35.00	10.04	14.56	8.03
35.00	10.17	14.55	8.09
35.00	9.99	14.54	8
35.01	9.84	14.54	8.05
35.00	9.77	14.52	7.95
35.00	9.66	14.53	8.01
35.00	9.70	14.52	7.98
35.00	10.26	15.75	8.03
35.00	9.99	15.75	8.09
35.00	9.82	15.75	8.21
35.00	9.82	15.74	7.83
35.00	9.71	15.74	7.97
35.00	9.70	15.74	8.08
35.00	9.71	15.75	8.1

Salinity ppt	DO mg/l	Temp ^{0}C	pH
34.99	9.66	15.75	8.01
35.00	9.72	15.75	8.07
35.00	9.74	15.75	8.05
34.99	9.50	15.75	8.07
34.99	9.63	15.75	8.1
34.99	9.87	15.75	8.09
34.99	10.18	15.75	8.07
34.99	9.86	15.75	7.85
35.00	9.88	15.75	8.18
34.99	9.97	15.75	8.15
34.98	10.24	15.75	7.68
34.99	10.21	15.75	8.16
34.98	10.52	15.75	8.28
34.98	10.26	15.75	7.95
34.98	10.57	15.75	7.63
34.98	10.34	15.75	8.18
34.97	9.96	15.75	7.99
34.98	10.17	15.75	8.29
34.98	10.16	15.74	8.21
34.97	10.21	15.75	7.86
34.98	10.07	15.74	8.09
34.98	9.95	15.74	8.3
34.97	10.22	15.74	7.8
34.97	10.12	15.74	7.73
34.98	10.46	15.74	8.12
34.97	9.92	15.74	8.23
34.97	9.85	15.74	7.92
34.98	10.41	15.74	7.7
34.97	10.22	15.74	7.94
34.97	10.50	15.74	7.99
34.97	10.44	15.74	8.2
34.97	10.67	15.74	8.05
34.97	10.36	15.74	8.12
34.96	10.33	15.73	7.95
34.96	10.39	15.73	8.13
34.96	10.31	15.73	7.95
34.96	10.62	15.73	7.96
34.95	10.43	15.73	7.98
34.95	10.41	15.74	8.02
34.96	10.28	15.74	8.21
34.96	10.06	15.73	8.2
34.97	10.38	15.73	8.03
34.97	10.25	15.73	8.03
34.95	10.29	15.73	7.78
34.95	10.54	15.72	7.73
34.96	10.67	15.73	8.02
34.95	10.41	15.73	8
34.95	10.52	15.72	7.96
34.95	10.18	15.72	7.98
34.95	9.99	15.72	8.13
34.95	10.57	15.72	7.99
34.95	10.37	15.72	7.85
34.96	9.98	15.72	8.2
34.96	10.13	15.72	8.35
34.95	10.13	15.72	7.95
34.95	10.33	15.72	7.93
34.95	10.54	15.72	8.02

Salinity ppt	DO mg/l	Temp ^{0}C	pH
34.95	10.37	15.72	8.03
34.94	10.53	15.72	8.09
34.95	10.17	15.72	7.97
34.95	9.97	15.73	8.04
34.95	10.34	15.72	8.09
34.94	10.12	15.72	8.05
34.95	10.32	15.72	8.07
34.94	10.49	15.72	8.02
34.95	10.32	15.72	8.09
34.95	10.41	15.72	8.33
34.94	10.12	15.72	8.02
34.95	10.23	15.72	8.14
34.95	10.35	15.72	8.33
34.95	10.56	15.72	7.99
34.94	10.39	15.72	8.01
34.94	10.36	15.72	7.84
34.95	10.10	15.71	8.22
34.94	9.71	15.71	7.78
34.94	10.22	15.72	8.12
34.94	10.34	15.72	8.02
34.94	10.55	15.71	7.99
34.94	10.21	15.71	8.02
34.94	10.17	15.71	8.1
34.94	9.92	15.70	8.11
34.94	9.88	15.70	8.01
34.93	10.12	15.70	7.86
34.94	10.21	15.70	7.87
34.94	10.17	15.70	8.25
34.93	10.09	15.69	7.98
34.94	10.16	15.70	8.13
34.94	9.91	15.70	7.92
34.94	9.65	15.70	8.06
34.94	9.93	15.70	8.2
34.94	10.04	15.69	8.1
34.94	10.43	15.69	8.08
34.95	10.33	15.69	8.09
34.95	10.06	15.69	8.11
34.94	10.21	15.69	8.07
34.94	9.80	15.69	8.08
34.94	10.13	15.69	8.02
34.94	10.24	15.69	8.13
34.95	9.93	15.69	8.09
34.95	9.74	15.69	8.04
34.94	9.90	15.69	8.09
34.94	9.82	15.69	7.94
34.95	9.69	15.69	8.05
34.95	10.04	15.69	8.08
34.95	9.77	15.69	8.06
34.95	9.99	15.69	8.07
34.95	10.20	15.70	8.05
34.94	9.69	15.70	8.03
34.94	9.60	15.70	8.05
34.94	9.60	15.70	8.06
34.94	9.66	15.70	8.08
34.94	9.67	15.69	8.08
34.95	9.97	15.69	8.1
34.94	9.61	15.69	8.05

