INVESTIGATIONS INTO THE SKELETAL MINERALOGY OF TEMPERATE AND POLAR BRYOZOANS

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Abstract

This study aims to explore the skeletal mineralogy of temperate and Polar bryozoans, investigating variability within and between species in relation to methodological, biological and environmental factors. Oceans are becoming warmer and more acidic and it is becoming increasingly important to increase our knowledge about the responses of marine calcifiers to environmental conditions. Bryozoans are important components of the benthic community globally, however prior to this study relatively little was known about their skeleton composition. This study has contributed over 1700 new mineralogical analyses to the field and provided new skeletal profiles for 115 bryozoan species. The study represents by far the most comprehensive regional profile of bryozoan mineralogy to date with 79% of Scottish species analysed. Targeted experiments have resulted in the documentation of impacts of curational procedures on skeletal mineralogy, resulting in recommendations which are pertinent across taxa in the wider fields of both curation and palaeoclimatography. Evidence is presented of mineral localization in specific skeletal features in bryozoan skeletons which adds to the growing body of data showing $MgCO_3$ localization for mechanical and ecological advantage in marine invertebrates. Prior to this study it was proposed that both "active" biological and "passive" environmental controls influence bryozoan skeletal mineralogy. Through the examination of both Polar and Temperate species, this study has provided evidence of biological control of bryozoan mineralogy, while finding no evidence of passive environmental control. This finding precludes the use of bryozoan mineralogy for palaeoclimatic interpretation, for the species included in this study, and it is recommended that future species are chosen carefully and thoroughly calibrated prior to their use as palaeothermometers. Further investigation into the effects of ecological specification on the temperature/mineralogy response may, however, prove an area for fruitful research, enabling prediction of climate change effects on the bryozoan skeletons of ecologically specialised species and providing insights into future changes in community composition.

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1.1 Calcium carbonate in the oceans

1.1.1 Marine calcifiers

Many marine invertebrates, such as the Porifera, Echinodermata, Mollusca, Foraminifera, Crustacea and Annelida, feature inorganic shells, tests and skeletons (Ruppert et al. 2004) with the majority constructing these from calcium carbonate ($CaCO_3$) deposited around an organic matrix (Ruppert et al. 2004). Rigid endoskeletons are common in exposed sessile and slow-moving benthic animals where a rigid skeleton may form a supportive platform or structure, as in stony corals; or as a protection against predation, as in barnacles and bivalves (Ruppert et al. 2004). In faster moving animals a shell or skeleton may aid movement or act as a buoyancy compensation device, as in cephalopods (Morton 1979). Some plants also incorporate $CaCO_3$ within their structure and organisms such as coccolithophores are major contributors to the high carbonate content of deep-sea sediment (Morse et al. 2007).

1.1.2 The oceans carbonate system

Unlike typical gases such as oxygen, the majority (98.5%) of pre-industrial CO_2 is found in the ocean rather than the atmosphere (1.5%) (Marinov & Sarmiento 2004). CO_2 is preferentially found in the ocean because it is highly soluble and undergoes a hydrolysis reaction to form carbonate and bicarbonate ions (Marinov & Sarmiento 2004). Calcium ions are also present in seawater (Taylor 2007) and these combine with carbonate to form calcium carbonate ($CaCO_3$) precipitate (Morse et al. 2007). Biogenic calcium carbonate ($CaCO_3$) deposition and dissolution are key biologically mediated regulatory components of the ocean carbon cycle (Murray 2004), a process called the carbonate pump (Volk & Hoffert 1985).

In nature $CaCO_3$ is deposited as three anhydrous polymorphs, vaterite, calcite and aragonite, which are chemically identical but form different crystal lattice structures with varying properties and stability (Morse et al. 2007). Vaterite is rare in marine invertebrates as it is the least stable polymorph (Gómez et al. 2003; Morse et al. 2007) with a dissociation constant (pK) of 7.913 +/- 0.02 at 25ºC (Plummer & Busenberg 1982).The

instability of vaterite in aqueous solutions usually results in its transformation into calcite or aragonite (depending on temperature) in a short period of time (Morse et al. 2007). Examples of vaterite in nature include its use as a precursor in pearl formation (Suzuki et al. 2009) and shell regeneration of marine and freshwater molluscs (Feng et al. 2011). Aragonite is more stable than vaterite to deposit, with a dissociation constant (pK) of 8.336 +/- 0.02 at 25ºC (Plummer & Busenberg 1982), and is common in marine invertebrates. Aragonite belongs to the orthorhombic crystal system (Fig. 1.1) and has a density of 2.94 g cm^{-3} (Nehrke 2007), making it the hardest of the three polymorphs with a Moh's scale hardness score of 3.5 – 4 (vaterite/calcite = 3 Moh's scale) (Chen et al. 2012). Aragonite is, however, thermodynamically more expensive to build than calcite (Anderson & Crerer 1993) and so is often less common in colder waters. Calcite is the most stable $CaCO_3$ polymorph, with a dissociation constant (pK) of 8.480 +/- 0.02 at 25ºC (Plummer & Busenberg 1982), and is widespread in nature (Morse et al. 2007). Calcite belongs to the trigonal crystal system (Fig. 1.1) and is less dense than aragonite (2.71 g cm^{-3}) (Nehrke 2007).

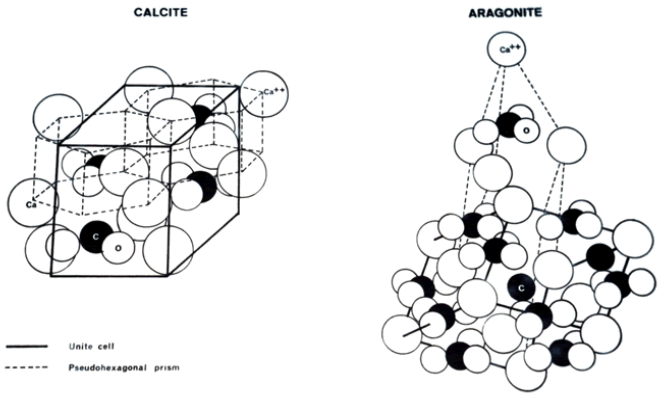


Figure 1.1:Molecular structure and crystal form of calcite and aragonite. Large hollow circles represent Ca^{2+}, small hollow circles represent O, black circles represent C. (William Pengelly Cave Studies Trust 2014)

Some organisms exclusively excrete aragonite or calcite but many have bimineralic shells or skeletons with aragonite and calcite secreted in distinct layers (Taylor et al. 2008).

Further biomineralization complexity is afforded by the differential incorporation of divalent metal ions within the aragonite or calcite lattice (Nehrke 2007). The Ca^{2+} ion in $CaCO_3$ can be replaced by another ion, usually Strontium (Sr^{2+}) or Magnesium (Mg^{2+}) (Morse et al. 2007).The type of mineral incorporated is predominantly determined by the fit of the ion within the lattice shape with the large Sr^{2+} (MW = 87.62) being the most

common incorporation in aragonite and small Mg^{2+} (MW=24.312) fitting snugly in the calcite structure (Nehrke 2007).

1.2 Ocean chemistry and climate change

1.2.1 Ocean acidification

Since the industrial revolution the increase in atmospheric CO_2 released from the burning of fossil fuels has had wide ranging climatic impacts worldwide (Hoegh-Guldberg & Bruno 2010). To date, the oceans have acted as a natural carbon and thermal sink, absorbing more than half of the world's anthropogenically produced CO_2 (Wood et al. 2009). Absorbed CO_2 acts to decrease the concentration of carbonate ions in seawater (Marinov & Sarmiento 2004) and increase H^+ ions, lowering the pH (Feely et al. 2010a) (Eq. 1.1). If global emissions of CO_2 from human activities continue to increase at current rates, then the average pH of the ocean could fall by a further 0.3 to 0.4 pH units within this century (Doney et al. 2009).

Equation 1.1: Ocean chemistry equilibrium equation. As CO_2 concentration in the ocean increases, it drives the equation to the right,.

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + H^+$$

Decreased carbonate saturation in the water may lead to reduced calcification rates (Feely et al. 2010a; Feely et al. 2010b; Ries et al. 2010), and acidification could cause dissolution of the more susceptible forms of calcium carbonate, aragonite and calcite with a high level of $MgCO_3$ incorporation (Smith et al. 2012; Smith & Garden 2012). The energetic stress experienced by organisms under future ocean acidification conditions is also expected to impact additional biological processes such as growth, reproduction (Orr et al. 2005; Findlay et al. 2008) and respiration (Blackford & Gilbert 2007) in susceptible marine animals (Fig. 1.2).

As the carbonate ion concentration in the ocean decreases it also reduces the ocean's capacity to absorb additional carbon (Marinov & Sarmiento 2004). Carbonate buffering means that only 85% of the anthropogenic carbon dioxide being adding to the atmosphere will eventually be dissolved into the ocean (Sarmiento et al. 1992) compared to 98.5% in pre-industrial times (Marinov & Sarmiento 2004) and the uptake capacity continues to decrease as more CO_2 is put into the atmosphere.

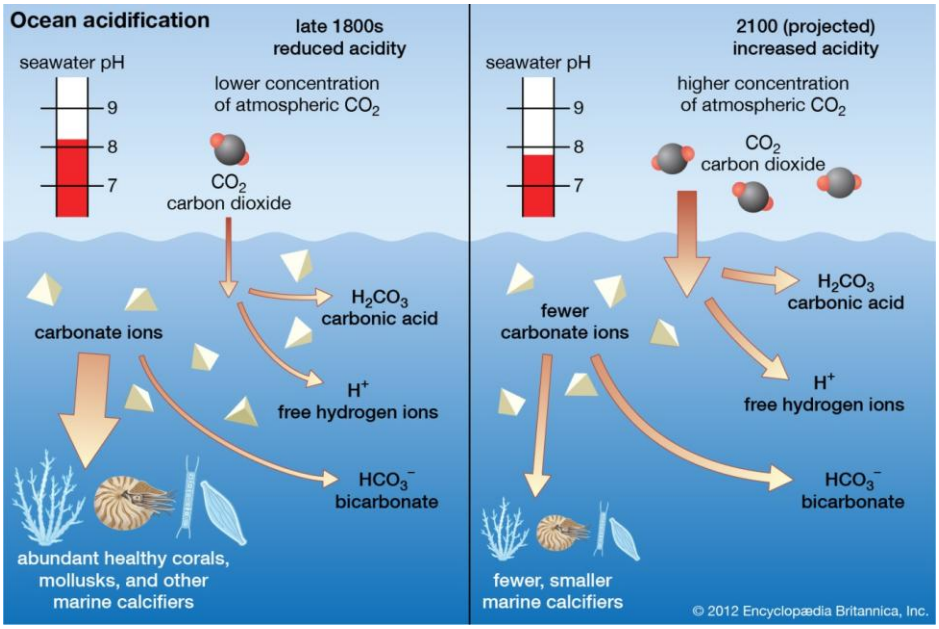


Figure 1.2: Figure showing potential impacts of ocean acidification on the marine environment comparing pre-industrial times to 2100 predicted levels (Encyclopedia Britannica 2012)

1.2.2 Ocean warming

Increasing CO_2 in the atmosphere is also resulting in an increase in global temperatures of ~ 0.2ºC per decade through the Greenhouse Effect (Hoegh-Guldberg & Bruno 2010) and the ocean acts as a sink for the majority of this anthropogenically produced thermal energy. As a result between 1961 and 2003 there was a global increase in oceanic mean temperature of 0.037ºC in the 0-3000m layer (Hoegh-Guldberg & Bruno 2010). Increasing ocean temperatures are expected to have a range of effects: increasing sea temperatures are expected to lead to increased vertical stratification, resulting in decreased nutrients and productivity (Behrenfeld et al. 2006) in the photic zone and a reduction in O_2 concentrations (Turley et al. 2007). The increase in ice-melt, especially in the Arctic, is predicted to cause a decrease in salinity (Meredith 2005) and changes in the temperature of the oceans are enabling range extension of species, altering community composition of the shallow oceans (Occhipinti-Ambrogi 2007). It is predicted that ocean acidification will work in concert with the absorption of thermal energy and controlled microcosm experiments have shown this double pressure to impact respiration (Blackford & Gilbert 2007), growth (Anlauf et al. 2011) and breeding (Findlay et al. 2008) in marine organisms.

CO_2 is less soluble in warmer waters, and it is predicted that warming will, therefore, further reduce the ocean's buffering capacity for anthropogenic carbon, raising atmospheric CO_2 and adding to the warming cycle (Turley et al. 2007).

1.2.3 Additional pressures

Further anthropogenic pressures in the ocean include the effects of fishing (Bank 1997; Turner et al. 1999) and trawling practices (e.g. Cranfield 2003; Henry et al. 2006), dredging and construction (e.g. Ohimain et al. 2008), pollution (e.g. Moran & Grant 1989) and the spread of non-native and invasive species (e.g. Bax et al. 2003; Occhipinti-Ambrogi 2007). All of these pressures may exacerbate the impacts of ocean acidification and warming to further stress marine communities (Cheung et al. 2009).

1.3 Calcium carbonate variation

1.3.1 Mechanisms of calcium carbonate variation

The calcium carbonate polymorph and the concentration of trace element measured within calcium carbonate can be influenced by thermodynamics, the composition of the solution within which the crystal is precipitated, and the growth rate of the crystal (Nehrke 2007).

Links between seawater temperature and calcium carbonate variation in marine organisms were first suggested by Clarke & Wheeler (1922), when correlations were observed between the inorganic constituents of marine organisms and local habitat temperature. In 1954 increased magnesium incorporation in calcium carbonate was recognised as a proxy for increasing seawater temperature by Chave (1954). In the same year Lowenstam (1954) identified a positive correlation between seawater temperature and aragonite content in bimineralic animals. Substitution of Mg^{2+} for Ca^{2+} in calcite has been studied through inorganic precipitation studies, which demonstrate that incorporation of magnesium within the calcite lattice is thermodynamically more favourable in warmer water (Chilanger 1962; Katz 1973; Burton & Walter 1985; Mucci 1987; Oomori et al. 1987). Similarly aragonite is more energetically expensive to build than calcite (Anderson & Crerer 1993), resulting in an expectation of preferential deposition of aragonite over calcite under warmer conditions (Chave 1954).

Seawater composition state has been shown to influence the deposition of abiotic calcium carbonate. Through geological time the seas have fluctuated between favouring low-magnesium calcite deposition, a seawater chemistry termed calcite seas, and favouring high-magnesium calcite and aragonite deposition, known as aragonite seas. Hardie (1996) asserted that past alternation between aragonitic and calcitic seas, was controlled by the fluctuation of Mg/Ca in the ocean. Inorganic studies into Mg/Ca ratios in seawater have been proven to drive differential incorporation of $MgCO_3$ in calcite with a higher ratio resulting in increased $MgCO_3$ and aragonite deposition (Stanley 2006).

Growth rate of calcium carbonate crystals also affects the composition and form of $CaCO_3$ deposited with slower growth favouring low-magnesium calcite (Kitano et al. 1969). Inorganic studies have shown crystal growth rate to be primarily controlled by seawater temperature and composition (Morse et al. 2007). Warmer waters and higher Mg/Ca ratios both increase crystal growth rate (Stanley 2006).

In the marine environment, however, biogenic calcium carbonate is often deposited which cannot be explained by thermodynamics or seawater chemistry; this behavior is usually ascribed to the effect of organic matter interactions (Morse et al. 2007). Kitano et al. (1969) investigated the influence of organic material on calcium carbonate precipitation and found that one type of organic compound induced calcite formation, while other compounds resulted in aragonite precipitation. Calcite deposition was found to be increased at slower reaction rates and was, therefore, preferentially deposited in the presence of organic compounds which inhibited calcium carbonate deposition (Morse et al. 2007). In well-studied phyla such as Mollusca, proteins have now been identified which act as nuclei for calcium carbonate growth and have been shown to be involved in the determination of either aragonite or calcite deposition (e.g. Glazer et al. 2010; Inoue et al. 2008; Suzuki et al. 2009; Furuhashi et al. 2010; Werkstoffe & Klopferspitz 2003).

It has also been observed, that changes in seawater chemistry do not strongly affect all marine taxa (Stanley 2006). Intracellular processes can also influence biogenic calcium carbonate deposition, as through the use of internal vesicles, many organisms are able to regulate the internal chemistry of the cell within which calcium carbonate is deposited. Marine calcifiers can, therefore, accomplish calcium carbonate compositions different to

those predicted from abiotic experiments (Morse et al. 2007) and sometimes contrary to the composition of seawater within which they live.

The relative influence of biological processes on calcium carbonate composition varies widely among marine organisms, with some passively depositing skeletal material in thermodynamic and chemical equilibrium with the seawater in which it resides, and others driving deposition through biological processes (Weiner et al. 2001).

1.3.2 Understanding the past, establishing a baseline for the present and predicting the future

Since the mid twentieth century the skeleton composition of biological groups, such as the Foraminifera (Dowsett et al. 2011) and Mollusca (Cohen & Branch 1992), have been used in palaeoclimatology, where historic and fossil specimens have been examined under the tenet that calcium carbonate holds a record of seawater chemistry and temperature from the time at which it was deposited (Lowenstam 1954). In recent years, it has also been hypothesised that animals with variable calcium carbonate chemistry, might act as a bellwether for climate change and ocean acidification (Fabry et al. 2009). It is anticipated that by quantifying what the mineralogical reaction will be for marine organisms to warming seas, it might be possible to predict which species will be 'winners and losers' in the future.

Positive correlations between wt% $MgCO_3$ in calcite and/or aragonite and seawater temperature have been observed in a number of phyla including Mollusca (Cohen & Branch 1992), planktonic Foraminifera (Barker et al. 2005; Martínez-Botí et al. 2011), benthic Foraminifera (Bohaty et al. 2012; Rosenthal et al. 1997), coccoliths (Ra et al. 2010) and corals (Chang et al. 2004), leading to their development as palaeoevironmental thermometers. Further, but less well studied, proxies relating mineralogy to environmental factors such as depth (Berger 1978) and Mg:Ca ratios in seawater (Morse 1997; Davis et al. 2000; Loste 2003; Ries 2005; Astilleros et al. 2010) have also since been suggested.

Mono-mineralic phyla such as coral, calcifying algae, Foraminifera, coccoliths and ostracods have proven most popular for the application of palaeoclimatic proxies due to their less complex mineralogy and, in many cases, proven true recording of environmental conditions. In recent years, however, interest has arisen in bi-mineralic organisms for the

investigation of climate change effects (Fabry et al. 2009) although work is required to assess their feasibility and, if proven useful, calibrate any resulting proxy.

1.4 The Bryozoa

1.4.1 Introducing the Bryozoa

Bryozoans are small filter feeding compound animals consisting of colonies of modular units termed zooids (McKinney & Jackson 1989). Bryozoans are sessile, inhabiting both fresh and marine water, although the majority of species are marine dwellers (Ryland 1970). Zooids are morphologically discrete units and although they are analogous to individual animals, they are interdependent for the function of the entire bryozoan organism (McKinney & Jackson 1989). Feeding zooids are termed autozooids and consist of a polypide feeding unit which extends a ring of tentacles termed the lophophore into the water column to capture food particles (Fig. 1.3 & 1.4).

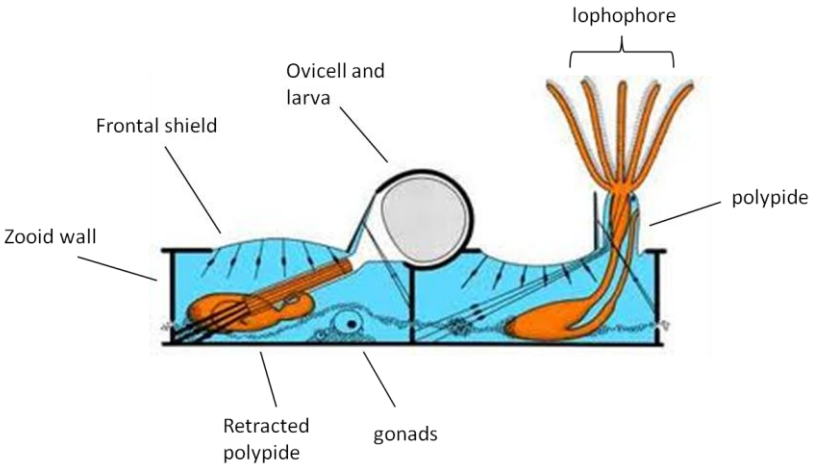


Figure 1.3: Schematic of cheilostome bryozoan. Adapted from figure by Dr Claus Nielsen (University of Copenhagen)

In calcifying bryozoans the full polypide can be retracted within the rigid zooidal skeleton, termed the zooecium (Fig. 1.3 & 1.4). Specialized zooids are common within the Bryozoa with a wide range of non-feeding heterozooids described. Heterozooids can perform a wide variety of functions including protection,

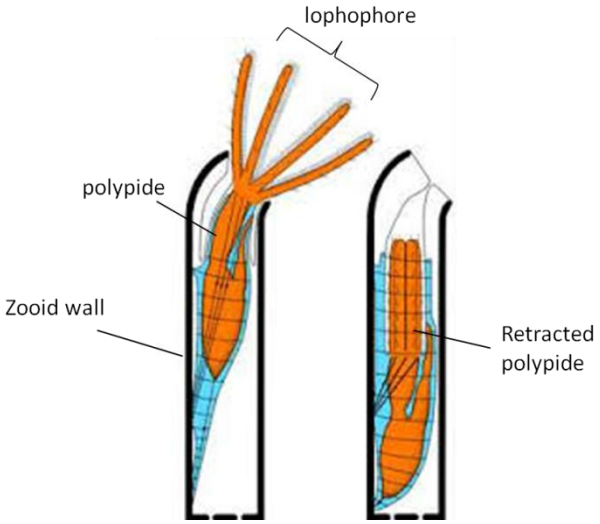


Figure 1.4: Schematic of cyclostome bryozoan. Adapted from figure by Dr Claus Nielsen (University of Copenhagen)

reproduction, locomotion, plumbing and structural support (McKinney & Jackson 1989). Avicularia are defensive heterozooids which are commonly found on cheilostome bryozoans. Avicularia feature a reduced polypide and modified orifice and operculum which are jaw-shaped and able to snap shut to deter or grasp predators (McKinney & Jackson 1989) (Fig. 1.5).

Figure 1. 5: Light microscopy images of birds head avicularia in Bugula. Reproduced from Viera at al. (2012)

To date over 6000 species of extant bryozoans have been described with the majority of these (>5500) falling within the Orders of Cheilostomatida or Cyclostomatida (Appeltans et al. 2012).

Bryozoa reproduce both sexually and asexually. Most zooids are hermaphroditic (Ryland 1970), although occasionally individual zooids within the colony are differentiated as female or male (Ostrovsky 2013), and recent research by Jenkins (2013) indicates that in some cyclostomes species entire colonies can be gender specific. Bryozoans are almost exclusively colonial hermaphrodites and although cross-fertilisation is the norm (Ostrovsky 2013), self-fertilisation has also been observed in some species (Hoare & Hughes 2001). Fertilisation occurs internally, with bryozoans gathering spermatozoa from the water column. Calvet (1900) differentiated between bryozoans which are oviparous or viviparous. Oviparous species release fertilised eggs into the water column where the larvae develop into planktonic cyphonautes (Ostrovsky 2013). Cyphonautes are both swimming and feeding larvae and grow considerably before settling and becoming fixed (Nielsen & Worsaae 2010). In viviparous species embryos are matured in an ooecium, which can be located either within the zooecium or in a calcified brood chamber, termed an ovicell in cheilostomes. During brooding, larvae either feed from internal yolk reserves or through a placenta-like extra-embryonic membrane (Ostrovsky 2013). When brooded larvae are released into the water column, they settle quickly to form a new colony. In general terms, the features of oviparous bryozoan reproduction are characteristic of an r-strategy and those of viviparous species of a K-strategy (Ostrovsky 2013). When a larva settles, it

metamorphoses into a primary zooid, termed an ancestrula, which initiates colony growth by asexual budding (Ryland 1970).

The majority of cyclostome and cheilostome bryozoans build colonies with shared calcium carbonate skeletons. These skeletons can take many diverse forms including small encrusting patches, free-living mobile discs, plant-like colonies and larger branching or foliose masses (Fig. 1.6).

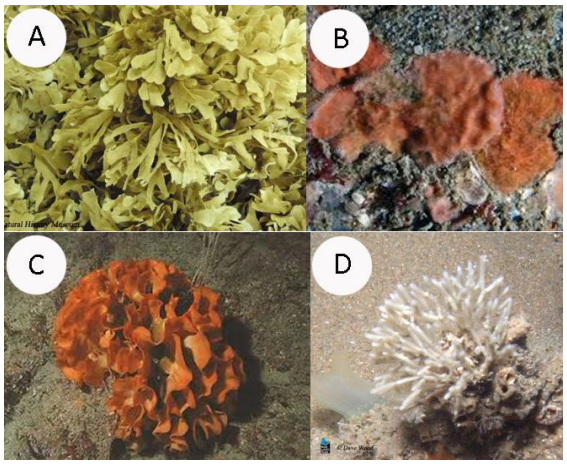


Figure 1.6: Diversity of bryozoan colony forms. A) Flustra foliacea (Linnaeus, 1758), an erect lightly calcified bryozoan; B) Patches of encrusting bryozoan; c) Pentaporia foliacea (Ellis & Solander, 1786), a large erect foliose colony; D) Cellaria sp. an erect, jointed twiggy bryozoans. Reproduced images (Wood 2012; Natural History Museum 2011; MarLIN 2014)

Bryozoans build their calcium carbonate skeletons extracellularly using epithelial cells to deposit crystallites of calcium carbonate skeleton along protein threads (Tavener-Smith & Williams 1972) around a chitinous organic cuticle/periostracum, which acts as a template and seeding surface (Fig. 1.7).

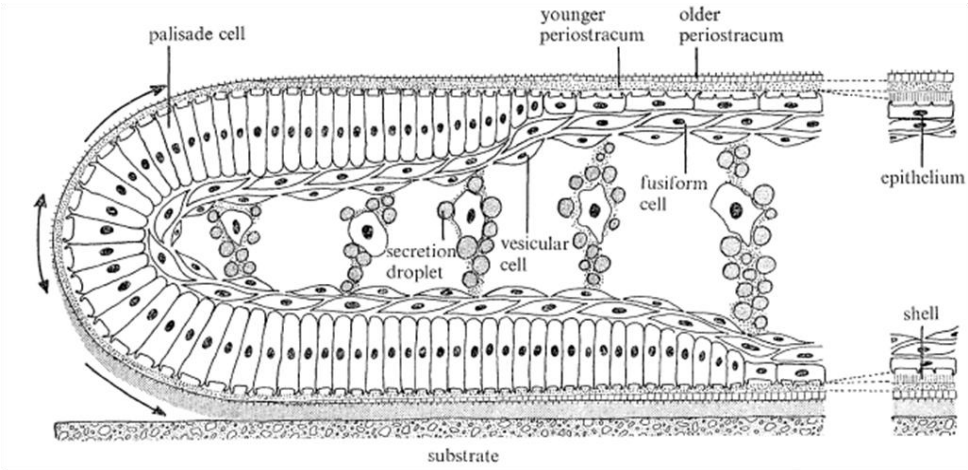


Figure 1.7: Growing tip of Membranipora membranicea (Linnaeus, 1767) showing relationship of periostracum and epithelial cells (here called palisade cells) and deposition of skeleton/shell (Tavener-Smith & Williams 1972)

1.4.2 History of mineralogical analysis and techniques applied to bryozoans

The very first mineralogical analyses on bryozoans were made by A. Schwager for J.Walther in 1885 on two specimens from the Bay of Naples (Walther 1885). These were followed in the early 20th century by a more comprehensive review by Clarke and Wheeler in (1917), and a subsequent revised edition (1922). These first mineralogical analyses were performed using a combination of chemical titration and staining. Elemental concentrations were accurately reported (including mol % $MgCO_3$ in calcite and organic matter) from titration and Clarke and Wheeler estimated the presence of aragonite using, the by then well-known, Meigen's stain (Meigen 1901) which stains aragonite purple while having no effect on calcite; this technique was qualitative rather than quantitative.

Lowenstam (1954) conducted the first X-ray diffraction studies of the Bryozoa and this technique had the advantage of providing quantitative results for the ratio of aragonite:calcite, as well as detecting the quantity of Mg incorporation in calcite through peak shift (Chave 1954). There followed a flurry of XRD studies by Schopf and Mannheim (1967), Rucker (1968), Rucker and Carver (1969), Siesser (1972) and others (Poluzzi & Sartori 1973; Poluzzi & Sartori 1974; Agegian & Mackenzie 1989; Bone & Wass 1990; Smith et al. 1998; Kuklinski & Taylor 2009; Taylor et al. 2009; Smith & Girvan 2010; Smith & Garden 2012) continuing through to the present day. Schopf and Allan (1970) were the first to use a microprobe on bryozoans and this was followed more recently by Schäfer and Bader (2008). A microprobe is able to give quantitative information on the spatial distribution of elements, although it is unable to detect the different calcium carbonate polymorphs.

Sandberg conducted a series of skeletal ultrastructure studies on the Bryozoa in the 1970s using scanning electron microscopy, drawing correlations between the crystal structure and chemical composition (Sandberg 1971; 1973; 1975; 1977; 1983). This was added to by a study from Healey (1979). Although offering interesting insights into the different mechanical properties the polymorphs may have, this is not a quantitative technique.

Recent publications have included mineralogical data obtained by Raman spectroscopy (Taylor et al. 2007; Taylor et al. 2009), which, like XRD, can distinguish between the calcium carbonate polymorphs in addition to quantifying wt% $MgCO_3$ in calcite. It offers the additional advantage of being non-destructive and providing spatial distribution of the

minerals at a small scale. Unfortunately, however, Raman spectroscopy is not considered as accurate as XRD (Taylor et al. 2007) and only provides data on surface mineralogy.

In the past decade there has also been a resurgence of the some of the original mineralogical techniques, such as staining bryozoans with Titan Yellow (staining $MgCO_3$ red) (Bone & James 1993; Smith & Girvan 2010; Smith & Lawton 2010), Meigen's solution (staining aragonite purple) (Taylor et al. 2007; Smith & Girvan 2010) and Feigl's solution (Sandberg 1971; Taylor et al. 2007) (staining aragonite black). These all offer information on the spatial distribution of minerals although they cannot be considered quantitative.

A more recent advance in mineralogical methodology is the analysis of skeletal mineralogy alongside phylogenetic position of taxa, also termed "phylomineralogy"(Smith et al. 2012). In many mineralogical studies samples are taken from multiple bryozoan lineages and, therefore, do not represent statistically independent samples (Grafen 1989; Felsenstein 1985; Blomberg et al. 2003). Some of the mineralogical differences between different species may be due to the divergent evolutionary history of the species and taxa. With the increasing availability of phylogenetic data mineralogical patterns can be assessed for a "phylogenetic signal", which can be quantified using measures such as Blomberg's K (Blomberg et al. 2003), and taken into account during subsequent analysis and discussion. Phylogenetic data can also be included in Bayesian computational tools (Hoff 2009) where a variable such as phylogeny can be assessed as a potential explanation for observed species traits. Examples of where this methodology has been applied to mineralogical traits of other taxa include Cairn & Macintyre's work on cnidarians (Cairns & Macintyre 1992) and recent publications by Smith et al. on serpulids (Smith et al. 2013) and coralline algae (Smith et al. 2012). To date no bryozoan mineralogical analyses have taken into account the phylogenetic signal, however, the recent publications of bryozoan phylogenies by Waeschenbach et al. (Waeschenbach et al. 2009; Waeschenbach et al. 2012) have opened this field for exploration.

There is a suite of mineralogical techniques available for the investigation of calcium carbonate, each with advantages and disadvantages. Used in conjunction these can collectively yield crucial information about the chemistry and organisation of bryozoan skeletons.

1.*4.3 Mineralogical variation in Bryozoa*

Bryozoan skeletons have been found to be entirely calcitic, entirely aragonitic or bimineralic, featuring both $CaCO_3$ polymorphs (Smith et al. 2006; Lombardi et al. 2008). There is also a wide variety of levels of Magnesium (Mg) naturally occurring in bryozoan calcite. This ranges from low (LMC, <4 wt% $MgCO_3$ in calcite), through intermediate (IMC, ≥4 wt% $MgCO_3$ in calcite to <8 wt% $MgCO_3$ in calcite) to high (HMC, ≥8 wt% $MgCO_3$ in calcite) (Kuklinski & Taylor 2009; Smith et al. 2006) with most bryozoans featuring LMC or IMC (Smith et al. 2006; Lombardi et al. 2008; Kuklinski & Taylor 2009; Taylor et al. 2009). Skeletal variability is exhibited at all taxonomic levels with the majority of variability occurring between different clades and species. In all species skeletal variation continues between different specimens within the same species. Why this wide spectrum of variation occurs is a question which has been puzzling scientists for nearly a century (Clarke & Wheeler 1917; 1922). There are a number of theories which have been proposed and discussed over the decades, with many still inciting debate today.

Evolutionary origin has been proposed to explain the variation in base mineralogy between taxonomic clades such as the orders of Cheilostomata and Cyclostomata. It is widely held that the ancestral mineralogy of the Bryozoa is LMC (Rucker 1968; Poluzzi & Sartori 1974; Boardman & Cheetham 1987; Borisenko & Gontar 1991; Taylor & Monks 1997; Taylor & Schindler 2004; Smith et al. 2006) and some authors assert that the older the evolutionary origins of the taxa (usually estimated by First Appearance Date (FAD) in the fossil record), the closer to this ancestral mineralogy the current mineralogy will be (i.e. LMC, no aragonite) (Smith et al. 1998; 2006). The fossil record indicates that there was a wide and rapid radiation in cheilostome species in the Cenozoic (65.5Ma-present) (Taylor et al. 2009), and some authors link the appearance of skeletal complexity to those taxa which first appeared after the late Cretaceous, during this time of rapid evolution (Smith et al. 2006; Taylor et al. 2009). An alternative theory is that mineralogy is less driven by the age of the taxa (FAD) but more by the seawater chemistry at the time of evolution. Bryozoa first evolved calcifying skeletons in the Lower Ordovician (Smith et al. 2006; Taylor et al. 2009), during a time of aragonitic seas, since then the oceans have cycled through calcitic seas and back to the aragonitic seas of the present day (Sandberg 1977; Taylor 2007). Aragonitic seas feature an Mg/Ca ratio which favours aragonite deposition and the formation of HMC/IMC. Calcitic seas have a much lower Mg/Ca ratio and favour calcite

deposition and the formation of IMC/LMC (Stanley & Hardie 1998; Taylor 2007); it is these seawater chemistry cycles which have been suggested as an explanation for the wide range in exhibited mineralogy in the Bryozoa (Sandberg 1977; Ries 2005; Taylor 2007; Taylor et al. 2009). Evolutionary origin of different species can be quantified using phylogenetic trees, which often use branch length to represent genetic distance of a species from their next nearest neighbour, and offer an alternative to the use of FAD.

Evolutionary/phylogenetic theories help to explain the differences in base mineralogy between taxonomic groups and species. Why then do we continue to see variability in mineralogy between specimens of the same species? The two main controls, which are exerted on bryozoan mineralogy within species, are summarised by many authors as biological and environmental control (Lowenstam 1954; Lowenstam 1964; Davis et al. 2000; Smith & Key 2004; Ries 2005; Steger & Smith 2005; Smith et al. 2006; Lombardi et al. 2008; Kuklinski & Taylor 2009), also known as "active" and "passive" control in publications by Schäfer & Bader (2005) and Bader & Schäfer (2008). Biological or "active" control indicates that calcium carbonate is deposited with a composition that is out of equilibrium with the seawater chemistry or temperature of the animal's habitat. Biological control in bryozoans usually refer to drivers such as astogeny, the thickening of secondary calcification in older zooids (Smith et al. 1998; Kuklinski & Taylor 2009; Smith & Girvan 2010), although it could also be considered to include growth rates (Barnes et al. 2007; Smith 2007; Kuklinski & Taylor 2008), breeding cycles, food availability (Bone & James 1993), physiological "wellness"(Stanley & Hardie 1998) and directed mineralization to confer a competitive advantage (Loste 2003; Borzęcka-Prokop et al. 2007; Smith & Girvan 2010). Environmental control, or "passive" control, indicates that skeletal mineralogy is driven by the seawater within which the bryozoan lives with little or no physiological involvement from the animal itself.

Past investigations into links between mineralogy and environmental conditions in the Bryozoa have been primarily concerned with temperature and have assumed predominantly environmental control of mineralogy. The earliest studies observed increasing aragonite in regions with warmer seawater (Lowenstam 1954; Carver & Rucker 1969). Some have investigated the use of stable oxygen isotopes as a proxy for seawater temperature (Smith & Key 2004; Lombardi et al. 2008; Knowles et al. 2010). Lombardi et al. (2008) compared

oxygen isotope and mean annual range of temperature (MART) derived temperatures against wt% $MgCO_3$ in calcite in an attempt to elucidate the accuracy of mineralogy as a temperature proxy.

Further studies have investigated the relationship between latitude and mineralogy (Smith et al. 2006; Kuklinski & Taylor 2009; Taylor et al. 2009; Smith & Girvan 2010; Loxton et al. 2012), with latitude usually considered a proxy for seawater temperature. Latitudinal studies have all either reinforced the expectation of a positive correlation between sea-water temperature and mineralogy in bryozoans (Smith et al. 1998; 2006; Taylor et al. 2008; 2009; Kuklinski & Taylor 2009; Loxton et al. 2012) or been inconclusive (Steger & Smith 2005; Smith & Girvan 2010). Other environmental factors, such as salinity, Mg/Ca ratio and aragonite compensation depth (ACD) also co-vary with latitude but have not been considered in any focused studies.

1.4.4 Known knowledge gaps and areas for advancement

Within the phylum Bryozoa there are still significant gaps in our knowledge of biomineralization. To date there have been ~2500 mineralogical analyses of bryozoan material covering less than 600 species, ~ 10% of known species. Globally, analyses have been limited to certain regions, centered primarily on the location of the scientists involved. Hotspots of mineralogy studies include New Zealand (Smith et al. 1998), Chile (Smith & Clark 2010), Mediterranean (Poluzzi & Sartori 1974) and the Polar regions (Borisenko & Gontar 1991; Kuklinski & Taylor 2009). The majority of mineralogy studies have involved single or low numbers of replicate analyses (Smith et al. 2006) and to date no studies have investigated seasonal changes in mineralogy. Indications of environmentally driven patterns in mineralogy have been detected, but as yet it has not been possible to quantify the effect of biological control on mineralogy or conclusively prove the existence of the vital effect. As such there is still uncertainty as to whether bryozoans can be used as "faithful recorders" of palaeontological conditions. Mineralogical studies on bryozoans to date have been heavily constrained by the mixed origin and limited availability of samples and associated metadata on environmental conditions. Taxonomic uncertainty around some species has also meant that comparing analyses between different publications can be problematic.

Although differential mineralogical composition of secondary calcification has been considered in some studies (Smith et al. 2006; Smith & Girvan 2010; Smith & Lawton 2010), differential deposition of skeletons at the zooidal scale has not been investigated in any studies. Localisation of minerals for mechanical or ecological advantage is yet to be considered for bryozoans.

Currently there is only a limited understanding of the biological process of mineralization in bryozoans. A schematic of calcification at the cellular level has been proposed, but as yet there have been no publications indicating genes, proteins or biochemical pathways which may control mineralogy in the phyla.

1.5 Aims and objectives

The aim of this study is to increase understanding of the skeletal mineralogy of Scottish and Polar bryozoans, investigating the level of variability within and between species in relation to:

- Specimen preservation and cleaning
- Taxonomic and phylogenetic relationships
- Spatial considerations including, latitude, depth, region and distance between sites
- Seasonal changes in environmental conditions
- Ecological adaptations
- Morphology at the ultra, micro and macro-scale

The study proceeds in eight chapters:

1. A general introduction to the thesis.
2. A controlled laboratory-based study investigating effects of specimen preservation and curational cleaning procedures on the skeletal mineralogy and morphology of *Flustra foliacea*. The study established a best practice for preservation and cleaning in subsequent chapters.
3. An extensive survey of the mineralogy of Scottish bryozoans. The purpose of this chapter is to establish a base-line for the bryozoan mineralogy of the region and

compare results with previously published regional studies. Latitudinal, taxonomic and phylogenetic patterns are also considered.

4. An ecological and mineralogical examination of bryozoan specimens collected through a two-year settlement panel study in the East, West and North of Scotland. Environmental data is collected for the duration of the experiment and the study enables the investigation of mineralogical patterns relating to seasonality, depth, temperature and site.
5. An investigation into intra-species variability and the comparative influence of environmental and biological factors of on the skeletal mineralogy of Antarctic and British bryozoans (Chapter 5). Samples are collected from the two regions in a twinned study, which aims to identify the impact of spatial scale between sites on mineralogy. The twinned study also allows comparison of the range of skeletal variability seen in species from stenothermal and eurythermal regions.
6. Utilisation of imaging and staining techniques to characterize the skeletal morphology of bryozoan specimens (Chapter 6). Morphology is investigated at the ultrastructure (crystal), zooidal and colony scale and the aim is to establish whether there are any patterns of differential localization of minerals and calcium carbonate polymorphs in skeletal features and discuss this in the context of ecological or mechanical advantage.
7. An in-depth case study applying all of the above techniques to the non-native bryozoan, *Schizoporella japonica* Ortmann, 1890 (Chapter 7).
8. An integration of the key findings obtained from the thesis experiments discussed in light of the stated aims. Chapter contributions are synthesized to identify overarching themes and implications. The thesis will conclude with recommended areas for future research (Chapter 8).

Chapter 2: The forgotten variables: The impact of common cleaning and preservation techniques on skeletal chemistry.

2.1 Abstract

For centuries bryozoan collections have been subjected to curational procedures involving a wide variety of preservation and cleaning techniques. To date, however, little consideration has been given to how cleaning and preservation may affect fragile or dissolution-susceptible calcium carbonate within the skeleton and consequently how this may influence any resulting mineralogical measurements.

The effects of cleaning procedures, including washing, bleaching and ultra-sonication, on the skeleton of a marine bryozoan are investigated in this study. Preparations comprised 26 treatments that were performed in triplicate on colony tips of *F. foliacea* prior to mass measurement and quantification of wt% $MgCO_3$ in calcite. The effects of preservation, using 96% ethanol, 70% IMS, 4% formal-saline and air-drying, were investigated on five bryozoan species. Preservation was undertaken for a minimum of two years, with samples extracted in triplicate at regular time-points to investigate wt% $MgCO_3$ in calcite.

Findings show that more mass and $MgCO_3$ in calcite is lost during cleaning with higher bleach concentrations, over longer durations and with use of ultrasonic cleaning. Specimens with higher initial Mg-calcite are especially susceptible to Mg-calcite loss during cleaning. Preservation experiments show that aragonitic and high Mg-calcite (HMC, >8 wt% $MgCO_3$ in calcite) species show no impact of preservation on wt% $MgCO_3$ in calcite over time but intermediate Mg-calcite species (IMC, >4 wt% $MgCO_3$ in calcite) both showed an increase in wt% $MgCO_3$ in calcite over time with preservation.

The interaction between bryozoan skeletal Mg-calcite and solutions, both cleaning and preservation, is found to be controlled by a combination of solution chemistry and reaction kinetics; preservation fluids in particular are highly dynamic and chemically complex.

In order to minimize the impact of cleaning and preservation on bryozoan Mg-calcite it is recommended that future specimens collected for X-ray diffraction analysis are rinsed in pH adjusted de-ionized water prior to air-drying on acid-free paper and long-term storage in an air-tight container.

2.2 General Introduction

2.2.1 Factors known to affect the mineralogy of calcium carbonate

During an animal's lifetime $CaCO_3$ mineralogy is deposited, reflecting both environmental conditions and biological factors such as astogeny (Smith et al. 1998; Kuklinski & Taylor 2009; Smith & Girvan 2010), growth rates (Smith 2007; Kuklinski & Taylor 2009), and physiological fitness (Stanley & Hardie 1998). It is this lifetime record which is of interest to scientists, however, following the death of the animal, further factors may subsequently influence the mineralogy of the $CaCO_3$ with the potential to confound environmental signals (Lombardi et al. 2010).

Following an animal's death, $CaCO_3$ material can be subjected to damage or alteration by ecological, bio-stratinomic or diagenetic processes. Early sea processes consist predominantly of the ecological factors of decomposition and scavenging (Zuschin et al. 2003) and the bio-stratinomic factors: abrasion and post-mortem transport (Smith et al. 1992; Zuschin et al. 2003). Decomposition and scavenging can damage the $CaCO_3$ material through breakage, scraping and crushing (Zuschin et al. 2003) and bacterial decomposition of organic material can expose the $CaCO_3$ to dissolution (Smith et al. 1992; Lombardi et al. 2010). Post-mortem transport can cause abrasion of fragile skeletal components (Chave 1964; Smith et al. 1992) or move the skeleton to different environments where different seawater composition or pH can subject the $CaCO_3$ to higher rates of dissolution (Chave & Schmalz 1966; Walter & Morse 1985; Burton 1993). Once the skeleton has been deposited as sediment, it can be subject to further mineralogical changes on the seafloor through diagenetic processes (Smith et al. 1992). Diagenesis covers everything that may happen after sediment is laid down until it becomes rock and can be mechanical (e.g. reworking, compaction), chemical (e.g. dissolution/precipitation, cementation) and organic (e.g. bio-turbation, bacterial action) (Smith et al. 1992; Zuschin et al. 2003). Chemical diagenesis is of particular importance for mineralogy as aragonite is either dissolved or transformed into calcite (Sandberg 1975; Canfield & Raiswell 1991) and structural gaps, left by dissolution or decay of organic material, can be filled with a high Mg-calcite cement (Bathurst 1975), especially in warm-water regimes (Smith et al. 1992).

In order to avoid the potentially confounding effects of taphonomy on mineralogical paleo-climate proxies it is recommended that recent specimens should be alive when collected (Smith et al. 1992; Lombardi et al. 2010).

2.2.2 Preservation of calcified organisms

Collecting invertebrates has been popular since the 17th century. The English botanist Samuel Doody (1656-1706) incorporated bryozoans in his herbarium collections, pressing them in books of heavy paper like leaves. A contemporary of Doody's at the Royal Society, James Petiver (1663-1718), was the proprietor of an apothecary shop in London, within which was assembled one of the largest and most varied collections of natural history specimens in England (Stearns 1952). In c1700 Petiver produced a single page folio offering advice on methods for preserving collections of "all natural curiosities" on voyages. In this pamphlet he recommended that larger marine animals collected on voyages be preserved in "Rack, Rum or Brandy" while "sea-mosses, coralls, corallines, sea-feathers, spunges etc" be "put altogether into any old box" or stacked in the manner of "a barrel of Colchester oysters"(Petiver 1700). Sir Hans Sloane (1660-1753) purchased both collections in the early 18th century and, unsurprisingly perhaps, Petiver's collections were found to be in a "poorly curated state" (Walker et al. 1999). When Carl Linnaeus (1707–1778) assembled his substantial collections, he also preserved marine invertebrates, including bryozoans, by drying and pressing them in herbarium books (Walker et al. 1999), a technique which, although simple, has resulted in collections which are still in an excellent state of preservation over 300 years later. During the later part of the 18th century amateur collecting increased, aided by the influx of unusual specimens from expanding empires and exploratory voyages overseas. John Ellis (1710-1776), a British linen merchant and naturalist, published detailed instructions on how to fix and preserve bryozoans in brandy (Ellis 1755) although unfortunately his collections, housed at the Royal College of Surgeons in London, were lost to a bomb in World War II. Spirit preservation, although in regular use for medical specimens during the time, was however, only rarely used by naturalists due to the prohibitively high cost of glass and distilled spirits. Consequently the majority of collections from this period were preserved by drying. In 1786 Abbé Manesse published the first book on specimen preservation aimed at the general collector entitled "Treatise on the Manner of Stuffing and Preserving Animals and Skins" (Manesse 1786).