Salinity ppt	DO mg/l	Temp ^{0}C	pH
34.95	9.67	15.69	8.02
34.95	9.38	15.69	8.06
34.95	9.63	15.69	8.02
34.95	9.92	15.69	8.05
34.95	9.67	15.69	8.03
34.95	9.89	15.68	8.02
34.95	9.70	15.68	8.01
34.94	9.49	15.68	7.96
34.94	9.62	15.67	8.02
34.94	9.75	15.67	8.09
34.94	9.86	15.67	8.03
34.94	9.80	15.67	8.01
34.94	9.71	15.68	8.05
34.94	9.58	15.69	8.03
34.94	10.21	15.69	8.04
34.94	9.80	15.69	8.01
34.94	9.69	15.69	8.1
34.94	9.80	15.69	8.1
34.94	9.74	15.69	8.09
34.94	9.96	15.68	8.04
34.94	9.66	15.68	8.08
34.94	9.44	15.68	8.03
34.94	9.58	15.68	8.05
34.94	9.71	15.68	8.11
34.94	9.65	15.68	8.12
34.95	9.85	15.68	8.05
34.94	10.24	15.68	8
34.95	9.81	15.68	8.02
34.95	9.64	15.68	8.13
34.95	9.64	15.68	8.07
34.94	10.02	15.67	8.11

Appendix 9.2

Table 33. Chapter 4. *Ciona Intestinalis* gonad and somatic tissue dry weight data

Specimen Number	Treatment	Gonad Weight (mg)	Somatic tissue Weight (g)
1	Air control	1.47	0.051
2	Air control	2.53	0.069
3	Air control	1	0.032
4	Air control	2.76	0.07
5	Air control	0.89	0.05
6	No Gas control	3.781	0.082
7	No Gas control	7.55	0.124
8	No Gas control	3.623	0.054
9	No Gas control	3.18	0.062
10	No Gas control	2.78	0.067
11	No Gas control	1.907	0.049
12	No Gas control	2.401	0.057
13	No Gas control	6.79	0.087
14	No Gas control	2.99	0.082
15	No Gas control	3.72	0.068
16	No Gas control	3.33	0.081
17	No Gas control	0.42	0.038

18	No Gas control	2.35	0.067
19	No Gas control	4.13	0.082
20	pH 100 years	3.05	0.071
21	pH 100 years	11.989	0.194
22	pH 100 years	5.28	0.057
23	pH 100 years	5.75	0.105
24	pH 100 years	3.411	0.077
25	pH 100 years	7.141	0.117
26	pH 100 years	5.24	0.109
27	pH 100 years	6.63	0.085
28	pH 100 years	3.847	0.071
29	pH 100 years	4.924	0.1
30	pH 100 years	0.45	0.034
31	pH 100 years	0.185	0.014
32	pH 100 years	0.87	0.033
33	pH 100 years	0.065	0.015
34	pH 100 years	0.155	0.016
35	pH 100 years	0.4	0.023
36	pH 100 years	0.26	0.031
37	pH 100 years	4.96	0.109
38	pH 100 years	1.55	0.039
39	pH 100 years	1.05	0.039

Table 34. Chapter 4. *Mytilus edulis* somatic tissue and shell weight data

Specimen Number	Treatment	Somatic Tissue Weight (mg)	Shell Weight (mg)
40	Air control	1.06	12.37
41	Air control	1.75	20.00
42	Air control	3.42	30.03
43	Air control	3.93	52.05
44	Air control	7.4	73.02
45	Air control	0.928	11.09
46	Air control	0.641	90.05
47	Air control	0.94	10.10
48	Air control	1.53	13.48
49	Air control	2.34	28.57
50	Air control	2.07	16.07
51	Air control	4.886	37.01
52	Air control	4.602	49.03
53	No gas control	0.429	60.10
54	No gas control	1.27	15.61
55	No gas control	1.847	24.38
56	No gas control	4.68	53.39
57	pH 100	3.05	21.86
58	pH 100	3.42	33.37
59	pH 100	5.27	45.24
60	pH 100	3.5	37.12
61	pH 100	4.67	70.05
62	pH 100	1.105	12.11
63	pH 100	3.21	22.03
64	pH 100	0.37	40.42
65	pH 100	6.46	61.12
66	pH 100	5.107	37.41
67	pH 100	0.26	33.15
68	pH 100	0.32	42.29
69	pH 100	0.42	57.42
31	pH 100	0.71	90.81