Controversially at the time (Lee 1823), Abbé Manesse advised against the use of the common arsenic soap for larger specimens and, after several trials, instead advocated the use of alcohol solutions; based on the clear spirits we now recognize as vodka, gin, rum or brandy (Anonymous 1831). Abbé Manesse recommended a solution of 20% alcohol made with distilled water and added salt, nitre and alum (Anonymous 1831). A few years later in 1802 the German chemist M. Nicolas recommended an increase in the composition to 33% alcohol with added "sulphite" or "alumina". In 1817 the British naturalist, George Graves (1784-1839), published his popular "Naturalist's Pocket-Book", which was widely used by naturalists throughout the early 19th century; in this he recommended a 50% alcohol solution with added alum (Graves 1817). Graves was the first naturalist to specify detailed preservation techniques for different phyla. For shells, corals etc. he recommended killing the animal in brandy, cleaning in warm water and then drying and wrapping in paper. He only recommended preserving in alcohol solution if the "recent state" was required, advise no doubt influenced by the heavy taxation of spirit and glass containers (Great Britain Parliament 1812). These guidelines were echoed by Sarah Lee for zoophytes (1823) and John Stark for polypi (1828) and was detailed thoroughly in the Manual of the Practical Naturalist (1831). Following the repeal of the Window Tax by Robert Peel in 1845, glass containers became more affordable again and in 1855 duty-free methylated spirit (IMS) was also developed, increasing the prevalence of spirit preservation. In 1859 formaldehyde was first synthesized, and was subsequently identified in 1867 by August Wilhelm von Hofmann (1818–1892). Formaldehyde had the dual benefits of being both cheaper and less flammable than spirit and largely replaced alcohol as the fixative of choice for the next 100 years (Moore 1999). In recent decades it has emerged, however, that DNA is not preserved in formaldehyde, unlike in spirits, and in combination with a rising awareness of the health risks associated with formaldehyde, this has prompted many museum curators to return to the use of alcohol and drying as the long term preservatives of choice for modern bryozoan collections. Formaldehyde is still in use today but almost exclusively as an in-field fixation technique for bulk samples, where its low cost and relative ease of transport prior to dilution are preferred over alcohol.

Many observations have been recorded with regard to the effect of preservation on calcium carbonate, in some cases resulting in method refinements.

Drying: Calcareous shells are susceptible to damage in the acid environment caused by the use of unsuitable paper or wood in storage cabinets (e.g. oak) (Stansfield 1992). The release of acid fumes causes Byres disease: efflorescence on specimens resulting from the reaction between calcium carbonate and organic acid fumes from wood or paper (Walker et al. 1999). An additional risk to dried specimens is that humidity and pests can disrupt the protein matrix within the calcium carbonate skeleton (Lincoln & Sheals 1979; Walker et al. 1999); this can cause crumbling or disintegration of the skeleton (Steedman 1976). As a result it is widely recommended that acid-free paper or cotton is used to wrap specimens and cabinets are constructed of low acid wood. Dry collections in museums are housed in dry, cool, pest-free environments (Walker et al. 1999).

Fluid preservations (general): The choice of water used for dilution in fluid preservatives can impact the properties of the preservative. Tap water, although commonly used (Cato 1990), can vary significantly between regions. Hard water can be alkaline and contain high concentrations of Ca^{2+} and Mg^{2+} ions, which can interact with calcium carbonate composition and cause deposits to accumulate (Steedman 1976; Oomori et al. 1987). Tap water can also be acidic, adding to the low pH of the preservation solution and potential skeleton dissolution (Cato 1990). Seawater is a common diluent for preservatives, especially formaldehyde, but can be highly variable in composition between regions. At high levels of saturation calcite and aragonite can be deposited from seawater onto the skeleton (Steedman 1976); high CO_2 saturation can increase specimen dissolution (Steedman 1976) and varying Ca^{2+} and Mg^{2+} concentrations can cause movement of Mg between solution and skeletal calcite (Oomori et al. 1987). Distilled water has been recommended for dilution since the 18^{th} century as it "contains no foreign matter" (Manesse 1786). Distilled and deionized water, however, are often acidic and may act to dissolve calcium carbonate during long-term preservation (Steedman 1976).

The recommended ratio for fluid preservation is 9:1, fluid:specimen (Steedman 1976; Cato 1990; Moore 1999). The Sorenson reaction determines that the more specimens present, relative to fluid volume, the greater will be the amount of acid produced. Evaporation or over-packing of jars can, therefore, cause a reduction of pH and increase the risk of skeleton dissolution (Steedman 1976). Leaching of organic fats into the preservation fluid can occur in all forms of preservation. Its effects are heightened with alcohols, especially

IMS (Moore 1999), and causes acidity in the preservation solution (Marte et al. 2003). This decreased pH, in turn, causes increased calcium carbonate dissolution (Waller & Simmons 2003) and a return to higher pH levels; pH fluctuations are, therefore, not always easy to predict or monitor. Cork bottle stoppers have been shown to become acidic with time, affecting the preservation solution and potentially causing dissolution of calcareous specimens (Moore 1999).

Alcohol: Specimens preserved in high concentration (≥75%) alcohol have been observed to be brittle (Steedman 1976). Alcohol is also prone to evaporation and monitoring and regular topping-up is required in order to maintain the recommended 9:1 ratio (Notton 2010). Fixing in high concentration alcohol can cause desiccation and shrinking of organic material and fibres that can distort the skeleton and, in severe cases, cause fracturing (Moore 1999). The instability of alcohol pH was proven in 1977, when the wet collections of the Basel and Munich museums were tested for pH; 14% were found to have a pH <6 and 14% pH >8 (Taylor 1977); topping-up with ethanol was found to have no effect on the pH (Cato 1990). Changes in the pH of alcohol have since been proved to cause damage, decomposition and decalcification (Moore 1999; Kotrba & Golbig 2009). Potential damage to the mineralogy of calcium carbonate by ethanol has only been investigated in fish otoliths to date, although effects on both wt% $MgCO_3$ in calcite and isotopes have been recorded (Milton & Chenery 1998; Edwards et al. 2002; Hedges et al. 2004). Alcohol is currently often viewed by museum curators as a safe refuge against using the wrong preservative for future analysis (Moore 1999). The high cost of alcohol and the flammability risks, however, mean it is not used ubiquitously.

Formaldehyde: The pH of formaldehyde is 2.8 to 5. The Cannizzaro reaction between formaldehyde and water drives production of formic acid and will always be operating to lower pH, whether organic material is present or not (Steedman 1976). As a result calcareous specimens preserved in un-buffered formaldehyde will dissolve within days. Dilution with seawater is the usual "quick and dirty" way to buffer formaldehyde to a pH of 8.2, however, this is only shown to remain constant for approximately one week before it begins to drop (Steedman 1976). Buffering reduces the fixation effect on biological material (Moore 1999). Levels of Ca^{2+} and Mg^{2+} have been shown to increase over time in the preservation solution of amphibians (Waller & Simmons 2003). If this also occurs in

marine invertebrates, then this could cause interactions with skeletal Mg-calcite (Oomori et al. 1987). Formal-saline has been shown to cause a significant increase in stable isotope measurements in invertebrate calcium carbonate, interpreted as a result of partial dissolution and re-deposition (Ganssen 1981). Conversely, however, Edwards et al. (2002) found carbon isotopes to reduce across samples following preservation in formal-saline.

Despite its pH disadvantages and carcinogenic properties, formaldehyde is still recommended in some curation manuals as the preferred fixative (Steedman 1976; Lincoln & Sheals 1979; Moore 1999; Smithsonian: National Museum of Natural History 2013), although it is usually suggested that specimens are transferred to alcohol for long-term preservation (Lincoln & Sheals 1979; Moore 1999; Smithsonian: National Museum of Natural History 2013). Unfortunately, scientists do not always take the time to buffer in-field formaldehyde beyond dilution with seawater, and subsequent transference into a long-term preservative sometimes takes place months or even years after collection with little thought to the effect on calcareous samples.

Beyond the effects of dissolution, the chemical interactions between preservation solutions and calcium carbonate are poorly understood and little studied.

2.2.3 Cleaning and preparation

The first cleaning procedure to be documented for marine invertebrates was a recommendation to wash specimens in warm water prior to preservation (Graves 1817). John Stark (1828) echoes this advice by detailing that "polypi" should be dipped in fresh water to remove salt prior to preservation. The Manual of the Practical Naturalist (Anonymous 1831) offers more detailed instructions for bryozoans (referred to here as zoophytes and coralline) and suggests that the more calcified forms should be "washed in wine" prior to washing in water and, "if very dirty" may be cleansed with a mixture of "soapsuds and pearlash". Pearlash, Potash and Lye were all derived from soaking ashes in water to produce an alkaline solution and have been in use for the bleaching of textiles since the dawn of history; this is, however, the first record of their use for the bleaching of bryozoan skeletons. In 1903 Norman (1903) discusses in detail the different methods of preparation he employed prior to taxonomic work on bryozoans. These include incineration, boiling in potash solution and placing in "Eau de Javille". Claude Berthollet (1748-1822) first produced sodium hypochlorite in 1789 in the village of Bleach, near the

Mill Javelle in Paris. The resulting liquid became known as “Eau de Javelle” or “Bleach” and was a weak (~5%) solution of sodium hypochlorite (Banta et al. 1973). Norman observes that “Eau de Javille” is an aggressive substance to organic material that destroys not only soft tissues but also dissolves chitin (Norman 1903), an often beneficial result for taxonomists. The high quality drawings and descriptions in earlier taxonomic works by Linneaus, Hincks etc., suggest that it is likely Norman’s predecessors from the 1900s also employed cleaning, bleaching and preparation methods, however, as observed by Banta et al (1973), these are rarely documented and rather passed down by word-of-mouth.

The method of “Calcining”, or incineration as Norman calls it, is documented in an article by Rogick (1945) and details the method of burning off the organic material prior to mounting on a slide. Downsides to incineration are noted by both Norman and Rogick. Norman notes that specimens may not survive the incineration process (Norman 1903) and Rogick (1945) indicates that “calcined” specimens do not have the longevity of unprepared specimens. Banta et al. (1973) critique the process harshly, documenting that the life-spans of specimens are often reduced to weeks or months by the process, attributing this observation to decomposition of the organic matrix of the skeleton and crystal changes in the calcium carbonate. Banta et al. (1973) write that neutral or alkali solutions are the only acceptable form of preparation and advocate the use of 5% bleach, “Eau de Javille”, overnight to prepare specimens. Bleaching continues to be used in the majority of bryozoan studies up to the present day. Ultrasonic cleaning was developed in the mid 20th century and works through the process of cavitation, the formation of bubbles or cavities in a liquid. It is the collapse of these bubbles which generates shock waves which impinge on the surface of submerged items and effectively scour them (Chedd 1970). The use of ultra-sonication in the cleaning of bryozoan specimens seems to have first come into use in the early 1980s, with the earliest paper detailing ultrasonic use from 1982 (Dyrynda & Ryland 1982). It has since been used in at least 56 bryozoan studies.

Since the late 20th century, some scientists have begun to suspect that bleaching and ultra-sonication might have negative impacts on bryozoan calcium carbonate skeletons. Sandberg (1971), observed that “overzealous ultrasonic treatment may result in disintegrated specimens” and Taylor & Weedon (2000) describe a delicate calcium carbonate structure which “in many specimens is wholly or partly lost during bleaching or

cleaning". Opinion remains divided in the literature, however, with Smith et al. (1998) asserting that neither ultrasonic or bleaching affects mineralogy results. Chedd (1970) explains that the effects of ultrasonic techniques on a material are poorly understood and remain at the "empirical suck-it-and-see level", although some studies have been conducted into the effects of ultrasound on calcareous specimens. In 30 minute experiments, Clark (1973) demonstrated that optimum cleaning of nano-fossils occurred very rapidly and continued ultrasound after this point caused only damage to calcareous structures. Hodgkinson (1991) summarises the method as a "damaging and largely uncontrolled cleaning" after observations that any weakness in foraminifera tests is almost immediately fractured using ultrasonic. Watanabe et al. (2001) assessed the effects of a range of pre-treatments, all of which included ultrasonic, on Mg/Ca in coral aragonite and found that in all cases Mg/Ca was reduced after treatment.

The effects of bleaching with sodium hypochlorite have been less well studied. In a study on scolecodonts in 1961, it was observed that Chlorox (main active ingredient: sodium hypochlorite) caused translucency of specimens and the dissolution of fragile components in some specimens (Tasch & Shaffer 1961). Keatings et al. (2006) examined the effects of bleaching on ostracod valve chemistry and found no change in Mg/Ca following bleaching. Effects of chemical oxidation on Mg-calcite have been somewhat more investigated. Both Marr et al. (2013) and Feldmeijer et al. (2013) found no difference in Mg/Ca for Foraminifera treated with short periods of chemical oxidation. Barker et al. (2003) however, saw reductions of up to 25% in Mg/Ca resulting from chemical cleaning, although specimens subjected to less than 20 minutes oxidative treatment are observed to display an elevated Mg/Ca. Watanabe et al (2001) also found Mg/Ca to be reduced in coral aragonite with all pre-treatments. Conversely, Mitsuguchi et al. (2001) report an increase Mg/Ca in coral aragonite following oxidation. Krause-Nehring et al. (2011) found a mixture of losses and gains of Mg/Ca following pre-treatment of the bivalve *Arctica islandica*. Overall, in the literature, there is no consensus as to the effect of bleaching or oxidation on Mg/Ca in calcium carbonate and the topic continues to incite debate.

2.3 Aim and Objectives

2.3.1 Background to purpose of study

The effects of cleaning and preservation on bryozoan $MgCO_3$ in calcite are under-studied and poorly understood. There is an abundance of historic samples stored in museum collections, which could potentially be used for mineralogical studies of palaeoclimate. In addition new specimens are being added to collections daily which may be used for future investigations into climate change effects. The potential importance of these specimens and their skeleton chemistry behooves the examination of the effects of different cleaning processes and preservation techniques, as well as the temporal effects of storage, on wt% $MgCO_3$ in the calcite of bryozoans.

2.3.2 Objectives of study

1. Investigate the effects of ultra-sonication and bleaching on the skeletal mineralogy of *Flustra foliacea.* Methodologies will test the individual and combined effects of ultra-sonication, bleach of varying concentrations, water source and the temporal effects of these treatments on wt% $MgCO_3$ in calcite.

2. Investigate the effects of specimen preservation using 96% ethanol, 70% industrial methylated spirits (IMS), 4% formal-saline and air-drying on the wt% $MgCO_3$ in the skeletal calcite of five bryozoan species over a two year period.

2.4 Materials and Methods

2.4.1 Experiment to investigate the effects of cleaning methods on Mg-calcite

All replicates for this experiment were taken from a single colony of *Flustra foliacea* collected by SCUBA on 27/10/2012 from a depth of 16m at Saulmore Point, West Scotland (56.455021N, 5.413649W). The specimen was dried as collected and processed within a month. An experimental flow chart is shown in Figure 2.1; all steps were conducted on triplicate samples. To ensure uniform surface area of samples for treatment a 65mm diameter punch was used to extract a disc. Each disc was weighed using a fine scale balance (accurate to 0.01mg) prior to treatment. The material remaining, following disc extraction, was subdivided into portions A and B (Fig. 2.1) which were used to establish a baseline for the total carbon content, ratio of organic:inorganic carbon and Mg-calcite for the untreated sample. Carbon content was obtained from portion A using CHN analysis following the protocol described in Section 2.3.4. X-ray diffraction was used to determine wt% $MgCO_3$ in calcite in portion B following the protocol described in Section 2.3.3.

Treatments were conducted on the extracted discs (C/D) as shown in Table 2.1.

Table 2.1: Treatment composition for treatment 1-24 and subsequent analysis. Ticks indicate the associated analysis was conducted for those treatments, crosses indicate the analysis was not conducted for treatments.

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
Treatment	Ultrasonic:	NO ULTRASONIC												ULTRASONIC											
	Water source:	DEIONIZED						TAP						DEIONIZED						TAP					
	Bleach conc'n (%):	0%		10%		78%		0%		10%		78%		0%		10%		78%		0%		10%		78%	
	Time (minutes):	10	480	10	480	10	60	10	480	10	480	10	60	10	480	10	60	10	60	10	480	10	60	10	60
Analysis	XRD before (B)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	XRD after (D)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	CNH before (A)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	CNH after (C)	✓	×	✓	✓	×	×	×	×	×	×	×	×	×	×	✓	✓	×	×	×	×	×	×	×	×
	ICP-AES (sol G)	✓	×	✓	✓	×	×	×	×	×	×	×	×	×	×	✓	✓	×	×	×	×	×	×	×	×
	staining before (E)	×	×	×	✓	×	×	×	×	×	×	×	×	×	×	×	✓	×	×	×	×	×	×	×	×
	staining after (F)	×	×	×	✓	×	×	×	×	×	×	×	×	×	×	×	✓	×	×	×	×	×	×	×	×

Ultrasonic treatment was conducted using an ultrasonic bath. Bleach used was Domestos™ containing 40.5g/l sodium hypochlorite. Dilutions were carried out with either deionised water or tap water (Kensington, London SW7) as specified. Kensington tap water ion content can be found online and is notable for its hardness and high natural magnesium and calcium content. Samples of tap water and deionised water were collected on the day of the experiment (9/11/2012), fixed using mercuric chloride at a 0.02% volume ratio (Dumousseaud et al. 2011) and analysed by the National Oceanographic Centre (NOC)

Carbonate Chemistry Facility for total Dissolved Inorganic Carbon (DIC), Total alkalinity (TA), pCO_2 and aragonite and calcite saturation states. Methods used to determine these factors are described in the Carbonate Chemistry Facility practical guide (Dumousseaud et al. 2011).

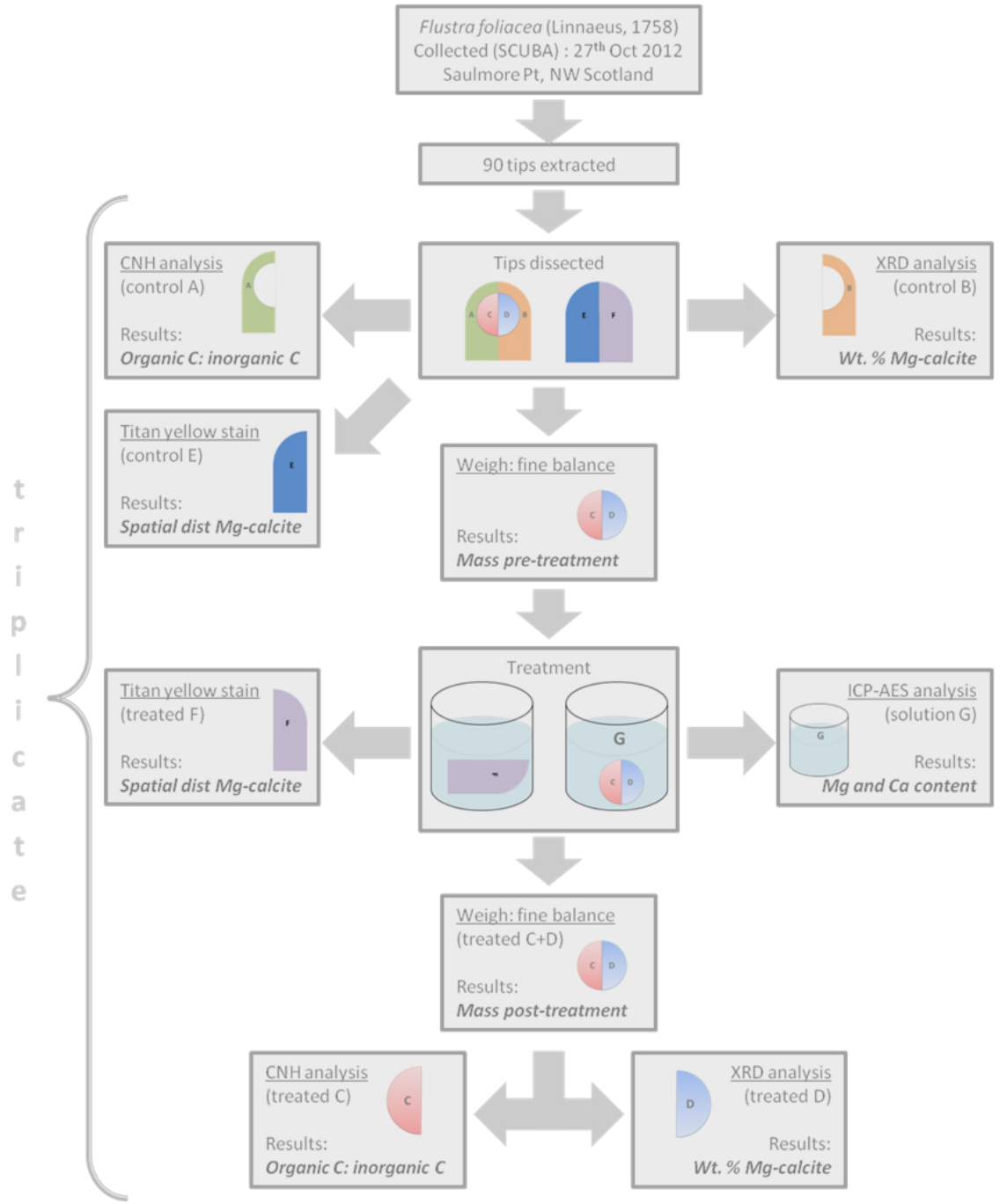


Figure 2.1: Flow-chart detailing methodology for cleaning experiment and subsequent analysis. All steps were conducted on triplicate samples.

Following treatment sample discs (C/D) were rinsed in water (either deionised or tap depending on treatment), thoroughly air dried and re-weighed. Discs were subdivided as shown in Figure 2.1 into portions C and D. Using the methodologies specified in Section 2.3.3 and 2.3.4 the wt% $MgCO_3$ in calcite in portion D was determined using XRD and the carbon in portion C was analysed using CHN for some treatments as detailed in Table 2.1. Some treatment solutions (solution G), detailed in Table 2.1, were retained for subsequent Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) analysis following the protocol described in Section 2.3.5. For selected treatments (Tab. 2.1) additional tips were divided in half lengthways to form portions E and F (Fig. 2.1). Prior to rinsing and drying, treatment was conducted on portion F, as detailed in Table 2.1. Portions E and F were etched in 5% acetic acid for 30 seconds, dried and then immersed in Titan Yellow dye (prepared as per Choquette and Trusell (1978)) for 20 minutes. Titan Yellow stain is specific for Mg-calcite, staining areas of higher Mg red. Staining was fixed using sodium hydroxide and specimens were imaged using a Zeiss light microscope and camera.

2.4.2 Experiment to investigate the effects of preservation on Mg-calcite

Specimens of British bryozoans were collected live from the West Coast of Scotland by SCUBA or wading as shown in Table 2.2.

Table 2 2: Specimen details. * indicates specimen was collected and preserved on macroalgae

Species	Date	Location
Membranipora membranacea (Linnaeus, 1767)*	19/1/2011	Clachan Siel, Scotland. (1m depth) 56.317084N, -5.583951W
Electra pilosa (Linnaeus, 1767) *	19/5/2011	Clashfarland Point, Scotland. (6m depth) 55.45567N, 0.453853W
Flustra foliacea (Linnaeus, 1758)	26/10/2011	Saulmore Point, Scotland (16m depth, 13ºC) 56.455021N, -5.413649W
Scrupocellaria reptans (Linnaeus, 1758)	26/10/2011	Saulmore Point, Scotland (16m depth, 13ºC) 56.455021N, -5.413649W
Crisia eburnea (Linnaeus, 1758)	26/10/2011	Saulmore Point, Scotland (16m depth, 13ºC) 56.455021N, -5.413649W

Specimens were rinsed in fresh water and then transferred immediately into preservative made up to the following recipes:

- 4% formal-saline: 10% formaldehyde (40% concentration) + 90% seawater
- 96% ethanol: 96% medical grade ethanol + 4% tap water
- 70% IMS: 70% Industrial Methylated Spirits + 30% tap water
- Air-dried specimens were allowed to dry naturally on tissue for at least 3 weeks before transferring into plastic bags or boxes.

All specimens were stored in a dark cupboard at room temperature. At each time-point three samples from the tip or growing edge were removed from each preservation/species. Samples were rinsed twice in tap water before bleaching for 1 hour in 10% bleach (Domestos™ bleach containing 40.5g/l sodium hypochlorite, diluted using tap water). Samples were rinsed a further two times and dried at 55ºC for 2 hours, and stored in gelatin capsules for not more than one week before XRD analysis to determine wt% $MgCO_3$ in calcite (see protocol in Section 2.3.3).

On the 16/10/2013 the pH of the preservation fluids was determined using Merck indicator strips (pH 4-7) which were read while moist and are indicated as being accurate to 0.2 pH units.

2.4.3 XRD analysis

Mineralogical analyses were conducted at the Natural History Museum in London (NHM) using a high-precision Nonius X-ray Diffractometer (XRD) with a position-sensitive detector and cobalt generated X-rays. To set up the machine fluorescent powder was used to visualize the X-ray beam (Fig. 2.2) and the X-ray beam width and substrate angle, μ, were adjusted to ensure tight beam focus (μ=5.9).

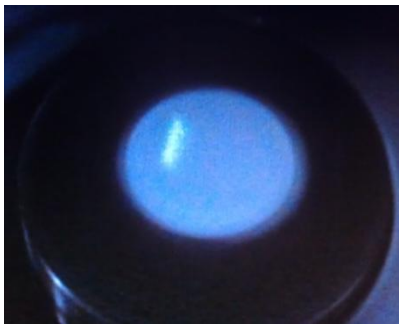


Figure 2.2: Adjusted X-ray beam shown on fluorescent powder.

Pure silica (Si) and silver behenate ($AgC_{22}H_{43}O_2$) on quartz substrate were used as the instrument calibration standards (Blanton et al. 1995; 2000) and the instrument was calibrated daily. Bryozoan samples were powdered using a quartz pestle and mortar and affixed using a drop of acetone to single quartz crystal substrates of 3.5mm depth.

Quantitative XRD analysis was undertaken to determine the wt% $MgCO_3$ in calcite. The wt% $MgCO_3$ in calcite is accurate to within 2% on a well-calibrated instrument (Kuklinski & Taylor 2009).

X-rays were generated by bombarding a target material, in this case cobalt, with electrons until X-rays were produced; CoK_α radiation has a wavelength (λ) of 1.788965Å. When the incoming X-rays met the sample, interference took place between the crystal lattice structure and the X-ray (Fig 2.3). The X-ray was subsequently diffracted and the angle of diffraction (2θ) and the intensity (counts) were detected and recorded as a spectrum (Fig. 2.4).

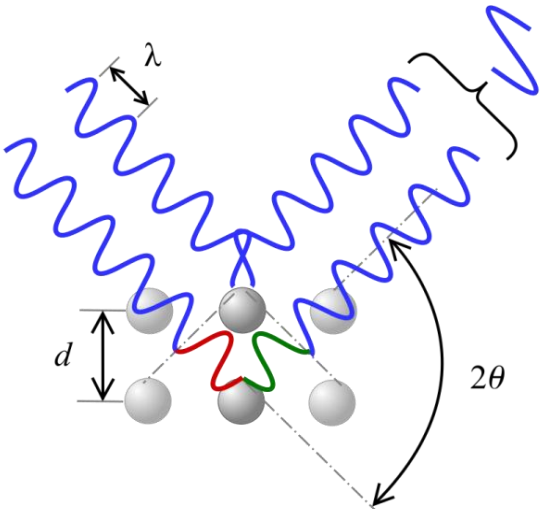


Figure 2.3: X-ray diffraction according to Bragg's law (Gregors 2013)

The peak height (measured in counts) of an individual spectra peak, reflects the intensity of the diffracted ray (y-axis) and the position on the x-axis is the angle of diffraction (2θ).

In order to calculate wt% $MgCO_3$ in calcite, the midpoint position of the d104 peak (approx. 34.5 on the x-axis), the most intense calcite peak, was measured at 50% of the maximum peak height to give the angle of diffraction, 2θ, for the sample (Fig. 2.4).

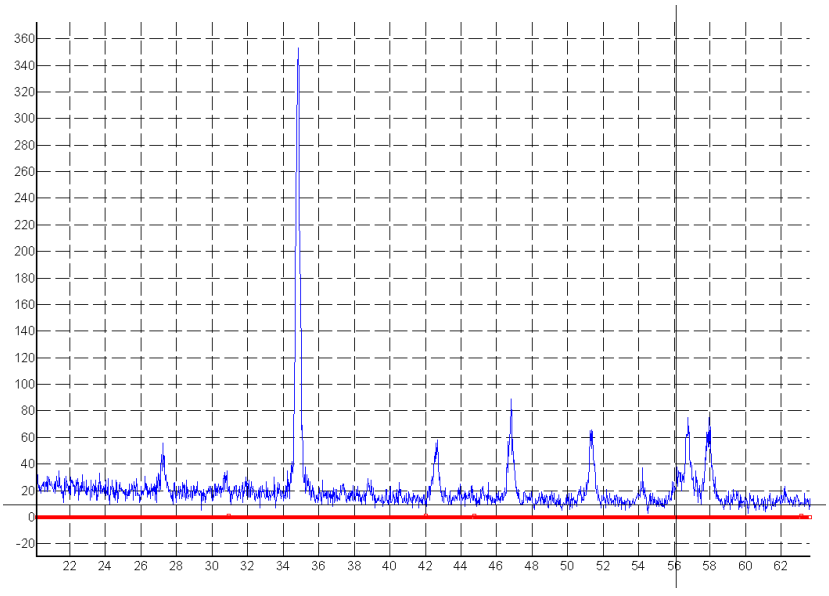


Figure 2.4: XRD spectrum for a calcitic bryozoan. y-axis shows counts as a gauge of peak intensity. x-axis shows 2θ.

Using the wavelength of the X-ray radiation (λ) and the 2θ from the spectra, Bragg's Law (Eq. 2.1) can be applied to calculate the d-spacing (d); the spacing between the planes of a crystal lattice (Fig. 2.3).

Equation 2.1: Bragg's law

$$n\lambda = 2dsin\theta$$

Crystals of pure $CaCO_3$ and those containing some $MgCO_3$ have different lattice structures (Fig. 2.5) caused by the different sizes of Ca and Mg ions, and subsequently have different d-spacing.

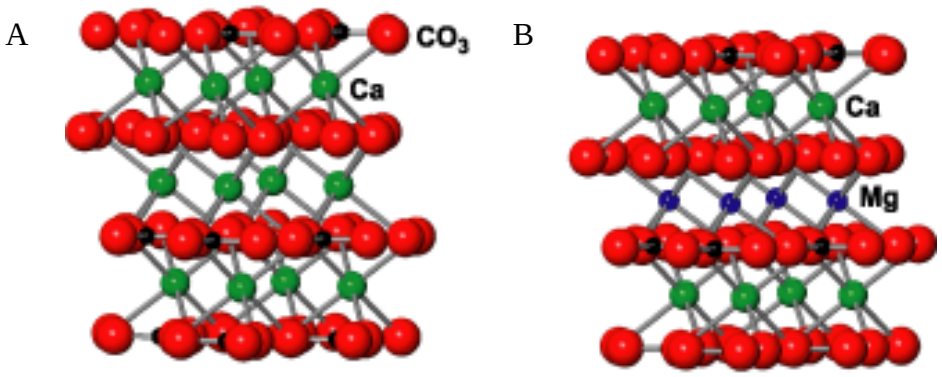


Figure 2.5: Crystal lattice structures of A) calcite ($CaCO_3$) and B) dolomite (~35 Mol. % $MgCO_3$). (Reeder 2013)

By assuming a linear interpolation between the d-spacing of $CaCO_3$ (3.035Å) and $MgCO_3$ (2.742Å) (Fig. 2.6), a calculation of the molecular percent (Mol. %) of $MgCO_3$ in calcite for the sample can be calculated from the d-spacing (d) of the sample using Equation 2.2.

Equation 2.2: calculation of the mol. % $MgCO_3$ in sample calcite from the d-spacing

$$\text{Mol.\% } MgCO_3 \text{ in calcite (sample)} = \frac{3.035 - d(sample)}{3.035 - 2.742}\ x\ 100$$

Skeletal carbonate mineralogy is reported in a variety of units in the literature, including % Mg, Mol. % $MgCO_3$ and wt% $MgCO_3$. In this study wt% $MgCO_3$ in calcite is used as it is a more commonly used term in biogeochemistry and is also more in fitting with other proportional mass measurements in this study such as mass loss, wt% calcite and organic:

inorganic carbon ratios. Conversion between units is simple and a chart detailing the method is found in Table 3 in Smith et al. (2013).

Mol. % $MgCO_3$ in calcite was converted to wt% $MgCO_3$ in calcite using the molecular weights of $CaCO_3$ (MW = 84.32135) and $MgCO_3$ (MW = 100.08935) (Eq. 2.3).

Equation 2.3: conversion of Mol. % $MgCO_3$ in calcite (Mol. %) to wt% $MgCO_3$ in calcite (wt%)

$$\text{wt\%} = \left(\frac{Mol.\% \; x \; 84.32135}{(Mol.\% \; x \; 84.32135) + \; ((100 - Mol.\%) x \; 100.08935}\right) x \; 100$$

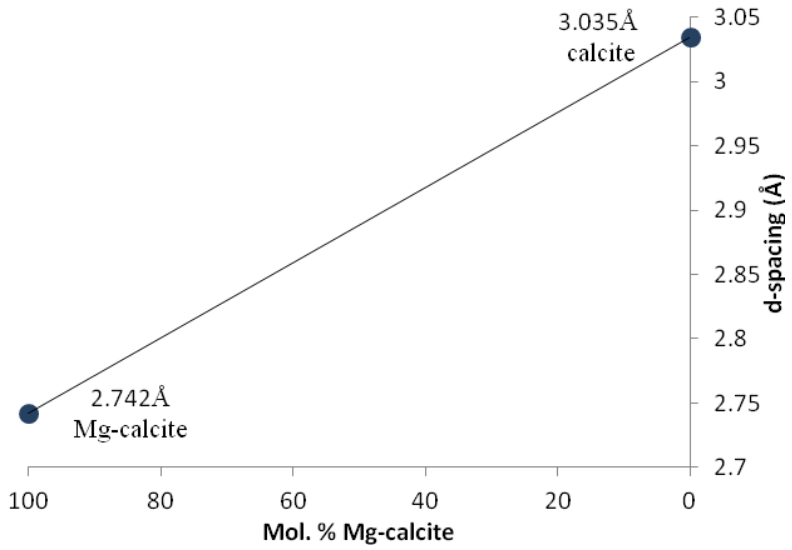


Figure 2.6: Linear interpolation between d-spacing of $MgCO_3$ and $CaCO_3$ when plotted against Mol. % $MgCO_3$ in calcite. Mg-calcite in this figure refers to calcite where every Ca ion has been replaced with Mg.

2.4.4 CHN analysis

The sample (C) was finely ground using a quartz pestle and mortar and divided into two quotients of approximately 5mg each. The first quotient was placed into a tin capsule and weighed. The second quotient was placed in a silver capsule, weighed and then repeatedly treated with dilute hydrochloric acid until all inorganic carbon had been dissolved (there was no more fizzing). Between acid treatments the quotient was allowed to dry on a heated ceramic plate. Following acid treatment, the second quotient was re-weighed.

A Thermo Finnigan EA112 Elemental Analyser was used to combust all quotients at temperatures exceeding 900ºC with a flow of pure oxygen; the resulting carbon oxides were quantified chromatographically. The results for the first quotient provided data on the

total carbon content in the sample, the second quotient provided data on the organic carbon in the sample; subtraction of the organic carbon from the total carbon allows calculation of the inorganic carbon in the sample. CHN accuracy was measured on multiple analyses of aspartic acid throughout the run; N, C and H variability was measured as <1%. A secondary standard, BBOT (2,5-(Bis)-5-tert-butyl-2-benzo-oxazol-2-yl)thiophene), was tested during the batch and gave a deviation of < l% from the expected values for carbon. Results are, therefore, considered accurate to within 1%.

2.4.5 Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES)

Quotients (9.8ml) of the solution (G) were digested using 2ml of concentrated HNO_3 and 0.5ml of concentrated H_2O_2. The solution was dried down on a heated ceramic plate prior to making up to 10ml with 2% HNO_3 solution. The resulting solutions were run through a Thermo iCap 6500 Duo machine. An inductively coupled plasma (ICP) is used to produce excited atoms from the sample, which emit electromagnetic radiation at wavelengths characteristic of the elements Mg and Ca. A spectrometer separates and resolves these lines and measures their strength in order to quantify content of Mg and Ca in the solution. An in-house standard was run alongside samples and found to be accurate to 9% for Ca and 5% for Mg. Results are, therefore, considered accurate to 10%.

2.4.6 Review of past cleaning and preservation methods

To my knowledge 33 papers have been published, where the mineralogy of >2500 bryozoans was investigated using XRD (Cheetham et al. 1969; Greeley 1969; Carver & Rucker 1969; Smith 1970; Sandberg 1971; Siesser 1972; Poluzzi & Sartori 1973; Poluzzi & Sartori 1974; Patzold et al. 1987; Agegian & Mackenzie 1989; Bone & Wass 1990; Borisenko & Gontar 1991; Bone & James 1993; Bone & James 1997; Rahimpour-Bonab et al. 1997; Taylor & Monks 1997; Smith et al. 1998; Crowley & Taylor 2000; Grischenko et al. 2002; Machiyama et al. 2003; Taylor & Schindler 2004; Steger & Smith 2005; Gordon et al. 2006; Lombardi et al. 2008; Schäfer & Bader 2008; Kuklinski & Taylor 2009; Taylor et al. 2009; Smith & Clark 2010; Smith & Girvan 2010; Loxton et al. 2012). These articles were examined to determine any preparation/cleaning techniques used in past studies.

2.4.7 Data analysis

Graphical representations of data were produced using the R programming language (R Core Team 2013) and Excel (Microsoft 2006). Plates were prepared using GIMP (Kimball & Mattis 2012).

Effects of cleaning (detailed in Section 2.3.1): wt% $MgCO_3$ in calcite and mass data were tested for normality using Anderson-Darling normality tests; Mg-calcite was found to be normally distributed whilst mass failed the normality test. Homogeneity of variance for Mg-calcite and mass were tested using Levine's test of equal variance; both passed Levine's test. As the criteria for parametric testing were satisfied, data were analysed using linear regression and generalised linear model (GLM) ANOVA with *posthoc* Tukey testing. The best-fit multiple regression model was determined through creation of the most complicated model (all factors and factor combination) and stepwise deletion of non-significant factors and factor combinations until full significance was achieved for all remaining factors/factor combinations. Bootstrapping was done with the package "boot" (Ripley 2013), re-sampling the full observation vectors for 1000 iterations. Relative importance of regressors in multiple regression was calculated using the "reclaimo" package (Groemping 2013) in R (R Core Team 2013) using the function calc.relimp. This function uses the *lmg* method to calculate R^2 partitioned by averaging over orders (Lindeman et al. 1980).

Effects of preservation (detailed in Section 2.3.2): wt% $MgCO_3$ in calcite data were tested for normality using Anderson-Darling normality tests and failed the normality test. Mg-calcite passed, when tested for homogeneity of variance using Levine's test of equal variance. As the criteria for parametric testing were satisfied, data were subdivided by species and preservation and tested for statistically significant (GLM) ANOVA relationships between Mg-calcite and preservation duration (time).

2.5 Results

2.5.1 Cleaning Methods

Effect of starting Mg-calcite concentration on subsequent Mg-calcite loss

There is a statistically significant linear relationship between the Mg-calcite lost during cleaning and the starting concentration of Mg-calcite in the sample ($p<0.001$, $R^2=68.14\%$) (Fig. 2.7)

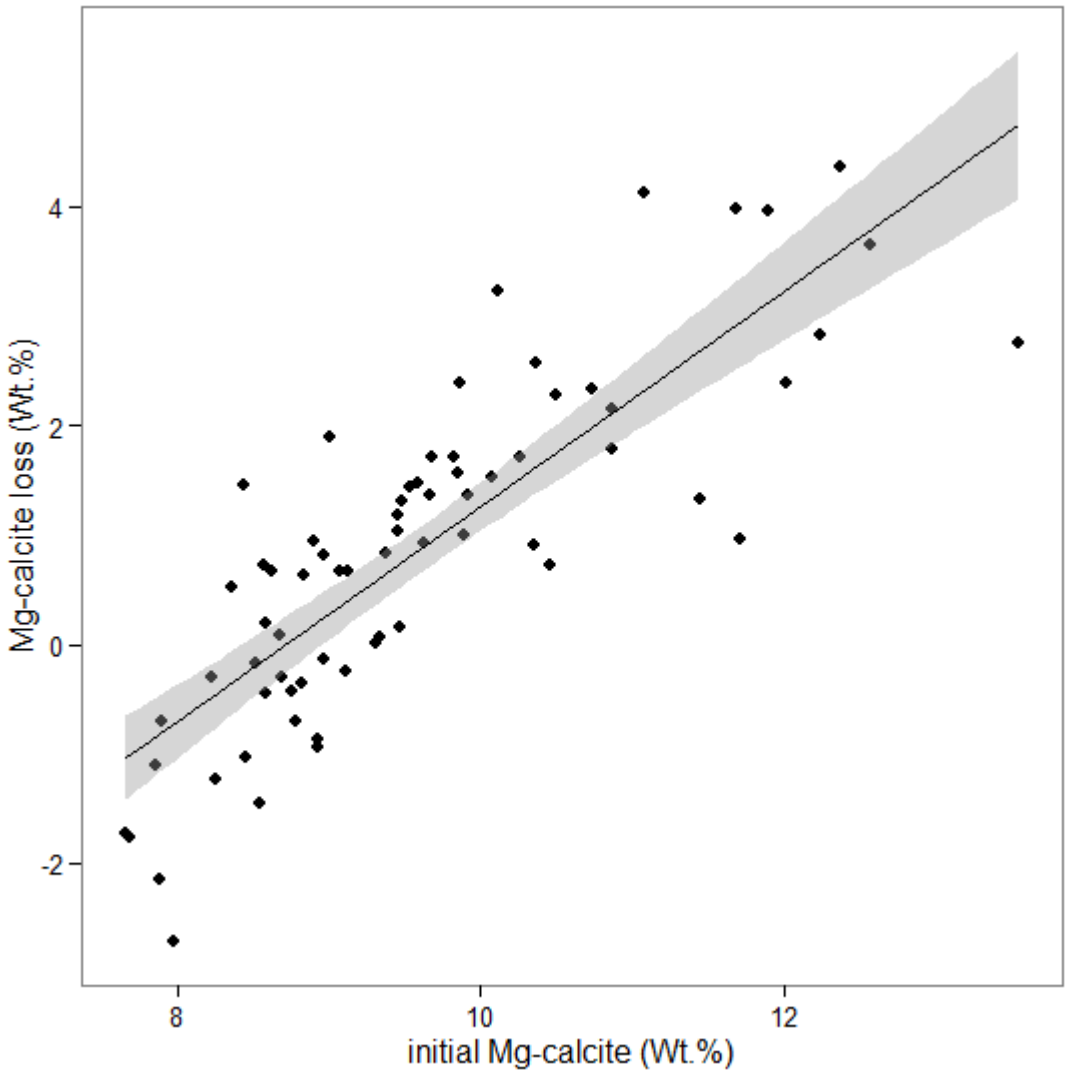


Figure 2.7: Scatter plot showing the wt% $MgCO_3$ in calcite lost from samples against their initial wt% $MgCO_3$ in calcite. All samples are shown. Linear relationship indicated by line +/- 5% error (grey shading)

The relationship between initial wt% $MgCO_3$ in calcite and wt% $MgCO_3$ lost from calcite is very similar for deionised water and Kensington tap water (Fig. 2.8). Deionised water has a

statistically significant linear relationship between initial wt% $MgCO_3$ in calcite and wt% $MgCO_3$ lost from calcite during cleaning ($p<0.001$, $R^2=70.15\%$) with the equation $y = -9.2996 + 1.0652x$. The line crosses 0 on the y-axis at 8.73 wt% $MgCO_3$ in calcite (Fig. 2.8 top). Kensington tap water has a statistically significant linear relationship between initial wt% $MgCO_3$ in calcite and wt% $MgCO_3$ in calcite lost during cleaning ($p<0.001$, $R^2=67.14\%$) with the equation $y = -8.2633 + 0.9471x$. The line intercepts 0 on the y-axis at 8.725 wt% $MgCO_3$ in calcite (Fig. 2.8 bottom).

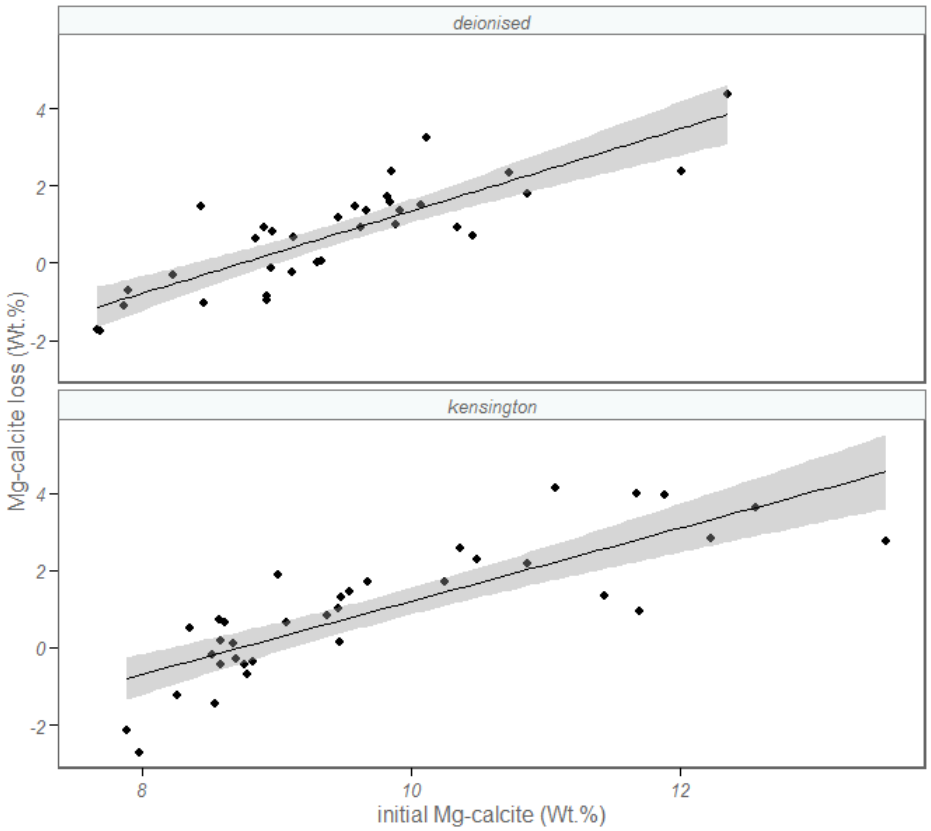


Figure 2.8: Scatter plots for deionised and Kensington tap water showing the Mg-calcite lost from samples against their initial Mg-calcite. Linear relationships indicated by line +/- 5% error (grey shading)

To try to reduce the confounding effect of this relationship on subsequent analyses, the percentage of initial wt% $MgCO_3$ lost from calcite, rather than a direct measure of wt% $MgCO_3$ lost from calcite, will be used to investigate the effect of other factors on wt% $MgCO_3$ in calcite.