Table 35. Chapter 4. *Mytilus edulis* shell dimension data. Missing values are due to cracked or broken shells.

Specimen Number	Treatment	Shell length (mm)	Shell Width (mm)	Shell Thickness - centre (mm)	Shell Thickness - bottom of dorsal scar (mm)
70	No gas control	7	4		0.08
71	No gas control	7	4		0.13
72	No gas control	8	5		0.60
73	No gas control	9	5		0.12
74	No gas control	13	8		0.24
75	pH 100	10	6	0.78	0.67
76	pH 100	11	6	0.00	0.22
77	pH 100	11	7	0.00	0.17
78	pH 100	12	8	0.72	0.66
79	pH 100	14	8	0.00	0.57

Appendix 9.3

Table 36. Chapter 5. *Ciona Intestinalis* gonad and somatic tissue data

Specimen Number	Treatment	Gonad Weight (mg)	Somatic tissue Weight (g)
1	Air control	5.5	0.104
2	Air control	0.298	0.021
3	Air control	0.277	0.033
4	Air control	1.4	0.05
5	Air control	1.54	0.087
6	Air control	0.527	0.055
7	No Gas control	6.3	0.107
8	No Gas control	6.41	0.084
9	No Gas control	2.57	0.077
10	No Gas control	1.9	0.06
11	No Gas control	2.68	0.065
12	No Gas control	1.677	0.046
13	No Gas control	2.298	0.071
14	No Gas control	2.057	0.083
15	No Gas control	6.78	0.107
16	No Gas control	1.723	0.065
17	No Gas control	9.57	0.142
18	No Gas control	7.32	0.104
19	No Gas control	1.8	0.07
20	No Gas control	4.02	0.082
21	No Gas control	3.183	0.0753
22	No Gas control	2.833	0.048
23	No Gas control	2.643	0.053
24	No Gas control	2.479	0.067
25	No Gas control	7.36	0.123
26	No Gas control	5.261	0.097
27	No Gas control	2.03	0.077
28	No Gas control	7.43	0.165
29	No Gas control	3.17	0.079
30	pH 100 years	7.643	0.161
31	pH 100 years	2.645	0.099
32	pH 100 years	4.82	0.073
33	pH 100 years	7.923	0.096
34	pH 100 years	2.52	0.095
35	pH 100 years	3.52	0.083
36	pH 100 years	0.584	0.029
37	pH 100 years	5.796	0.088
38	pH 100 years	7.536	0.1
39	pH 100 years	2.663	0.054
40	pH 100 years	5.767	0.095
41	pH 100 years	1.694	0.049
42	pH 100 years	5.824	0.093
43	pH 100 years	1.646	0.029
44	pH 100 years	0.552	0.026

Table 37. Chapter 5. *Mytilus edulis* somatic tissue and shell weight data

Specimen Number	Treatment	Somatic Tissue Weight (mg)	Shell Weight (mg)
45	Air control	62	669.60
46	Air control	3.9	70.70
47	Air control	5.3	82.70
48	Air control	4.2	84.00
49	Air control	3.5	57.70
50	Air control	10.3	158.80
51	Air control	49.1	843.50
52	Air control	42.7	688.90
53	Air control	47.9	556.20
54	Air control	44.1	576.20
55	Air control	6.6	117.60
56	Air control	7.4	100.00
57	Air control	16.6	184.30
58	Air control	38.7	624.40
59	Air control	41.9	830.00
60	Air control	63.4	967.20
61	Air control	44.3	579.80
62	Air control	45.3	497.50
63	Air control	51.6	646.60
64	Air control	9.9	138.20
65	Air control	20.2	163.90
66	Air control	11	103.70
67	Air control	6.5	58.30
68	Air control	2.6	30.40
69	No gas control	63.2	705.20
70	No gas control	30.3	523.80
71	No gas control	23.6	359.80
72	No gas control	19.4	254.10
73	No gas control	37.4	456.50
74	No gas control	0.3	
75	No gas control	3.1	50.80
76	No gas control	4.6	55.00
77	No gas control	2.3	34.70
78	No gas control	9.3	123.40
79	No gas control	5.1	70.50
80	No gas control	9.8	123.40
81	No gas control	48	832.20
82	No gas control	56.6	767.70
83	No gas control	7	80.60
84	No gas control	1.2	15.70
85	No gas control	2.6	31.90
86	No gas control	10.1	120.90
87	No gas control	60.7	611.60
88	No gas control	37.6	436.30
89	No gas control	38.9	807.10
90	No gas control	85.6	1052.00
91	No gas control	37	451.80
92	No gas control	24.4	269.60
93	pH 100	12.3	183.50
94	pH 100	4.5	86.10