Water: Tap water was found to contain more dissolved inorganic carbon (DIC), carbonate ions and dissolved CO_2 (pCO_2) than deionised water and, subsequently had a higher pH, total alkalinity (TA) and saturation of calcite and aragonite ($\Omega_{calcite}$ and $\Omega_{aragonite}$). Deionised water contained a higher concentration of OH ions and was subsequently more acidic than tap water (Tab. 2.3).

Table 2.3: Results of carbonate analysis of tap water and deionised water used in experiment 2.4.1

	pH	*TA*	*DIC*	*pCO2*	*HCO3*	*CO3*	*OH*	*W calcite*	*W aragonite*
tap water	8.1	4295.1	4208.6	918.8	4031.6	131.2	0.7	1.87	1.11
deionised	6.53	33.5	27.8	1.5	24.8	2.9	2.3	0.03	0.02

There is no statistical difference for either mass loss or wt% $MgCO_3$ lost from calcite following cleaning treatments (n=13) relating to the use of deionised versus tap water (two way ANOVA, mass loss = water*treatment, F=1.331, p=0.253; Mg-calcite loss = water*treatment, F=0.324, p=0.697). Results for both deionised and tap water treatments were, therefore, combined to create a larger dataset, prior to statistical analysis for other factors.

Ultrasonic: There is a strong statistically significant difference between mass loss observed following cleaning treatments (n=6) using ultrasonic versus those not using ultrasonic (2-way ANOVA, sonic*treatment, F=5.339, p<0.001). *Posthoc* analysis, however, shows this to only be significant for treatment 6, 78% bleach x 60 mins (Tukey, p<0.001)

There is no statistical difference between wt% $MgCO_3$ lost from calcite following cleaning treatments (n=13) using ultrasonic versus those not using ultrasonic (2-way ANOVA, sonic*treatment, F=0.370, p=0.867).

Bleach concentration: There is a strong statistically significant difference between mass loss observed following cleaning treatments (n=4) using different concentrations of bleach (0%,10%,78%), (2-way ANOVA, bleach concentration*treatment, F=25.19, p<0.001). Tukey *Posthoc* analysis shows this to be significant between treatments conducted with 0% and 10% bleach (p<0.001) and with 0% and78% bleach (p<0.001). There is no significant difference between treatments conducted with 10% and 78% (p=0.426).

There is no statistical difference between wt% $MgCO_3$ lost from calcite following cleaning treatments (n=13) using different concentrations of bleach (2-way ANOVA, bleach concentration*treatment, F=1.432, p=0.238).

Time: There is a strong statistically significant difference between mass loss observed from cleaning treatments (n=6) conducted for different lengths of time (10mins, 60mins, 480mins) (2-way ANOVA, time*treatment, F=67.347, p<0.001) (Fig.2.9).

Linear analysis for the effect of time for individual treatments shows this to be significant for treatments using 10% bleach without ultrasonic (Treatment 3, p<0.001, R^2=84.7%, $y = 0.72163 + 0.00384x$) and with ultrasonic (Treatment 4, p<0.001, R^2=87.05%, $y = 1.058 + 0.0224x$); and treatments using 78% bleach both without ultrasonic (Treatment 5, p<0.001, R^2=92.73%, $y = 0.6807 + 0.0443x$) and with ultrasonic (Treatment 6, p<0.001, R^2=93.39%, $y = 0.5467 + 0.0702x$). There is no significant relationship with time for treatments conducted with 0% bleach (Treatments 1 and 2).

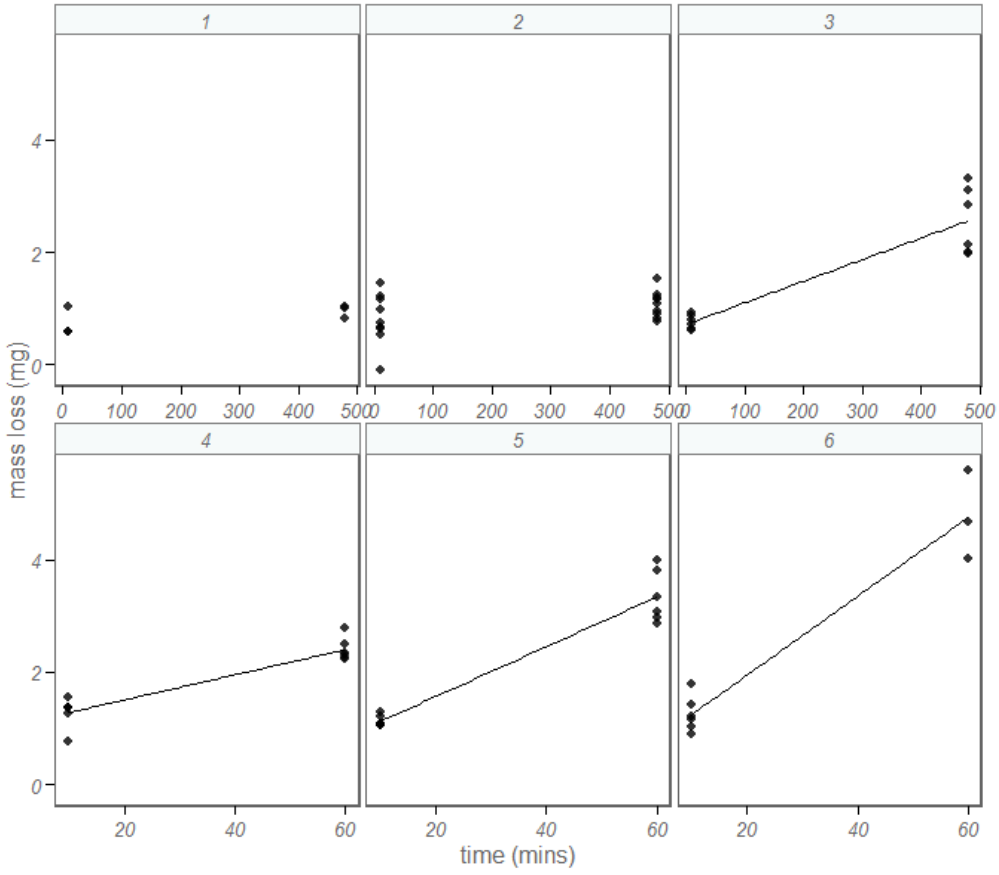


Figure 2.9: Scatter plots and linear regressions show effect of time on treatments. Both deionised and tap water presented. Treatments are: 1: 0% bleach; 2: 0% bleach + ultrasonic; 3: 10% bleach; 4: 10% bleach + ultrasonic; 5: 78% bleach; 6: 78% bleach + ultrasonic.

There is no statistically significant difference between Mg-calcite loss observed from cleaning treatments (n=6) conducted for different lengths of time (two way ANOVA, time*treatment, F=1.801, p=0.127).

Combined effects of factors: Multiple regression modeling indicates that five factors/ factor combinations are significant in explaining 85.44% of mass loss during treatment (Fig. 2.10):

- bleach concentration*time*initial wt% $MgCO_3$ in calcite*ultrasonic (LMG method, p<0.001, explains 77.86% of variance)
- bleach concentration*initial wt% $MgCO_3$ in calcite (LMG method, p=0.004, explained 6.72% of variance)
- bleach concentration (LMG method, p=0.003, explained 6.64% of variance)
- bleach concentration*ultrasonic (LMG method, p<0.001 , explained 6.51% of variance)
- initial wt% $MgCO_3$ in calcite*ultrasonic (LMG method, p<0.001 , explained 2.28% of variance)

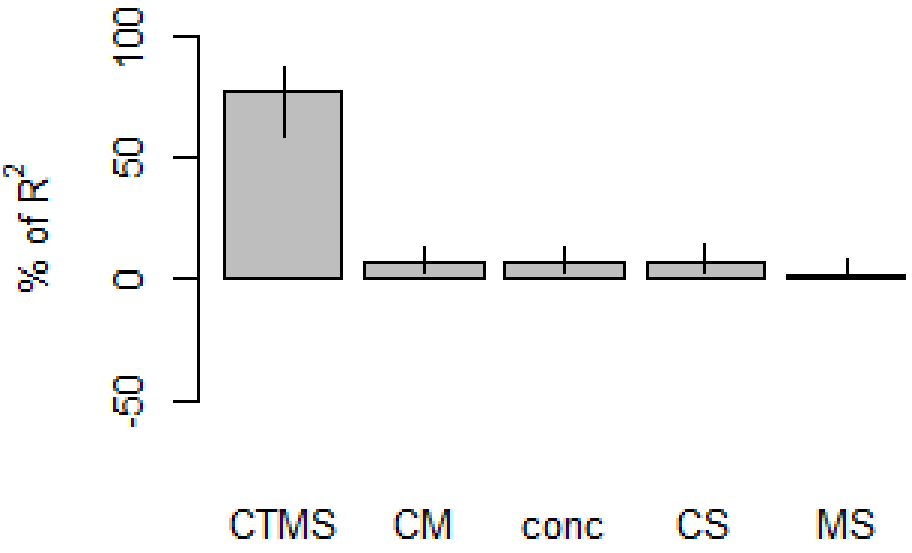
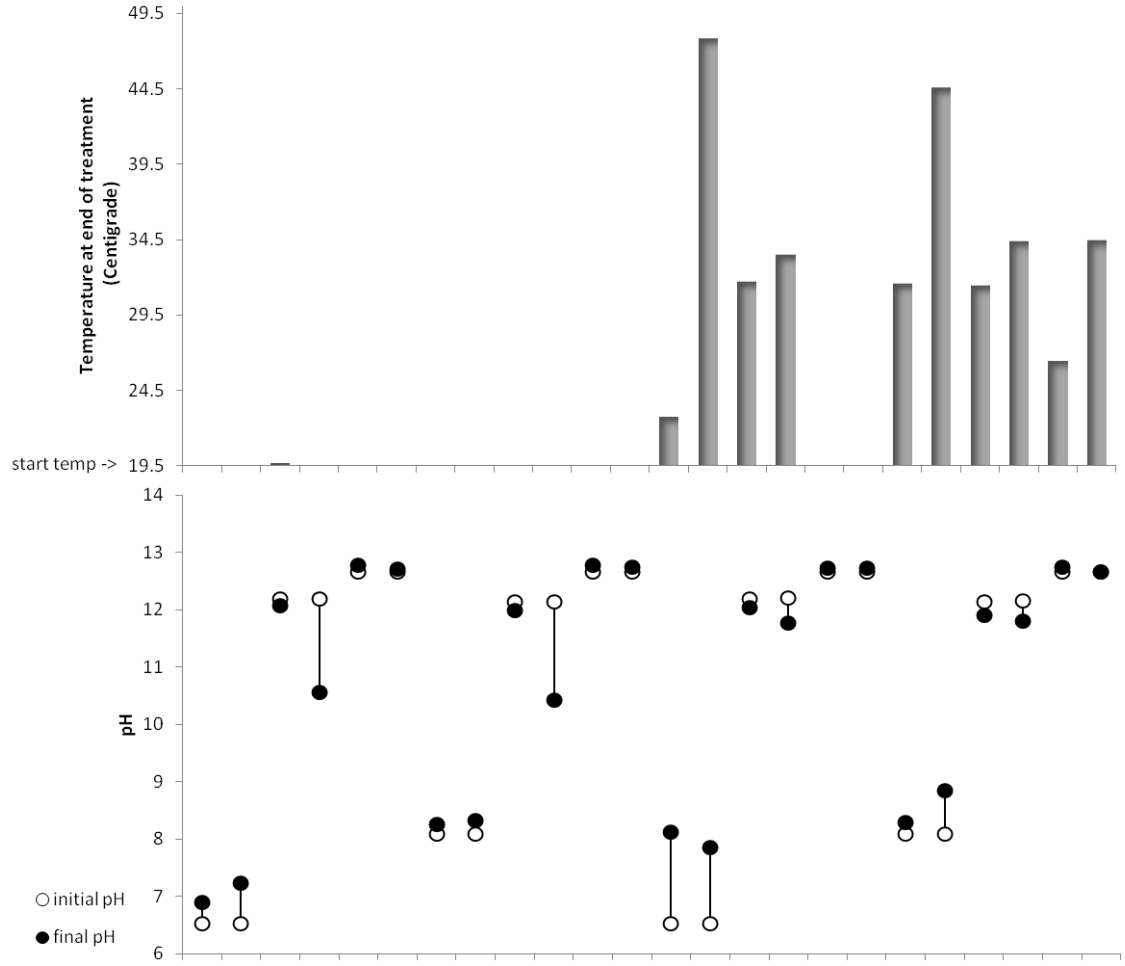


Figure 2.10: relative importance (LMG method) for mass-loss with 95% bootstrap confidence levels. R^2 = 85.44%, metrics are normalised to sum 100%. CTMS: combined effects of bleach concentration, time, initial Mg-calcite and ultrasonic; CM: combined effect of bleach concentration and initial Mg-calcite; conc: bleach concentration; CS: combined effect of bleach concentration and ultrasonic; MS: combined effect of initial Mg-calcite and ultrasonication.

Multiple regression indicates that two factors explain 77.45% of wt% $MgCO_3$ in calcite loss during treatment: initial wt% $MgCO_3$ in calcite of samples ($p<0.001$) and the combined effect of concentrations*time ($p<0.001$). The relative importance of these factors is shown to be 83.25% and 16.75% respectively (LMG method).

pH and temperature changes caused by cleaning treatment:



Treatment:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Ultrasonic:	NO ULTRASONIC												ULTRASONIC											
Water source:	DEIONIZED						TAP						DEIONIZED						TAP					
Concentration (%):	0%		10%		78%		0%		10%		78%		0%		10%		78%		0%		10%		78%	
Time (minutes):	10	480	10	480	10	60	10	480	10	480	10	60	10	480	10	60	10	60	10	480	10	60	10	60

Figure 2.11: Top graph: temperature (ºC) at the end of the treatment Note/ Temperature data missing for treatments 17 & 18. Bottom graph: pH at beginning (hollow circles) and end (filled circles) of treatments. Table shows treatment details. Concentration (%) indicates bleach concentration.

ADDITIONAL VARIABLES – SUBSET OF DATA (SEE TAB. 2.1)

Organic vs inorganic Carbon before and after treatment

There is significantly less organic carbon after treatment than before for all treated specimens (Paired T-test, $p<0.001$, T-value=7.99) and the amount of organic carbon lost is statistically different for all treatments ($F=6.95$, $p=0.006$)

There is no statistically significant difference in the amount of inorganic carbon before and after treatment.

Mg and Ca in cleaning solution following treatment

There is a statistically significant correlation between Mg in solution and the Mg lost from the sample in 3/5 of the treatments (Trt) (Fig. 2.12D). Trt1, $p=0.009$; Trt2, $p=0.011$; Trt3, $p<0.001$. There is no statistically significant correlation between Mg in solution and lost from sample for treatments 4 or 5. There is a statistically significant correlation between the Mg in solution and the mass lost from the sample in 4/5 of the treatments (Fig. 2.12A & D). Trt1, $p=0.042$; Trt2, $p=0.009$; Trt3, $p=0.019$; Trt5, $p=0.006$. There is no statistically significant correlation between Mg in solution and mass lost following treatment 4. There is no statistically significant correlation between the Ca in solution and the Ca lost from the samples in any of the treatments (Fig. 2.12C). There is a statistically significant correlation between Ca in solution and the mass lost from the sample in 2/5 of the treatments (Fig. 2.12A & C) Trt3, $p=0.020$; Trt5, $p=0.007$. There is no statistically significant correlation between Mg in solution and lost from sample for treatments 1, 2 or 4. There is a statistically significant relationship between the treatment and the amount of Mg and Ca in solution (MANOVA, $F=7.206$, $p<0.001$) (Fig. 2.12C & D).

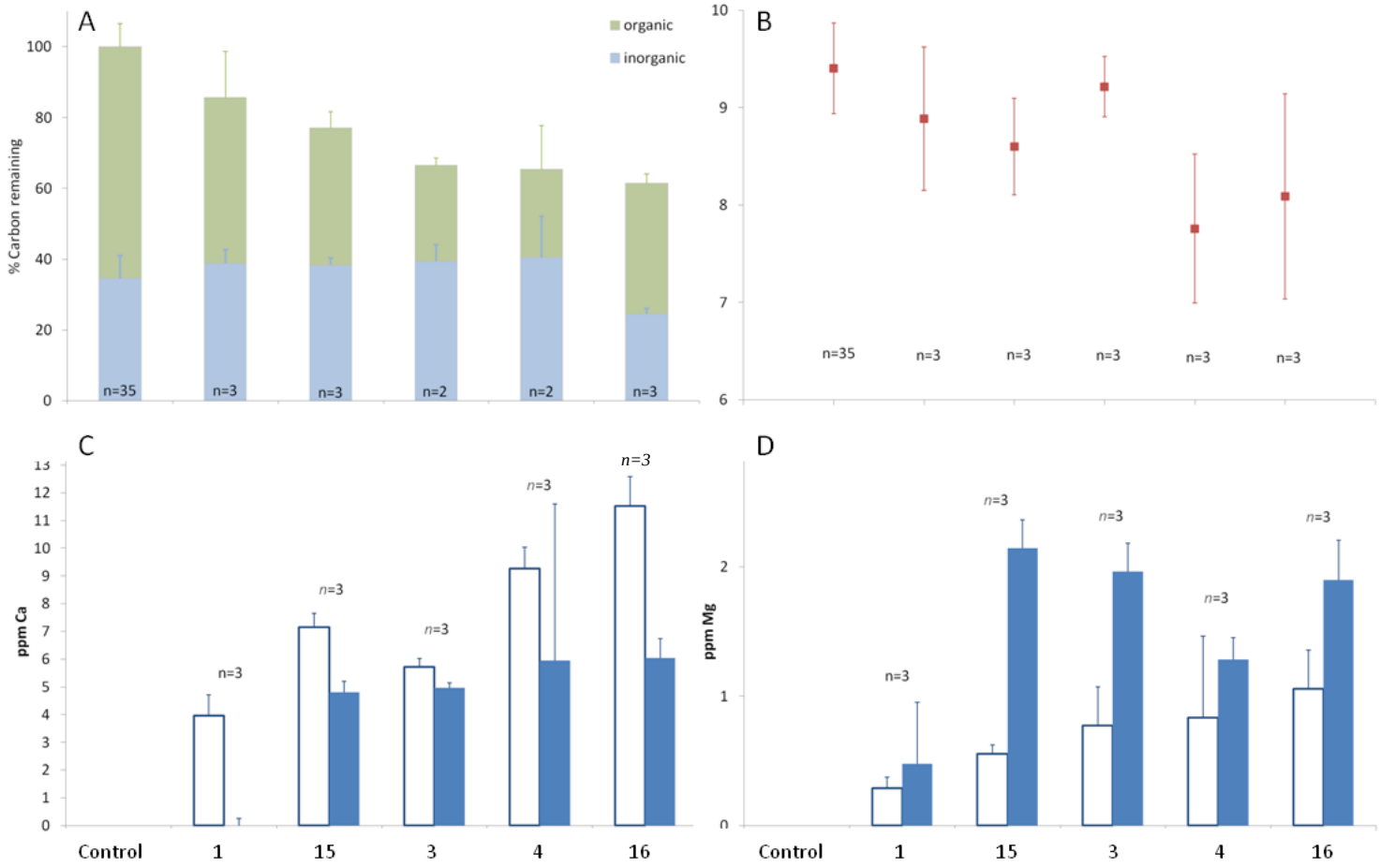


Figure 2:12 A-D: Array of graphs for treatments 1 (0% bleach, 10 minutes), 15 (10% bleach + ultrasonic, 10 minutes), 3 (10% bleach, 10 minutes), 4 (10% bleach, 480 minutes), 16 (10% bleach + ultrasonic, 60 minutes) plotted against the control, before samples. 2:12A: stacked bar graphs of mean % of organic carbon (green) and inorganic carbon (blue) remaining after treatments, error bars show standard deviation. 2:12B: Mean wt% $MgCO_3$ in calcite after treatment, error bars show standard deviation. 2:12C: Mean calcium (ppm) detected in solution G (blue) and lost from sample (hollow), error bars show standard deviation. 2:12D: Mean magnesium (ppm) detected in solution G (blue) and lost from sample (hollow), error bars show standard deviation.

Staining of specimens

Staining of untreated specimens (Fig. 2.13.A) shows that $MgCO_3$ is predominantly located in the spines of *F. foliacea* with the zooid walls covered in an organic layer. After 1 hour of 10% bleach and sonic (treatment 16) most spines have been lost and much organic material has been removed exposing the high Mg-calcite zooid walls (Fig. 2.13B). After 8 hours of 10% bleach (no ultrasonic) (treatment 4), all spines have been lost, high Mg-calcite walls are exposed and showing damage and thinning (Fig. 2.13C).

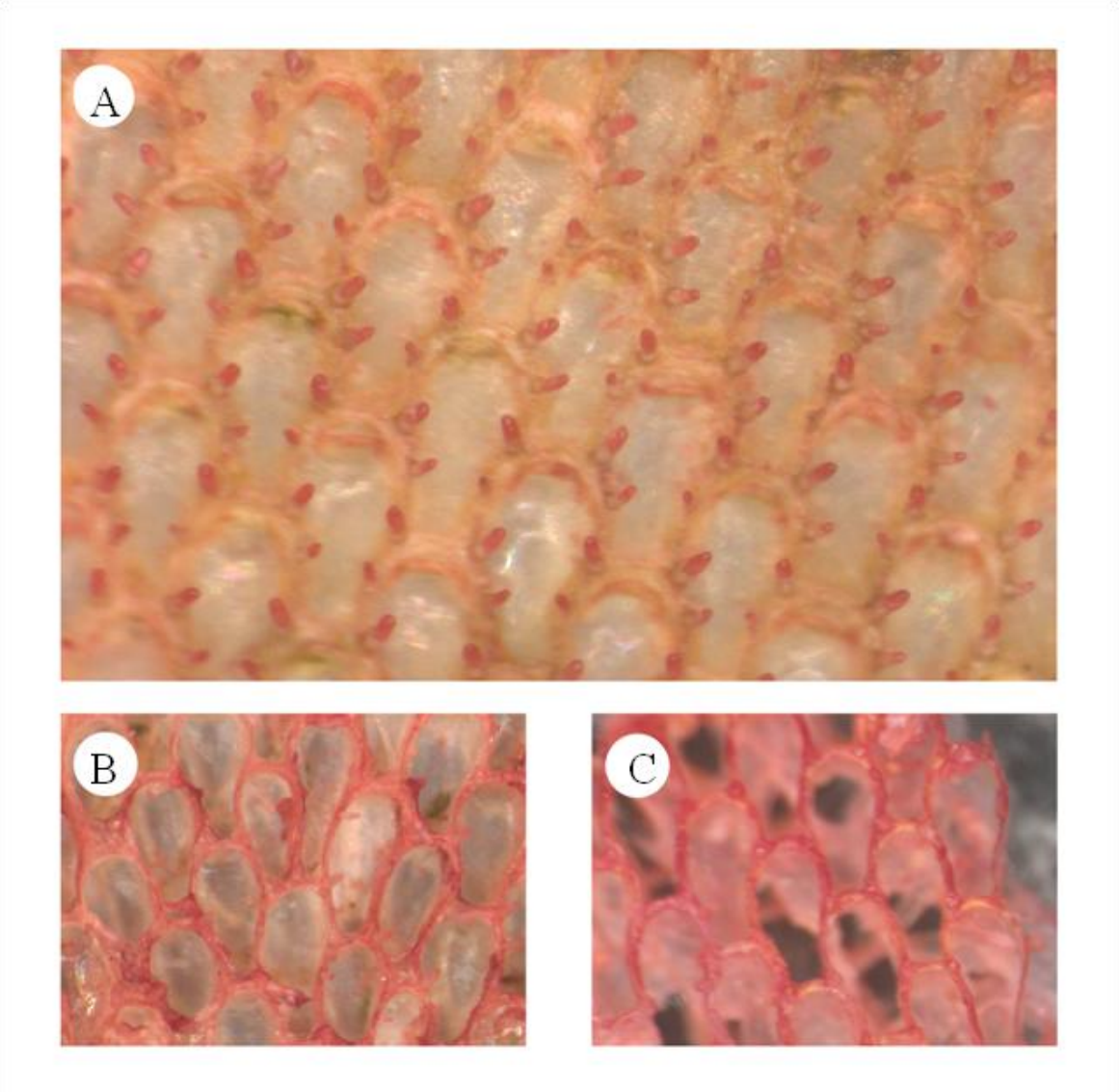


Figure 2.13 A-C: Titan Yellow staining of specimens of *F. foliacea* highlighting the spatial distribution of Mg-calcite (red). A: untreated sample, B: Specimen cleaned for 1 hour in 10% bleach and ultrasonic (Treatment 16); C: specimen cleaned for 8 hours in 10% bleach (treatment 4)

PAST RECORDS OF CLEANING BRYOZOANS PRIOR TO XRD ANALYSIS.

Of the 33 published studies on bryozoan mineralogy examined using XRD, 70% detailed whether or not any cleaning had been conducted prior to XRD analysis. Where detailed, 49% of studies used no cleaning. Where cleaning was used, bleaching was the most common method (Fig. 2.14), although the concentration of bleach used was not specified for 64% of samples (Fig. 2.15). When specified, 10% domestic bleach was the most common concentration used. Bleaching time was only specified for 3% of samples which underwent treatment. In only three studies were both bleach concentration and time specified. Intensity of cleaning ranged from none to 28 hours in full concentration domestic bleach.

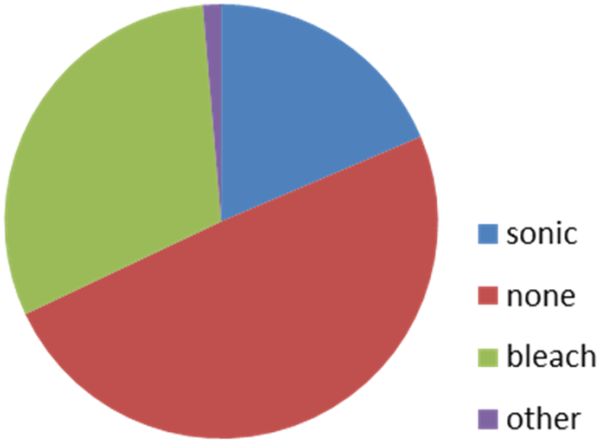


Figure 2.14: cleaning processes used in past studies

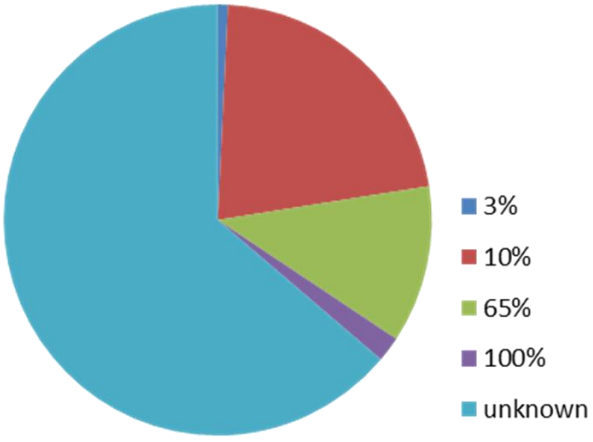


Figure 2.15: Bleach concentration used in past studies

2.5.2 Preservation

Membranipora membranacea

Specimens of *M. membranacea* were preserved for 552 days with samples extracted at 50, 279, 429 and 552 days for analysis. Trace calcite (<5%) was detected in 4 samples out of the 48 samples analysed; following examination of the specimens, this was attributed to trace *spirorbid* contamination and disregarded. All samples showed no change in mineralogy over time with any of the preservation treatments and remained 100% aragonite throughout.

Electra pilosa

Specimens of *E. pilosa* were preserved for 675 days with samples extracted at 0, 160, 268, 433, 501 and 675 days for analysis. Mean wt% $MgCO_3$ in calcite +/- standard deviation (SD) was found to be 9.217 +/- 0.874. No statistically significant difference in Mg-calcite was detected for samples preserved using different methodologies or durations (Fig. 2.16).

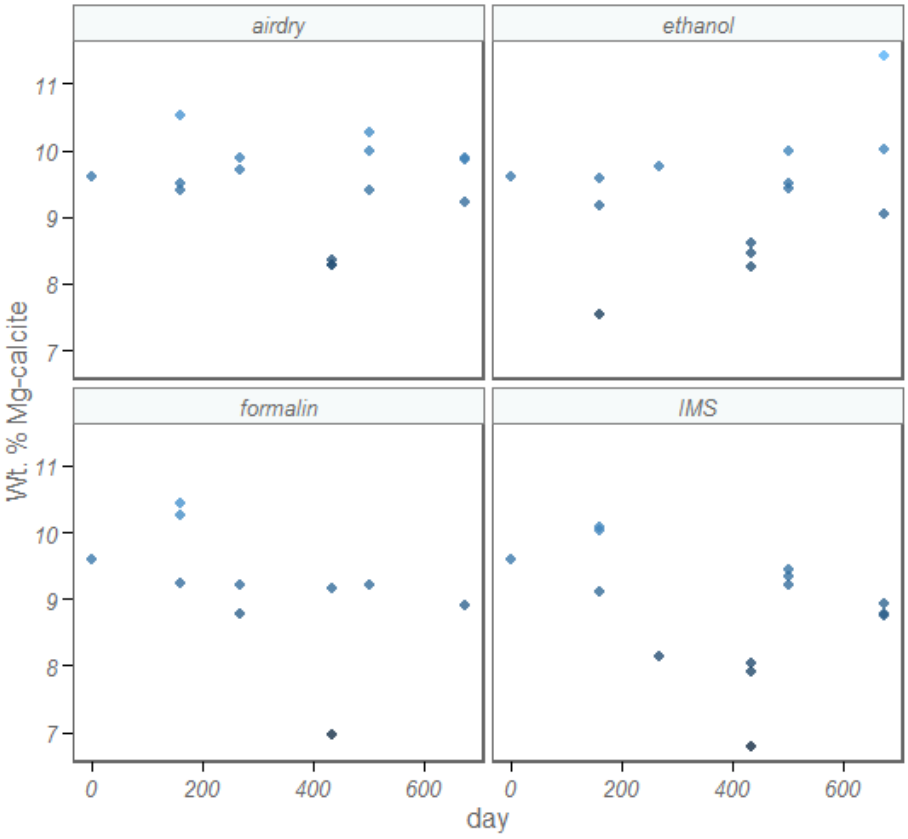


Figure 2.16: Variation in wt% $MgCO_3$ in calcite over time in *E. pilosa* samples preserved by air-dry, ethanol, formal-saline and IMS.

Flustra foliacea

Specimens of *F. foliacea* were preserved for 536 days with samples extracted at 0, 130, 253, 362 and 536 days for analysis. Mean wt% $MgCO_3$ in calcite +/- SD was found to be 8.815 +/- 1.085. No statistically significant difference in wt% $MgCO_3$ in calcite was detected for samples preserved using different methodologies or durations (Fig. 2.17).

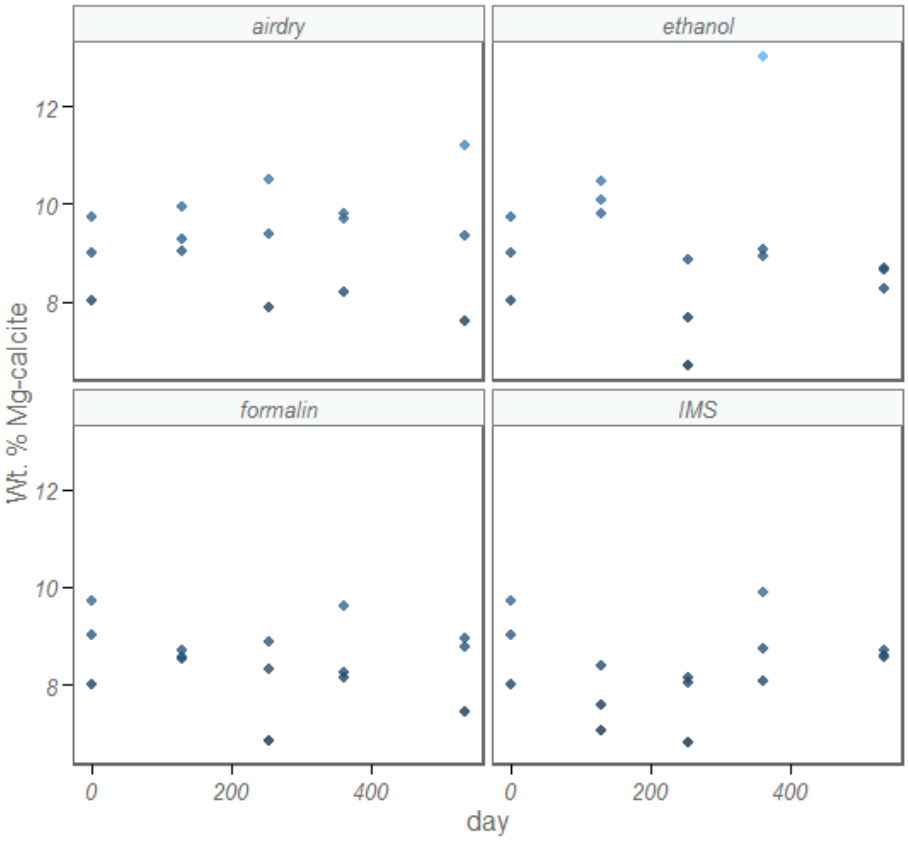


Figure 2.17: Variation in wt% $MgCO_3$ in calcite over time in *F. foliacea* samples preserved by air-dry, ethanol, formal-saline and IMS

Scrupocellaria reptans

Specimens of *S. reptans* were preserved for 537 days with samples extracted at 0, 130, 253, 363and 537 days for analysis. Mean wt% $MgCO_3$ in calcite +/- SD was found to be 4.646 +/- 0.868. Wt% $MgCO_3$ in calcite was found to have a statistically significant positive linear relationship with preservation duration for all preservations types (Linear model: 98% ethanol, p=0.042, R^2=35.32%; 4% formal-saline, p=0.002, R^2=67.96%; 60% IMS, p=0.009, R^2=59.23%; airdry, p=0.015, R^2=46.23%). In all cases wt% $MgCO_3$ in calcite increased with preservation duration (Fig. 2.18).

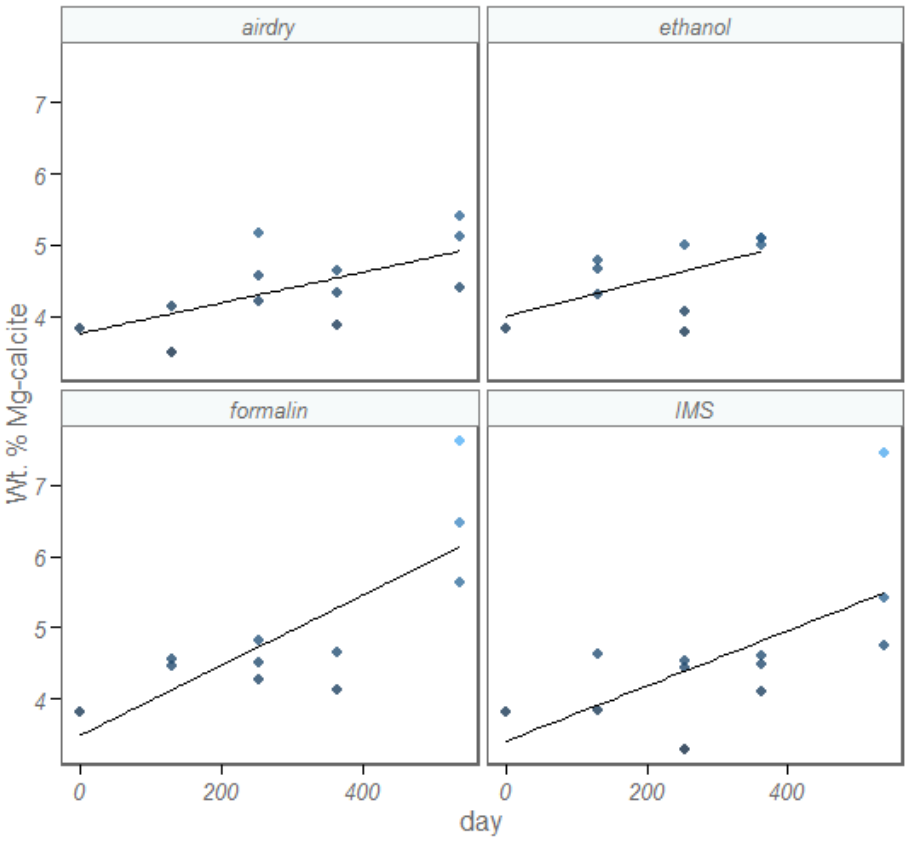


Figure 2.18: Variation in wt% $MgCO_3$ in calcite over time in *S. reptans* samples preserved by air-dry, ethanol, formal-saline and IMS

Crisia eburnea

Specimens of *C. eburnea* were preserved for 537 days with samples extracted at 0, 148, 253, 363 and 537 days for analysis. Mean wt% $MgCO_3$ in calcite +/- SD was found to be 5.059 +/- 0.741 wt% $MgCO_3$ in calcite was found to have a statistically significant positive relationship with preservation duration for airdried specimens only (Linear model: $p=0.008$, $R^2=51.7\%$). No statistically significant relationship difference between Mg-calcite and preservation duration was detected for samples preserved using ethanol, formal-saline or IMS. (Fig. 2.19)

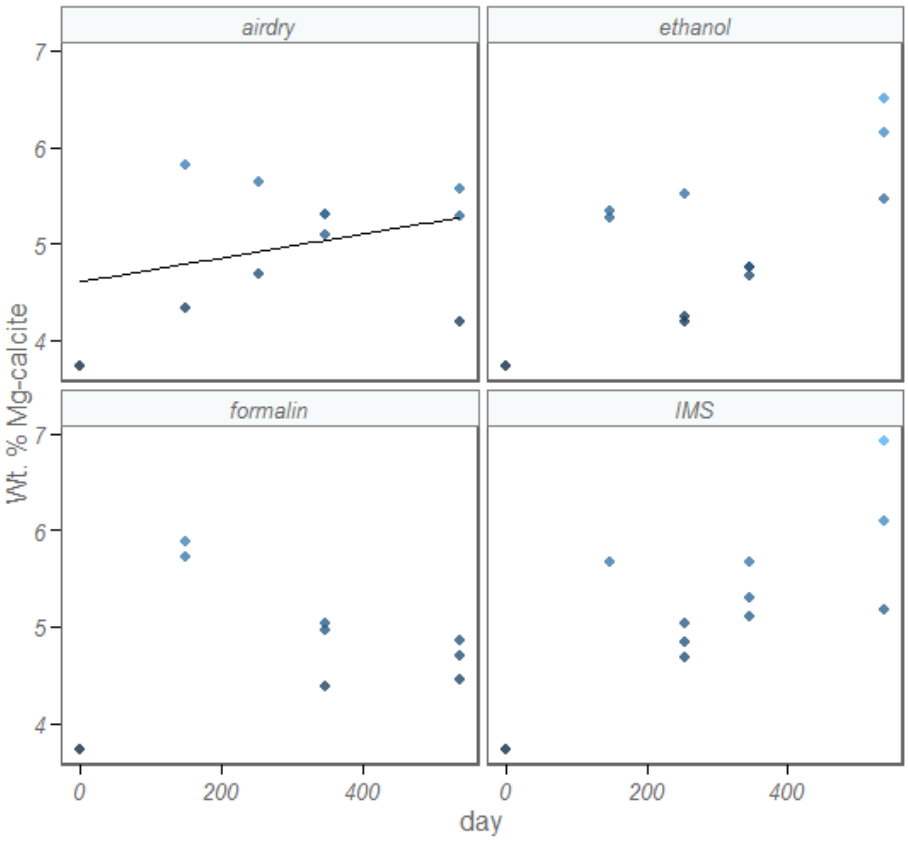


Figure 2.19: Variation in wt% $MgCO_3$ in calcite over time in *C. eburnea* samples preserved by air-dry, ethanol, formalin and IMS

pH at end of preservation

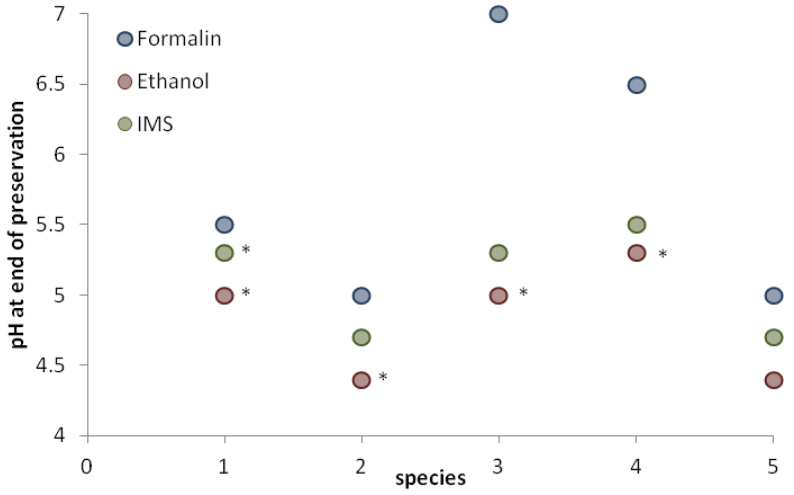


Figure 2.20: pH at end of experiment. * indicates more than 50% of preservation fluid evaporated. Species: 1 = *M. membranacea*; 2 = *E. pilosa*; 3 = *F. foliacea*; 4 = *S. reptans*; 5 = *C. eburnea*

For all species formal-saline has the highest pH and ethanol the lowest pH. All preservation fluids were acidic by the end of the experiment. Species 2 and 5 had the most acidic preservation fluids. Species 3 was the least acidic (Fig. 2.20).

DOCUMENTATION OF PRESERVATION OF BRYOZOANS PRIOR TO XRD ANALYSIS.

Of the thirty-three published studies on bryozoan mineralogy examined using XRD, 11 (33%) detailed the type of preservation used on the specimens.

Where detailed, 82% of examined specimens were preserved in ethanol prior to analysis. No studies detailed the length of time the specimen had been preserved (Fig. 2.21).

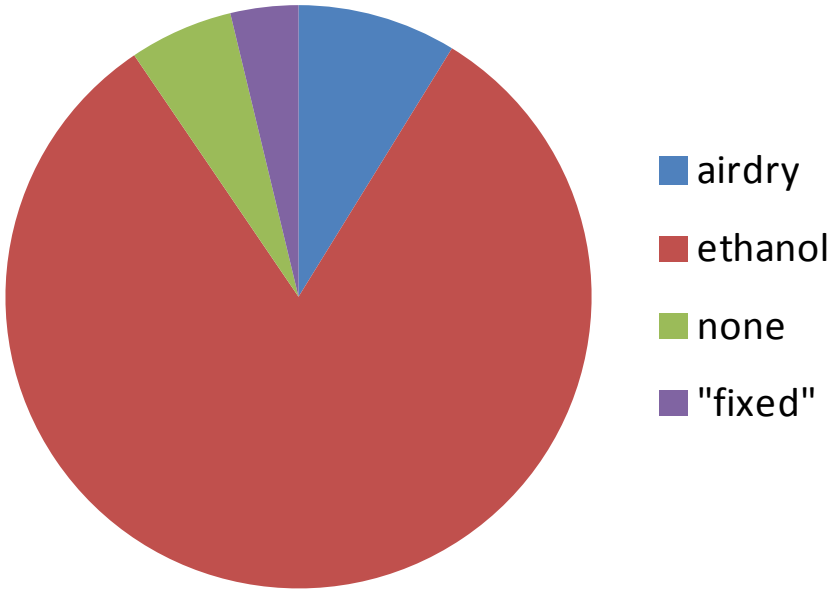


Figure 2.21: preservation from past studies

2.6 Discussion

2.6.1 The effect of cleaning on bryozoan mass and skeletal Mg-calcite

The objective of this experiment was to investigate the use and effects of cleaning procedures on bryozoan skeletons. Experimental methodologies aimed to test the individual and combined effects of ultrasonication, bleach of varying concentrations, water source and the temporal effects of these treatments on the mass and skeletal wt% $MgCO_3$ in calcite in *F. foliacea*.

CLEANING PROCEDURES USED IN PAST STUDIES PRIOR TO XRD ANALYSIS OF BRYOZOAN SKELETONS.

Studies contributing nearly half of the XRD analyses documented a conscious choice not to clean specimens, beyond rinsing in water, prior to XRD analysis (Fig. 2.14). Over 80% of the analyses, which deliberately used no cleaning, took place in the last decade and this may be an indication of increasing awareness of the potential risk of cleaning procedures to mineralogical results. 10% bleach continues to be the most common form of cleaning methodology (Fig. 2.14). The use of bleach has been documented for taxonomy for over a century (Norman 1903) and passed down by word-of-mouth from teacher to student (Banta et al. 1973). In the early mineralogical studies, it is likely that the preparation procedures chosen were the general methods advocated in bryozoan taxonomy rather than a technique modified for mineralogy. In the majority (65%) of the reviewed studies, documentation of cleaning procedures prior to XRD analysis was either missing or incomplete (Fig. 2.15) and this is a concern with regards to comparability of reported Mg-calcite values between studies. It should be noted that even where cleaning procedures were specified they referred only to procedures conducted as part of the documented study. To date no consideration has been given to cleaning procedures which may have been conducted on specimens prior to preservation or during the curational history of museum specimens, probably because this information itself is seldom documented.

THE EFFECT OF CLEANING ON BRYOZOAN MASS

Greater mass loss during the cleaning procedure was found with increasing bleach concentration, use of ultrasonic, longer treatment time and higher initial wt% $MgCO_3$ in calcite of the sample. These factors were found to be statistically significant both

individually and in combination. Water used for dilution and rinsing was found to have no effect on sample mass.

Bleach concentration: More mass was lost from samples during treatment with higher concentration bleach. Bleach acts through the dissociation of sodium hypochlorite (NaOCl) with water to form a strong oxidizing agent (HOCl) and raise pH (Eq. 2.4). At higher concentrations more oxidizing agent is produced resulting in a greater statistical chance of collisions with organic material and a faster rate of reaction (Moore 2012) .