95	pH 100	10.7	128.50
96	pH 100	3.2	41.30
97	pH 100	41.6	420.20
98	pH 100	11.9	159.10
99	pH 100	41.3	674.50
100	pH 100	55.1	829.30
101	pH 100	19.2	314.50
102	pH 100	12	261.10
103	pH 100	51	495.30
104	pH 100	106.6	798.90
105	pH 100	84.1	734.40
106	pH 100	92	834.00
107	pH 100	18.7	180.80
108	pH 100	7.6	80.70
109	pH 100	13.3	160.20
110	pH 100	9.4	**113.00**
111	pH 100	6.7	69.30
112	pH 100	3.3	39.70
113	pH 100	28.4	307.10
114	pH 100	3.7	59.00
115	pH 100	8.9	98.25
116	pH 100	2.7	56.70
117	pH 100	17.1	362.20

Table 38. Chapter 5. *Mytilus edulis* shell dimension data. Missing values are due to cracked or broken shells.

Specimen Number	Treatment	Shell length (mm)	Shell Width (mm)	Shell Thickness - centre (mm)	Shell Thickness - bottom of dorsal scar (mm)
117	Air control	27	27	0.68	0.68
118	Air control	34	19	0.69	0.59
119	Air control	40	24	0.71	0.58
120	Air control	62	32	0.93	0.65
121	Air control	63	34	1.07	0.77
122	Air control	66	36	0.96	0.80
123	Air control	70	38	0.61	0.68
124	Air control	35	20	0.71	0.62
125	Air control	35	21	0.80	0.82
126	Air control	43	24	0.73	0.61
127	Air control	68	39	0.99	0.65
128	Air control	68	38	0.75	0.57
129	Air control	24	14	0.00	0.24
130	Air control	27	14	1.12	0.75
131	Air control	29	18		0.51
132	Air control	35	20		0.09
133	Air control	37	24	1.01	0.58
134	Air control	40	24		0.35
135	Air control	59	33		0.18
136	Air control	60	34		0.67
137	Air control	66	31		0.11
138	Air control	69	36	0.92	0.92
139	No gas control	13	9	0.91	0.95
140	No gas control	23	14	1.05	0.99

141	No gas control	27	16	0.89	0.75
142	No gas control	29	16	1.12	0.55
143	No gas control	45	27	1.04	1.00
144	No gas control	49	38	1.07	0.93
145	No gas control	53	35	1.09	0.95
146	No gas control	65	35	0.90	0.93
147	No gas control	73	36	0.85	0.68
148	No gas control	13	8		0.59
149	No gas control	30	17	0.59	0.51
150	No gas control	35	21	0.70	0.66
151	No gas control	66	36	1.10	0.85
152	No gas control	14	9		0.46
153	No gas control	18	13		0.47
154	No gas control	22	13		0.23
155	No gas control	35	19	0.83	0.69
156	No gas control	47	25		0.56
157	No gas control	50	27		0.29
158	No gas control	51	30	0.88	0.89
159	No gas control	58	34	0.00	0.33
160	No gas control	66	35	0.94	0.70
161	No gas control	78	47		0.57
162	pH 100	24	14		0.58
163	pH 100	31	17	0.70	0.59
164	pH 100	40	23	0.76	0.61
165	pH 100	40	23	0.72	0.64
166	pH 100	47	24	0.88	0.59
167	pH 100	54	27	0.86	0.75
168	pH 100	61	33	0.85	0.68
169	pH 100	66.5	38.5	1.06	0.74
170	pH 100	73	35	1.18	0.82
171	pH 100	5.5	30		0.15
172	pH 100	16	9	0.84	0.68
173	pH 100	23	14	0.00	0.16
174	pH 100	30	18	0.91	0.63
175	pH 100	30	16		0.16
176	pH 100	41	22		0.51
177	pH 100	41	23		0.15
178	pH 100	64	35		0.24
179	pH 100	66	38		0.55
180	pH 100	70	37		0.15
181	pH 100	27	17	0.64	0.79
182	pH 100	27.5	16.5		0.55
183	pH 100	32	17	0.68	0.67
184	pH 100	51	27	0.60	0.67
185	pH 100	52	27	0.75	0.75

Table 39. Chapter 5. *Balanus crenatus* shell dimension data.