Equation 2.4: Action of sodium hypochlorite

$$NaOCL + H_2O \rightarrow HOCl + Na^+ + OH^-$$

The higher pH works to disrupt cell walls (Alberts et al. 2010) while the oxidizing agent breaks carbon bonds in organic material to denature proteins (Klein 2012). Together these act to remove organic material from a sample, the loss of which is the main contributor to total mass loss during treatment in this experiment (Fig. 2.12A). Some inorganic material was also lost during bleach treatments (Fig. 2.12A), an observation also reported by Taylor & Weedon (2000) in bryozoans and Tasch & Shaffer (1961) in scolecodonts. Bryozoan calcium carbonate is deposited in a complex with protein threads around a template of organic cuticle and periostracum (Tavener-Smith & Williams 1972; Hall et al. 2002) . In the Mollusca (Zuschin et al. 2003) and Crustacea (Inoue et al. 2008) this intraskeletal protein matrix has been shown to hold the calcium carbonate skeleton together; loss of this organic matrix during treatment could result in calcium carbonate loss (Banta et al. 1973) .

Ultrasonic: The production of shock waves through cavitation has been shown to disrupt and lyse cells, and has an abrasive action on a sample surface (Chedd 1970). This could explain the increased mass loss, particularly organic carbon, when ultrasonic is used in treatments (Fig. 2.12A). The friction of the sound waves moving through solution (Chedd 1970) caused warming of the treatment solution in all cases, increasing temperature up to 48.5ºC; 28ºC above room temperature (Fig. 2.11 top). Warming generally increases reaction rates as particles have greater energy and the number of collisions is increased, in addition more of the colliding particles have sufficient activation energy for a reaction to occur (Moore 2012); this can increase the rate of protein denaturation and cell lysis (Alberts et al. 2010). Sjoberg & Rickard (1984) found that increased temperature increased the dissolution rate of calcite through alteration of the reaction kinetics. The process of

cavitation causes constant small explosive movements in the treatment solution (Chedd 1970), increasing the rate of movement of solution over the substrate surface. The impact of temperature and solute movement on calcium carbonate dissolution kinetics may explain the inorganic carbon loss seen in this experiment (Fig. 2.12A).

Initial wt% $MgCO_3$ in calcite in sample: It was observed during the experiment that the higher the initial wt% $MgCO_3$ in calcite in the sample, the more mass was lost during treatment. Titan Yellow staining (Fig. 2.13A) showed that the fragile spines of *F. foliacea* are made from high Mg-calcite. The higher wt% $MgCO_3$ in calcite in some samples, therefore, may indicate that they have more of these spines intact prior to treatment. Spines are one of the first skeletal features to be lost during treatment (Fig. 2.13B) and so samples with more spines to lose may, therefore, exhibit greater mass loss than those with less spines to start with. This may also partially explain the correlation between mass-loss and Mg in solution. An alternative explanation could be the different solubility product constants (K_{sp}) for $CaCO_3^{calcite}$ ($K_{sp} = 3.36 \times 10^{-9}$) and $MgCO_3$ ($K_{sp} = 6.82 \times 10^{-6}$), which determine that Mg-calcite is more susceptible to dissolution than calcite (Arvidson et al. 2003). This, however, would only explain the small proportion of mass loss which is from inorganic calcium carbonate (Fig. 2.12A).

Time: Sample mass loss was shown to be statistically increased with increasing treatment duration (Fig. 2.9). This may also be related to reaction kinetics – during a longer treatment duration more collisions would occur between particles, and although the reaction rate would be unaffected (Moore 2012), increased time would result in more total bond breaks in organic material and greater subsequent organic mass loss. The relationship between treatment duration and organic mass loss has been observed in bryozoans since the turn of the 20^{th} century (Norman 1903). Increased treatment duration has also been observed to cause increased loss of calcium carbonate in experiments by Tasch and Shaffer (1961) , an observation also seen in this experiment (Fig. 2.12A).

Water: The use of tap or deionised water for treatment dilution and rinsing made no statistical difference to mass loss during treatment. A difference in mass loss may have been expected due to the difference in starting pH of the two water sources (Fig. 2.11 bottom, Tab. 2.3), with more acidic pH expected to cause greater organic mass loss and calcium carbonate dissolution (Hodgkinson 1991). The pH of the treatments, however, did

not continuously reflect that of the starting water. Treatments containing no bleach showed a decrease in acidity during the treatment, which was greater for deionised water than for tap water. Addition of any bleach to the treatment also overrode any initial pH difference caused by the water (Fig. 2.11 bottom). As a result, the pH was almost equal between treatments using tap or deionised water and this may explain why it had no subsequent impact on mass loss.

Combined effects: In addition to affecting mass loss individually, the combined effect of bleach concentration, ultrasonication, initial Mg-calcite and treatment duration was found to explain 78% of mass lost during treatment (Fig. 2.10). Bleach concentration, ultrasonication (and associated increase in temperature and solution agitation) and the treatment duration could be working together to kinetically increase the oxidation reaction of bleach with organic compounds (Moore 2012). In addition, cavitation and shock waves caused by ultrasonication, would physically abrade specimens, disrupt cells and cause damage and loss of high Mg-calcite features, such as spines.

Less calcium was found in treatment solutions (Fig. 2.12C) than was lost from the sample. Calcium could be released from both the organic and inorganic components of a sample; Ca^{2+} is a vital component of cells, found within organelles for purposes including cell communication and triggering muscle contraction (Alberts et al. 2010). Ca^{2+} is, however, only found in fairly low concentrations in marine invertebrates (5.24 mg Ca^{2+} ions/kg of lobster muscle (Robertson 1961). The concentration of Ca^{2+} ions released from cell breakage would, therefore, be expected to be a minor contributor to calcium in the treatment solution. The main source of calcium in the treatment solution would be from $CaCO_3$. It is possible that part of the $CaCO_3$ may have been lost from the fragile treated specimen during the rinsing process and this could explain why there was less calcium in the solution than expected.

THE EFFECT OF CLEANING ON BRYOZOAN MG-CALCITE

Initial Mg-calcite in sample: There was a strong linear relationship between the amount of Mg-calcite lost during treatment and the initial Mg-calcite of samples (Fig. 2.7), explaining 83.25% of Mg-calcite loss. Higher initial Mg-calcite samples lost a greater amount of Mg-calcite and samples below 8.73 wt% $MgCO_3$ in calcite were more likely to gain Mg-calcite during treatment. This relationship was found to be the same regardless of whether tap or

deionised water was used (Fig. 2.8). This is best explained by solution equilibrium chemistry. The chemical equilibrium for movement of Mg^{2+} between calcite and solution is shown in Equation 2.5.

Equation 2.5: movement of Mg^{2+} between calcite and solution, x = mole fraction of Mg in solid phase, **(Oomori et al. 1987)**

$$Ca_{(1-x)}Mg_xCO_{3\,(solid)} \leftrightarrow (1-x)Ca^{2+}_{(soln)} + xMg^{2+}_{(soln)} + CO^{2-}_{3\,(soln)}$$

If the concentration of Mg in calcite on the left of the equation is higher than the concentration of Mg^{2+} ions in solution, then the reaction will be driven to the right, causing a reduction in wt% $MgCO_3$ in calcite. Conversely, if there are more Mg^{2+} ions in solution than in calcite, then the reaction will be driven to the left, increasing the wt% $MgCO_3$ in calcite (Oomori et al. 1987). Fig. 2.8 indicates that the Initial wt% $MgCO_3$ in calcite at which Mg-calcite will be neither lost or gained during treatment (0 on the y-axis) is remarkably similar for both tap (8.725 wt% $MgCO_3$ in calcite) and deionised water (8.73 wt% $MgCO_3$ in calcite). This may be an indication that equilibrium in this equation is reached when x = 8.73.

Combined effects: The combined effect of bleach concentration and treatment duration explain 17% of the Mg-calcite lost during treatment. During the reaction of bleach with water, OH^- ions are produced (Eq. 2.4); these can then capture Mg^{2+} ions from the treatment solution, as shown in Equation 2.6, pulling them out of solution and into a precipitate; this is the principle of water softening using bleach (Casiday et al. 2013). Reaction kinetics determine that the reaction will occur faster with stronger concentrations of bleach and more Mg^{2+} will be chelated during longer treatments (Moore 2012).

Equation 2.6: Removal of Mg^{2+} from water by bleach, the principal of water softening (Casiday et al. 2013)

$$Mg^{2+}_{(soln)} + H_2O + NaOCL \leftrightarrow HOCl + Na^+ + Mg(OH)_{2\,(solid)}$$

The removal of Mg^{2+} ions from solution would have the effect of driving the reaction discussed in Equation 2.5 to the right, removing Mg^{2+} ions from calcite and reducing overall wt% $MgCO_3$ in calcite, as evidenced in the experiments.

Water and Ultrasonication: Water was observed to have no effect, probably due to the pH equalizing effect of bleach discussed in Section 2.1.3. Ultrasonic treatment had no effect on the wt% $MgCO_3$ in calcite following treatment, possibly because the primary effects of cavitation are physical rather than chemical (Chedd 1970). It may be that increased

temperature and solution movement, caused by ultrasonic treatment, could be contributing to the reaction kinetics of Equation 2.6, however, if it is, it is too small a contribution to show as being statistically significant.

More magnesium was detected in treatment solutions than was lost from Mg-calcite (Fig. 2.12D) suggesting an additional source of Mg^{2+} ions in the solution. Mg^{2+} is present in high concentrations in cells (Alberts et al. 2010), bound primarily to G-actin, the protein responsible for cell movement (Barden & Remedios 1985), and found in high quantities in bryozoan larvae (Santagata 2007). As a result high concentrations of Mg^{2+} could be expected to be released from organic material during cell lysis and protein disruption, increasing the Mg^{2+} in solution beyond that released from Mg-calcite alone. Reinforcing this theory, there was a strong correlation between total mass loss and magnesium detected in solution.

RECOMMENDATION FOR CLEANING OF BRYOZOAN SPECIMENS PRIOR TO XRD ANALYSIS

The majority of impact to Mg-calcite occurs when the sample is in solution. As a result it is recommended that samples are rinsed in pH adjusted deionised water to remove salt, prior to drying before analysis. Specimens should not be allowed to soak. If organic material causes background noise on the XRD spectra, making it hard to differentiate peaks, then it is recommended that the sample run length is increased. If bleaching must be used, then it is recommended that samples be exposed to a maximum 10% bleach solution, made with pH adjusted deionised water, for up to ten minutes and ultrasonication should be avoided.

2.6.2 The effect of preservation on bryozoan skeletal Mg-calcite

The objective of this experiment was to investigate the use and effects of preservation on bryozoan skeletons. Experimental methodologies aimed to test the effects of specimen preservation using 96% ethanol, 70% industrial methylated spirits (IMS), 4% formal-saline and air-drying on the skeletal Mg-calcite of 5 bryozoan species over a 2-year period.

PRESERVATION PROCEDURES USED IN PAST STUDIES PRIOR TO XRD ANALYSIS OF BRYOZOAN SKELETONS.

Only a third of studies documented the preservation used on samples prior to XRD analysis (Fig. 2.21) and no studies specifically reported for how long specimens were preserved. Where preservation was specified, the most common method (82%) was ethanol. The

discovery of the health risks of formaldehyde, combined with the growing prevalence of genetic studies, has led to alcohol being considered the "safe" option for preservation (Moore 1999) and this appears to be reflected here. As in the case of cleaning (Section 2.5.1), the majority (66%) of the reviewed studies failed to document preservation at all, and the remaining studies rarely specified concentration or preservation duration. As with cleaning, this is a concern with regards to comparability of reported Mg-calcite values between studies.

Comparative effect of preservation on Mg-calcite for different species

Preservation, or duration of storage, was not observed to have any impact on the mineralogy of *M. membranacea,* an aragonitic species. It is possible that preservation may have affected total skeletal mass, or strontium incorporation over time in *M. membranacea,* however, neither of these factors were measured in this experiment. Neither *E. pilosa* nor *F. foliacea,* both high Mg-calcite, highly variable species (Figs. 2.16 and 2.17), showed any relationship between preservation type/duration on wt% $MgCO_3$ in calcite. In contrast the Intermediate Mg-calcite, less mineralogically variable species *S. reptans* (Fig. 2.18) and *C. eburnea* (Fig. 2.19) showed statistically significant relationships between duration of storage and wt% $MgCO_3$ in calcite for one or all forms of preservation. The differential reactions, to preservation by species, may be caused by the different initial wt% $MgCO_3$ in calcite of species, as discussed in Section 2.5.1, with lower Mg-calcite species being more susceptible to changes in their wt% $MgCO_3$ in calcite, primarily through exchange with the preservative. *C. eburnea* and *S. reptans* both feature small, fragile, complex skeleton morphologies in comparison to *E. pilosa* (encrusting) and *F. foliacea* (erect bilaminar). This increased surface-to-volume ratio would increase the relative amount of the skeleton exposed to the preservative and would have the effect of increasing the kinetic rate of reaction (Moore 2012). The smaller size of these species also means that the specimen: solution ratio would be lower in fluid-preservatives, increasing the fixative or preservation power (UNESCO 1966). *M. membranacea* and *E. pilosa* were both preserved on macroalgae and *F. foliacea* has a high organic content (Fig. 2.12A) compared to *C. eburnea* and *S. reptans*; this may be interacting with the preservative and reducing the impact of preservative on the calcium carbonate skeleton in these species.

WHY DOES WT% $MgCO_3$ IN CALCITE INCREASE WITH PRESERVATION DURATION?

Fluid preservatives: The environment in fluid preservatives is highly dynamic with pH and chemical components fluctuating with time (Steedman 1976; Marte et al. 2003). The predicted cause of this fluctuation and resultant effect on specimens is represented in Figure 2.22. The initial solution for both IMS and ethanol is a mixture of water and CH_3CH_2OH. Hard tap water contributes Mg^{2+}, Ca^{2+} and carbonate ions to a preservation solution (Steedman 1976; Oomori et al. 1987), and Edinburgh tap water, used as a diluent for both the ethanol and IMS preservatives, is reported to be slightly alkaline with a mean annual pH of 8.0 (Scottish Water 2012). Formal-saline is made up using sea-water, with a pH of ~8.2 and high natural concentration of Mg^{2+}, Ca^{2+} and carbonate ions. Given the chemical composition of the diluent, initial pH of all preservation solutions is expected to be >pH 7 (Steedman 1976).

Figure 2.22 Step 1: Ethanol is dehydrating, and initial osmotic shock can cause cell lysis (Alberts et al. 2010) in the organic matrix of the sample and subsequent release of intracellular Mg^{2+}, Ca^{2+} (Robertson 1961) into the preservative solution. More Mg^{2+} is released than Ca^{2+}, in accordance with the differential concentration of the ions within cells and proteins (Robertson 1961; Alberts et al. 2010). The alkali conditions of all the preservative solutions also adds to the leaching of proteins and fatty acids from the organic component (Kotrba & Golbig 2009); The Sorenson reaction (Steedman 1976) explains that the breaking down of proteins from preserved material into amino acids results in a drop in pH. In formal-saline the Cannizzaro reaction causes formaldehyde and water to react to cause formic acid, even in the absence of preserved material, and results in an acidic preservative solution within a week of preparation (Steedman 1976)

Figure 2.22 Step 2: As pH drops below 6.5 in all preservative fluids calcium carbonate is subject to dissolution (Waller & Simmons 2003; Kotrba & Golbig 2009), with Mg-calcite more susceptible than calcite (Oomori et al. 1987). This releases Mg^{2+} (and some Ca^{2+}) ions and HCO_3^-, which initially buffers the preservation solution (Steedman 1976) before raising it to slightly alkaline conditions. It is expected that the overall wt% $MgCO_3$ in calcite of the specimen would drop at this stage as Mg-calcite is dissolved in greater proportions that calcite.

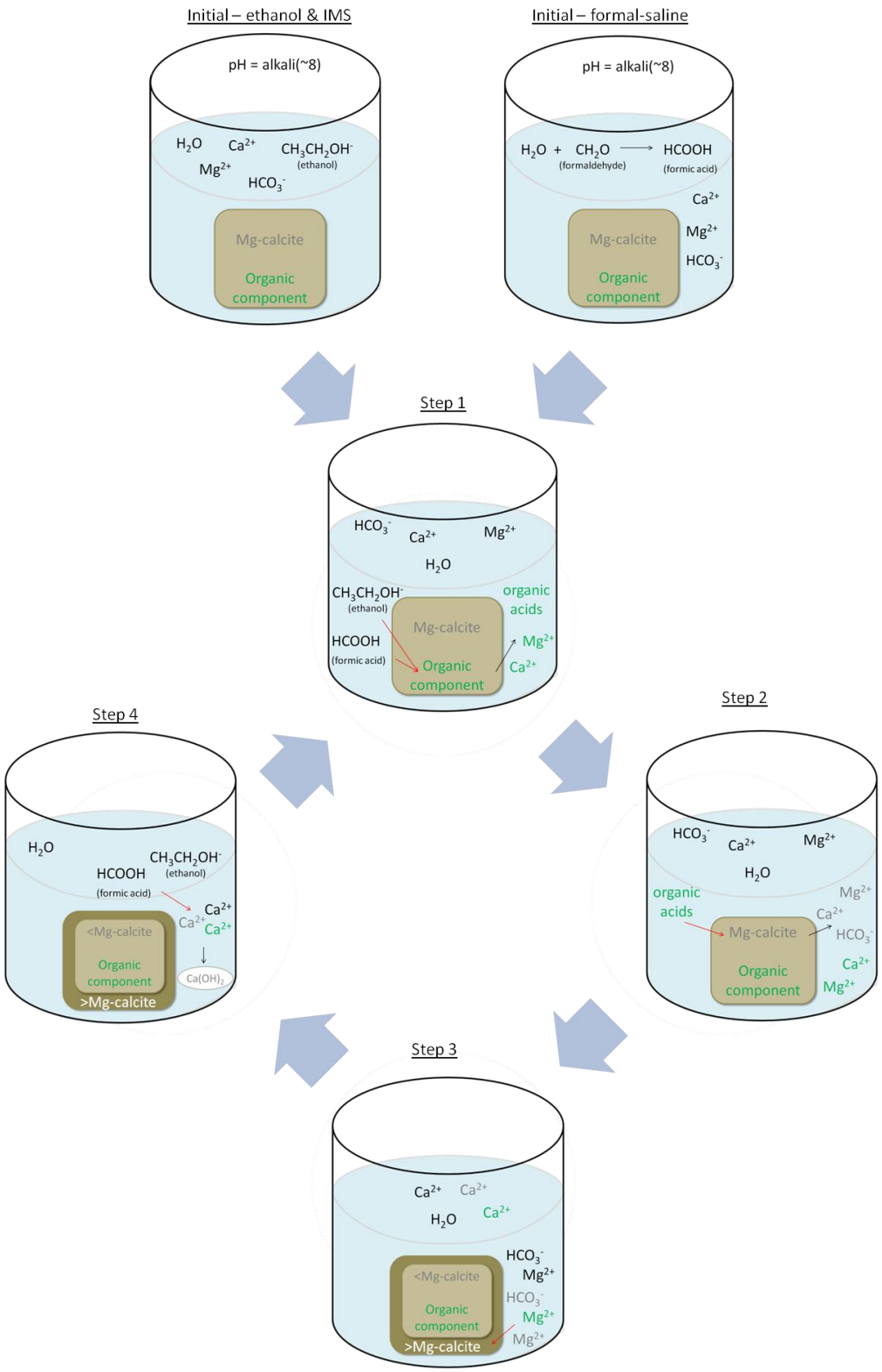


Figure 2.22: Flow diagram of predicted process of pH and chemical fluctuations in fluid-preservatives. Green compounds orginate from the organic component of the sample, grey from the inorganic component of the sample and black from the diluent. Equations and formulas are representative and not stoichiometrically correct. See text of chapter for details of each step.

Figure 2.22 Step 3: The alkaline conditions could trigger deposition of carbonate from solution onto the outer surface of the specimens. The preservative solution contains a higher concentration of Mg^{2+} ions than Ca^{2+} ions, derived from the initial diluents, organic material and dissolve Mg-calcite. As a result, according to Equation 2.7, it would be expected that Mg-calcite deposition be favoured over calcite (Oomori et al. 1987). The overall wt% $MgCO_3$ in calcite of the specimen would rise above the starting concentrations at this stage. As the carbonate ions are removed from solution, the pH would begin to neutralize.

Equation 2.7: Distribution coefficient, D, of Mg^{2+} between calcite and solution. M = concentration

$$D_{Mg^{2+}}^{(calcite)} = \frac{M_{Mg^{2+}}^{(calcite)} \Big/ M_{Ca^{2+}}^{(calcite)}}{M_{Mg^{2+}}^{(soln)} \Big/ M_{Ca^{2+}}^{(soln)}}$$

Figure 2.22 Step 4: Hydroxyl ions, released from ethanol or formic acid, could be expected to "mop up" any remaining Ca^{2+} ions in solution, either from the initial diluents, organic material (step1) or calcite dissolution (step 2), to form the flocculate $Ca(OH)_2$ which would settle at the bottom of the preservation jar. The removal of these ions would further lower pH. The alkali conditions of step 3 would also trigger the Sorenson and Cannizzaro reactions and preservation solutions would return to the acidic conditions of step 1.

The kinetics of reactions discussed in steps 1-4 will be susceptible to acceleration by increases to temperature or solute concentration (Notton 2010). The temperature at the commencement of preservation was measured to be 19ºC and is expected to have remained constant within the laboratory at Heriot-Watt University during the experiment duration. Evaporation in the ethanol and IMS preservations was observed, with greater evaporation in the more concentrated ethanol preservative. This would have increased the concentration of ions in solution and the rate of the reactions (Moore 2012) and may explain the lower final pH observed in ethanol and IMS preservations, than in formal-saline (Fig. 2.20). The fluctuating pH in fluid preservation, and the tendency for ethanol evaporation, have been

previously observed by a number of authors (Steedman 1976; Cato 1990; Moore 1999; Edwards et al. 2002; Marte et al. 2003; Waller & Simmons 2003; Kotrba & Golbig 2009; Garrigos et al. 2013). Previous investigations into the potential effect of this on calcium carbonate have also observed increasing wt% $MgCO_3$ in calcite caused by preservation of fish otoliths in ethanol (Milton & Chenery 1998; Hedges et al. 2004) and stable isotopes have been shown to increase in Foraminifera preserved by formal-saline (Ganssen 1981). This study adds further evidence to this pattern of increasing wt% $MgCO_3$ in calcite with fluid preservation in some marine invertebrates.

Air-drying: Two species showed wt% $MgCO_3$ in calcite increasing over time during preservation by air-drying. This may be explained by the phenomena of Byne's disease (Byne 1899). Byne's disease is an efflorescence resulting from a reaction between organic acid fumes, released from substances such as paper, reacting with calcium carbonate (Walker et al. 1999). The paper used for drying the specimens in this experiment was normal tissue-paper (not acid-free) and, therefore, may have been a source of acid fumes. An experiment by Tennent and Baird (1985) showed that in 35 cases, efflorescence was all found to be calcium compounds. If efflorescence is calcium specific, then this would have the effect of removing calcite from the specimen, increasing the proportion of Mg-calcite and subsequent wt% $MgCO_3$ in calcite; a reaction shown in Equation 2.8.

Equation 2.8: Reaction showing predicted effect of efflorescence on $MgCO_3$ in calcite. Efflorescence product shown is just one possible carbon salt **(Tennent & Baird 1985)**

$$H_2O + CaMgCO_3 + CH_3COOH \rightarrow Ca(CH_3COO)_2 + CO_2 + MgCO_3$$

The incidence of Byne's disease on mollusc shells has been observed to be enhanced by humid environments and residual salts on the specimen (Tennent & Baird 1985). The specimens used in this experiment were quickly rinsed prior to drying and may have, therefore, had a weak coating of residual salts. They were also dried and stored in a working laboratory, and may have been subject to some humidity. These combined effects may have enhanced the incidence of Byne's disease on these samples, increasing calcite lost through efflorescence and subsequently measured wt% $MgCO_3$ in calcite.