Specimen Number	Treatment	Shell base diametres (mm)	Shell base radius (mm)	Shell top width (mm)	Shel Height (mm)
185	Air control	11	5.5	4	6
186	Air control	11	5.5	4	6
187	Air control	11	5.5	4	5
188	Air control	10	5	3.5	5
189	Air control	10	5	4	4
190	Air control	8	4	2.5	2.5
191	Air control	11	5.5	4	6
192	Air control	10	5	4	4
193	Air control	13	6.5	6	6
194	Air control	12	6	4	4
195	Air control	9.5	4.75	3	5
196	Air control	12	6	5.5	7
197	Air control	8	4	2.5	4
198	Air control	11	5.5	4	5
199	Air control	8	4	4	5
200	Air control	11	5.5	4	5
201	No gas control	15	7.5	5	5
202	No gas control	14	7	6	7
203	No gas control	11	5.5	5	6
204	No gas control	13	6.5	4.5	5
205	No gas control	8	4	3.5	4
206	No gas control	11	5.5	4	5
207	No gas control	11	5.5	4	4.5
208	No gas control	10	5	3.5	4.25
209	No gas control	15	7.5	6.5	7.5
210	No gas control	5	2.5	2	2
211	No gas control	6	3	3	3.5
212	No gas control	8	4	3	4
213	No gas control	7	3.5	2	3
214	No gas control	8	4	2	4
215	No gas control	10	5	4	5
216	No gas control	10	5	3	5
217	No gas control	11	5.5	5	6
218	No gas control	10	5	4	4
219	No gas control	12	6	4	5
220	No gas control	9	4.5	3.75	4.5
221	No gas control	10	5	3.5	5
222	No gas control	10	5	4	5
223	pH 100	10	5	4	4.5
224	pH 100	10	5	2.5	4
225	pH 100	11	5.5	4	5
226	pH 100	10	5	6	3
227	pH 100	11.5	5.75	4	5
228	pH 100	11	5.5	3	5
229	pH 100	8	4	2.5	4
230	pH 100	9	4.5	4	4
231	pH 100	10	5	3.5	4
232	pH 100	7.5	3.75	2.5	4
233	pH 100	11	5.5	3	5

234	pH 100	11	5.5	4	5
235	pH 100	9.5	4.75	3	4
236	pH 100	13	6.5	4	5
237	pH 100	11	5.5	3.5	5
238	pH 100	9.5	4.75	3.5	3.5
239	pH 100	7	3.5	3	3
240	pH 100	10	5	3.75	5
241	pH 100	11	5.5	3.5	5

Appendix 9.4

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(56) Documents Cited:
WO 2008/102343 A1 **SU 001030255 A1**
US 20050087117 A1

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Other: **EPODOC, WPI, TXTE**

(54) Abstract Title: **Anti-fouling apparatus using a gas permeable membrane and a gas**

(57) An anti-fouling apparatus 1 comprises a gas permeable element 3 attachable to a surface and means 4 and 5 to introduce gas 6 to the gas permeable element. The gas is released into a micro-environment immediately adjacent the free surface of the gas permeable element and the gas is selected to modify at least one condition in the micro-environment, such that the micro-environment is rendered unfavourable to the accumulation of foulants therein. The gas may contain carbon dioxide, nitrogen, chlorine, ozone or a noble gas. The gas may react with sea water 2 to reduce the pH of the sea water in the micro-environment. The gas permeable element may include one of a gas permeable membrane, a micro-porous layer, a gas permeable solid, a gas chamber, a perforated pipe or an air stone. A structure protected by the anti-fouling apparatus and a method for its use, are also disclosed.

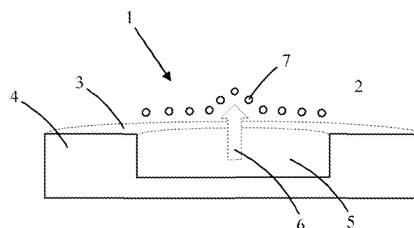

Figure 1

GB 2459854 A

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