RECOMMENDATIONS FOR PRESERVATION

The main cause of impact on wt% $MgCO_3$ in calcite from fluid preservatives is the fluctuation of pH during the duration of preservation. The use of buffers to stabilize pH is recommended in many early text books for alcohol preservation (Manesse 1786; Graves 1817; Anonymous 1831), although rarely practiced today. Similarly buffering of formaldehyde with substances to reduce the acidity has been recommended since 1921 with the earliest suggestions, including addition of $MgCO_3$ or borax (Atkins 1921); borax continued to be recommended through the 20th century (Steedman 1976) up to the present day (Smithsonian: National Museum of Natural History 2013). Addition of $MgCO_3$ in an acid solution would cause deposition onto the specimen and affect measured wt% $MgCO_3$ in skeletal calcite. Borax is a water softener and acts by capturing and binding Mg^{2+} and Ca^{2+} in a solid. This would impact the equilibrium equation (Eq. 2.5) and the distribution coefficient (Eq. 2.7), resulting in leaching of Mg from calcite and an overall reduction in wt% $MgCO_3$ in calcite. The potential impacts of the buffers themselves on the chemistry of preservation, suggests further research is necessary prior to the addition of any of these buffers to preservation fluids. If ethanol preservation is necessary for future genetic work, then it is recommended that the sample should be split, half undergoing ethanol preservation and half dried. The problem of evaporation can be reduced with secure fitting lids, and specimens should be regularly monitored to detect any evaporation early and implement "topping up" if required. It should be noted, however, that topping up does not resolve pH issues (Marte et al. 2003). To ensure consistency between samples, and reduce the impact of the Sorenson reaction, it is recommended that all fluid preservation is conducted in the 9:1 fluid:sample ratio as recommended in the literature (Steedman 1976; Moore 1999; Marte et al. 2003).

The problem of Byne's disease, associated with air-drying, can more easily be prevented. Thorough rinsing of specimens in pH neutral deionised water prior to drying on acid-free tissue paper, would prevent the production of acid fumes. Storage of specimens, packed in fresh acid-free paper in humidity proof plastic boxes would further prevent efflorescence from forming. Due to the available mitigations to potential Mg-calcite impacts, air-drying is, therefore, recommended for preservation of bryozoans prior to mineralogical analysis.

It has been noted with increasing regularity in collection management manuals and preservation guidelines that it is important to document the fixing and preservation history of specimens (Moore 1999; Huber 1998; Hedges et al. 2004; Kotrba & Golbig 2009), in order that consideration can be given to the effects of preservation during the curational history of museum specimens. Understanding the effects of specimen preservation on skeletons is complex, due to the variety of preservatives, methods of fixation, and storage conditions that have been used during the last two centuries. Specimens are also often subjected to different preservatives and storage conditions during their time in the collections and, unfortunately, information on the specific chemical treatments applied is rarely recorded in museums' records (Garrigos et al. 2013). An improvement in documentation is also indicated from this study, and it is further suggested that preservation should be documented in research articles, so that the potential effects of preservation can be considered where comparing mineralogy results between publications.

Next Steps in understanding the effects of preservation on Mg-calcite

This study offers indications that in some cases Mg-calcite can be increased by preservation in a similar way to that seen in fish otoliths (Milton & Chenery 1998; Hedges et al. 2004) and Foraminifera (Ganssen 1981). The presented study was conducted under an initial experimental design, which could be improved upon in future studies. It is recommended that a further study is conducted in a controlled environment (e.g. a museum collection room), and that a thorough baseline of initial mineralogy is conducted prior to preservation. In addition, it would be advantageous to use pH adjusted deionised water as a diluent for ethanol and IMS, and determine seawater composition prior to formal-saline dilution. Extracts of preservation fluid, collected at each time point and analysed by ICP-AES to establish elemental content, would add valuable information about the chemical process occurring in fluid preservatives. The ratio of 9:1 preservative:sample should be more closely observed and it would be beneficial to collect data on pH and temperature during the duration of the experiment. The results of the cleaning experiment in this chapter indicate that no cleaning of preserved specimens, beyond rinsing in pH adjusted deionised water, should be conducted prior to either preservation or XRD analysis. In light of these recommendations, a long-term study at the Natural History Museum was initiated in 2012 on *F. foliacea*, the results of which it is anticipated will build on this pilot study.

2.7 Summary and Conclusions

Wt% $MgCO_3$ in calcite in bryozoans was found to be susceptible to change by both cleaning and preservation procedures. The results of the experiments showed that:

- Samples with higher initial wt% $MgCO_3$ in calcite are more susceptible to mass loss and mineralogical impact during cleaning.
- Stronger concentrations of bleach (sodium hypochlorite) cause a greater loss of mass and Mg-calcite during cleaning.
- Longer cleaning procedures result in higher mass and Mg-calcite loss.
- Not all mass lost during cleaning is organic.
- Aragonitic and High Mg-calcite species showed no impact of preservation on wt% $MgCO_3$ in calcite over time. Intermediate Mg-calcite species all showed an increase in wt% $MgCO_3$ in calcite over time for some or all preservation types.
- The acidity of all preservation fluids increased with time.

The interaction between bryozoan skeletal Mg-calcite and solutions, either cleaning or preservation, is found to be controlled by a combination of solution chemistry and reaction kinetics. Preservation fluids in particular are highly dynamic and chemically complex due to interactions between organically released ions and compounds, inorganic dissolution and re-deposition, ionic components in diluents and Sorenson and Cannizzaro reactions.

In order to minimize the impact of cleaning and preservation on mineralogy, it is recommended that future specimens collected for mineralogical analysis are rinsed in pH adjusted deionised water prior to air-drying on acid-free paper and long-term storage in an air-tight plastic box. No further cleaning should be required prior to XRD analysis.

It is recommended that both cleaning and preservation processes undertaken during the full curational history of the specimen are considered and documented in future mineralogical studies.

Chapter 3. Mineralogy of Scottish Bryozoans

3.1 Abstract

This study describes the mineralogy of 156 species of the phylum Bryozoa within Scotland. This represents 79% of the species which inhabit Scotland and is a greater number and proportion of extant species than any previous regional study. The study is also of significance globally where the data augment the growing database of mineralogical analyses and offers first analyses for 26 genera and four families.

Specimens were collated through a combination of field sampling and existing collections and were analysed by X-ray diffraction (XRD) and micro-XRD to determine wt% $MgCO_3$ in calcite and wt% aragonite. Species distribution data and phylogenetic organisation were applied to understand distributional, taxonomic and phylo-mineralogical patterns.

Analysis of the skeletal composition of Scottish bryozoans shows that the group is statistically different to the neighbouring Arctic fauna but features a range of mineralogy comparable to other temperate regions. As has been previously reported, cyclostomes feature lower Mg-calcite and aragonite than cheilostomes and this is explained here by the lower phylogenetic distance (PD = 2.5) of cyclostomes from the low/intermediate calcitic ancestral skeleton when compared to cheilostomes (PD = 4.3).

At lower taxonomic levels the dual-calcite genus, *Cellaria*, the high Mg-calcite species, *Flustra foliacea*, and the aragonitic species, *Anarthropora monodon* and *Membranipora membranacea*, utilize the mechanical properties of their skeleton mineralogies to enable them to inhabit challenging ecological niches.

Scotland is a highly variable region, open to biological and environmental influx from all directions, and bryozoans exhibit this in the wide range of intra-species mineralogical variability they present. It is suggested that skeletal plasticity is driven by a combination of phylogenetic predisposition and environmentally driven physiological factors (e.g. growth rate).

3.2 Introduction

3.2.1 Bryozoan studies in Scotland

The first collections of bryozoans from Scotland are documented from the 18th century by two keen naturalists from Edinburgh - Robert Brown (1773-1858), who was a student at Edinburgh University, and Patrick Neill (1776-1851), a fellow of the Royal Society of Edinburgh (Rouse 2010). Early bryozoans were classified as Zoophyta, intermediates between plants and animals, and were often stored in museum herbarium departments. Research in Edinburgh continued into the 19th century with Charles Darwin, whose investigation into *Flustra* was written into a paper by his mentor, Robert Grant, in 1827 (Barrett 1977). Collections escalated from 1840 onwards with many independent naturalists collecting and supplying specimens to Canon Alfred M. Norman and Reverend Thomas Hincks, notable taxonomists of the time. One of these naturalists, George Johnston, published details of his significant collection in 1838, which included many type specimens from Scotland (Johnston 1847). In the 1860s the first widespread dredging collections began, particularly in the far North around Shetland. These were reported on by Alfred Norman (1868) and form a significant collection which is still lodged at the Natural History Museum (NHM) in London, and used today. Sir John Murray undertook survey work of the Clyde Sea area during the late 19th century (Chumley & Murray 1918) but there follows a gap before the initiation of the Marine Conservation Review, 1987-1998, led to the recommencement of collecting and recording bryozoans (Rouse 2010). The publication of taxonomical synopses in the second half of the 20th century has widened the range of marine biologists able to confidently identify species and in the last decade there has been a continued increase in the specimens and records of Scottish bryozoans, with the majority of these coming from marine surveys by government agencies. In the last few years there has been growth of “citizen science” groups such as Seasearch (Marine Conservation Society 2012) and this, combined with open data-sharing options such as OBIS (Intergovernmental Oceanographic Commission (IOC) of UNESCO 2010), has allowed for the amassment of an unprecedented volume of distribution records for Scottish Bryozoa.

3.2.2 Regional studies of mineralogy

Since the beginning of mineralogy studies on the Bryozoa there have been regional specific publications. The majority of these are of limited use due to the very low specimen numbers analysed, e.g. South Africa (Siesser 1972). Hawaii (Agegian & Mackenzie 1989), Talbot Shoal (Poluzzi & Sartori 1973), Naples (Walther 1885). There have, however, also been a handful of studies where the coverage of regional bryozoans has been great enough to draw meaningful conclusions. These studies are summarised in Table 3.1.

Table 3.1: Summary of published regional studies of bryozoan mineralogy.

Region	Year	Species analysed	Reference	Regional species estimate	% species coverage
Mediterranean	1974	94	(Polluzi & Sartori 1974)	300 (Zabala & Maluquer 1988)	31%
Antarctica	1991	21	(Borisenko & Gontar 1991)	300 (Barnes & Hillenbrand 2010)	7%
New Zealand	1998 & 2006	49	(Smith et al 1998; 2006)	953 (Gordon et al. 2010)	5%
Chile	2010	23	(Smith & Clark 2010)	267 (Moyano 1983)	9%
Arctic	2009	76	(Kuklinski & Taylor 2009)	300 (Kuklinski & Taylor 2009)	25%

Regional studies can offer a useful insight into the region-specific environmental, evolutionary and biological influences on mineralogy and also allow comparisons with other parts of the world's oceans and a context in which local patterns can be assessed.

3.2.3 Why a regional study in Scotland?

Scotland was chosen for this regional study for its ecological and biological diversity. Scotland is a point of convergence for the Atlantic Ocean and the North Sea and this

provides both a wide range of ecological niches with regards to temperature and depth (Scottish Government 2011) and a source for both Northern and Southern bryozoan species (Rouse 2010). Many bryozoans are at their most Southern or Northern extent in Scottish waters and this allows for comparative study between the mineralogical analysis of species in the centre of their distribution and at their eco-physiological limits.

3.2.4 Previous mineralogical analyses of Scottish species

There have been seven published mineralogical studies (Clarke & Wheeler 1917; 1922; Schopf & Allan 1970; Poluzzi & Sartori 1974; Borisenko & Gontar 1991; Smith et al. 2006; Taylor et al. 2009) featuring bryozoan species which are present in Scotland (see Appendix A). The earliest of these were conducted with chemical titration (Clarke & Wheeler 1917; Clarke & Wheeler 1922) with the remainder being conducted with XRD, and, in a few cases, Ramen spectroscopy (Taylor et al. 2009). The publications summarise the analysis of 41 species (n=148). Specimens collected from Scottish waters were used in the analysis of 5 species (n=8).

3.3 Aims and Objectives

3.3.1 Summary of purpose of study

The aim of this study is to conduct mineralogical analysis on the skeletons of over 75% of Scottish calcifying bryozoan species (n=197, known species to date).

3.3.2 Objectives of study

The key objectives of this study are to:

1. Build a baseline of skeletal mineralogy for Scottish Bryozoa.
2. Classify analysed species into high, low or intermediate Mg-calcite and calcitic, aragonitic or bimineralic mineralogy.
3. Apply species distribution data in conjunction with mineralogical data to investigate latitudinal and distributional patterns in mineralogy.
4. Compare the mineralogy of Scottish Bryozoa to data from Global, Polar and temperate publications.

3.4 Materials and Methods

3.4.1 Sample collection

This study is based on the mineralogical examination of 282 specimens of 154 bryozoan species collected from Scottish waters. Existing collections were investigated and field sampling undertaken in order to collect samples of as many species as possible; these are described below. Full details of all specimen sources can be found in Appendix B.

The Bryozoa collections of the Natural History Museum (NHM) in London and the National Museum of Scotland (NMS) were searched for Scottish species. 38 species were sourced from the NHM collection and these date back as far as 1792. 43 species were sourced from the NMS collection and these dated back as far as 1897. All NHM specimens selected for analysis were from the air-dried collection and all NMS specimens were preserved in 74% Industrial Methylated Spirit (IMS). No specimens were recorded as having undergone previous treatments which may affect mineralogy. The private collections of Dr Joanne Porter, Heriot-Watt University and Dr Jim Drewery, Marine Scotland (Rockall collection) were investigated. Preservation technique, collection site and date were noted where available.

Additional sampling was conducted, for the purpose of this study, in Scottish waters. Sampling was conducted through intertidal shore collecting, marina/pontoon survey, SCUBA diving and grabs/dredging. A map showing localities and type of sampling conducted is presented in Fig. 3.1. Collected specimens were either air-dried or preserved in 98% ethanol within hours of collection.

29% of the specimens analysed in this study were sourced from museum collections and were of varying age (up to 220 years old). Specimens collected for this study were preserved in 98% ethanol or air-dried within 24 hours of collection to minimise preservation effects (see Chapter 2). To mitigate possible effects of preservation on skeletal mineralogy of older samples the majority of analyses will be conducted on the discrete mineralogy data categories of LMC, IMC, HMC & calcite, mixed mineralogy and aragonite, rather than continuous data. Specimens were not exposed to any additional treatment (e.g. bleaching) prior to analysis and care was taken to apply a consistent methodology throughout to limit impact of curational procedures.

Figure 3.1: Map of Scotland showing sampling sites. Marina survey points are shown in red, grabs/dredges in yellow, SCUBA collection in purple and shore collection sites in green. Modified from Wikimedia Commons (Eric Gaba 2010)

Specimens were identified under a dissection light microscope using Field Studies Council Guides by Hayward and Ryland (1985; 1998; 1999). Where the features were too small or indistinct for confident identification using this technique, SEM was employed (see Section 3.4.3).

Samples were extracted from the tip of erect colonies and the growing edge of encrusting colonies. A minimum of 5 zooids were extracted for each sample. As far as possible care was taken to ensure that no substrate (e.g. coralline algae) or epibionts were included within the sample as they could potentially contaminate results through their added mineralogies.

Rare, figured, type or holotype specimens were not sampled but were analyzed whole using non-destructive micro-XRD (see Section 3.4.3).

3.4.2 Data Sources

DISTRIBUTION DATA

Distribution data of cheilostomes and cyclostomes were downloaded from Ocean Biogeographic Information System (OBIS) (Intergovernmental Oceanographic Commission (IOC) of UNESCO 2010). 53,423 records of calcifying bryozoans from latitude 35-85°N and longitude 10°W-30°E, were extracted. Only datapoints identified to species level from scientifically reliable sources have been included. Taxonomy was corrected to match the World Register of Marine Species (Appeltans et al. 2012).

Membranipora membranacea (Linnaeus, 1767) is an exceptional, aragonitic species which is unusually well recorded in the UK due to sampling bias towards intertidal zones. The inclusion of this species would smother any true patterns of mineralic distribution; therefore it has been excluded from distribution data.

PREVIOUS MINERALOGICAL ANALYSES

Seven studies covering 41 Scottish species (n=148) were conducted between 1917 and 2009. The majority (~95%) of the specimens analyzed in these studies, were not sourced from Scotland, however, all feature species which are found within Scottish waters. Only eight analyses of five species were conducted on specimens sourced from Scotland. A table detailing these analyses and data sources can be found in Appendix B.

Additional comparative studies from the Mediterranean (Poluzzi & Sartori 1974), New Zealand (Smith et al. 1998; 2006), Chile (Smith & Clark 2010), the Arctic (Kuklinski & Taylor 2009), the Antarctic (Borisenko & Gontar 1991) were used in this thesis for regional comparisons (Table 3.1).

3.4.3 Analysis Techniques

X-RAY DIFFRACTION (XRD)

Mineralogical analyses were conducted at the NHM, London using XRD as described in Section 2.3.3 to determine wt% $MgCO_3$ in calcite. In order to determine the proportions of aragonite and calcite in bimineralic species, peak intensities were fitted to standard patterns generated from 100% aragonite and 100% calcite. The error associated with this method is estimated to be 2% based on repeatability studies of samples with a known aragonite proportion (Kuklinski & Taylor 2009).

SCANNING ELECTRON MICROSCOPY (SEM)

Where required to confirm identification, SEM was undertaken with a low-vacuum instrument (LEO 1455- VP) capable of imaging large, uncoated specimens using back-scattered electrons.

3.4.4 Data Processing and Presentation

DATA PRESENTATION

Geographical Information Systems (GIS) maps were prepared using ArcGIS (v.9.3) geographical information system (ESRI 2011). Graphical and tabular representations of data were prepared in Excel (Microsoft 2006). To allow for direct comparison with previous Global work many of the graphical and tabular presentations are modelled on those by Smith et al. (2006; 2013) .

Range of carbonate mineralogy in a group of bryozoans specimens is described by its biomineralogical "space", a term introduced by Smith et al. (2006). Biomineralogical or mineralogical "space" is the area described by the ranges of wt% Mg-calcite and wt% calcite for a particular species or taxonomic group. It is usually expressed as a percentage of the possible space available for biomineralization (0-22 wt% Mg-calcite and 0-100 wt% calcite, a possible space of 2178 $wt\%^2$).

Plates of images were prepared using GNU Image Manipulation Program (GIMP)(Kimball & Mattis 2012)

STATISTICS AND DATA ANALYSIS

Wt% $MgCO_3$ in calcite data for all species were tested for normality using Anderson-Darling normality tests. Criteria for parametric *posthoc* testing were not met due to unequal sample sizes and heterogeneous variance between datasets. To elucidate differences between taxonomic, spatial, evolutionary and ecological groupings non-parametric Mann-Whitney U-tests were therefore carried out. To confirm trends associated with distributional range and latitudinal distribution of mineralogy, linear regression testing was undertaken. Data for temperate regions (Scotland, Chile and New Zealand) were analysed using a generalised linear model (GLM) ANOVA. The factor used was region (fixed) and the response wt% $MgCO_3$ in calcite. Criteria for parametric *posthoc* testing were not met due to unequal sample sizes between datasets, therefore potential differences between regions were elucidated by Mann-Whitney *Post-hoc* testing. The p-values for all analyses were calculated; this is the measure of the strength of evidence and is based on the traditional probability of error of 0.05. Only when the p-value is less than 0.05 is the evidence considered statistically significant. Statistics and data analysis were conducted using MINITAB release 14 software (2004).

PHYLOGENETIC COMPARATIVE METHODS

Phylogenetic data and branch lengths were taken from the recent publication of Waeschenbach et al. (2012). Branch length of the phylogeny indicates the number of substitutions per site based on Bayesian multi-gene analysis of a concatentated 7-gene dataset *(ssrDNA, lsrDNA, rrnL, rrnS, cox1, cox3, cytb*) constructed using BEAST under the random logical clock and GTR+I+G model (Waeschenbach et al. 2012). The phylogeny was input into the R statistical language (R Core Team 2013) using Newman coding. Kembel's (2010) methodology for Comparative Phylogenetic Methods was followed. Comparative phylogenetic diversity and phylogenetic distance between cyclostomes and cheilostomes were calculated using the "pd" and "cophenetic" functions in the Picante package for R (Kembel et al. 2010; 2013). Blomberg's K (Blomberg et al. 2003) values for cyclostomes, cheilostomes and all Scottish bryozoans were calculated as a measure of underlying phylogenetic signal in the traits of wt% MgCO3 in calcite and wt% calcite

using the "multiPhylosignal" function in the Picante package for R (Kembel et al. 2010; 2013). Blomberg's K was also calculated as a measure of underlying phylogenetic signal for the trait of morphology.

MARKOV CHAIN MONTE CARLO GENERALIZED LINEAR MIXED-MODEL (MCMCGLMM)

For model compatibility the phylogenetic tree from Waeschenbach et al. (2012) was ultra-metrically scaled for evolutionary time using the "chronos" function in the APE package for R (Paradis et al. 2013).

All mineralogical measurements underwent weighted average transformation in the context of beta regression (Eq. 3.1) following the methodology of Smithson and Verkuilen (2006).

Equation 3.1: Weighted average transformation in the context of beta regression. n=total data points

$$\frac{y(n-1)+0.5}{n}$$

This initial transformation ensured that no data points equalled 0 or 1 and prepared the data for subsequent transformation using the logit function (Equation 3.2). The logit function takes account for the fact that the mineralogical measurements used are proportions (p) (Warton & Hui 2011).

Equation 3.2: logit function

$$logit\ (p) = \ln\left(\frac{p}{[1-p]}\right)$$

The MCMCglmm library (Hadfield 2009), for the R statistical language (R Core Team 2013), was used to model the comparative effects of phylogeny and latitude on skeletal mineralogy. For the mineralogical measurements of wt% $MgCO_3$ in calcite and wt% calcite three generalised linear mixed-models were fitted; a mixed model including effects for phylogeny and latitude, and two further models looking at phylogeny or latitude alone as predictors.

MCMCglmm generates model parameters using a Markov chain Monte Carlo (MCMC) algorithm (Sorensen & Gianola 2002) which is then applied to the generalized linear mixed-model. For each model 65,000 iterations were run and mixing and convergence were assessed using the methods implemented in the CODA package (Cowles 2013). All

models showed consistent parameter estimates and low values for autocorrelation, indicating that the models were well mixed and had reached convergence. The fit of the models to the mineralogical data was compared using the deviance information criterion (DIC); models with low DIC are preferred (Hadfield 2012; Smith et al. 2013).

DATA CATEGORIZATION

Each species was given two mineralogy categorizations, the first to indicate the dominant calcium carbonate polymorph, the second to gauge the scale of magnesium incorporation within calcite. These categories are shown in Table 3.2.

Table 3.2: Mineralogy categorisations

Mg-calcite	Low Mg-calcite	LMC	0-4 Wt. % $MgCO_3$
	Intermediate Mg-calcite	IMC	4-8 Wt. % $MgCO_3$
	High Mg-calcite	HMC	8-12 Wt. % $MgCO_3$
	Very High Mg-calcite	VHMC	>12 Wt. % $MgCO_3$
Biomineral presence	Calcitic	C	100% calcite
	Calcite dominated mix	CD	>75% calcite
	Bimineralic	BI	25-75% calcite
	Aragonite dominated mix	AD	<25% calcite
	Aragonitic	A	0% calcite

3.5 Results

3.5.1 Scottish Bryozoans

PHYLUM BRYOZOA IN SCOTLAND

282 specimens covering 154 species were analyzed with a mean wt% calcite of 84.42. The most common mineralogy was found to be 100% calcite: 93 species (60%) were formed entirely from calcite. Bimineralic species, containing a mixture of the two polymorphs, were the second most common type (55 species, or 36% of those tested) and these were spread fairly evenly across the range from 1 wt% to 99 wt% calcite (Fig. 3.2). Entirely aragonitic species were by far the least common with just 6 species (4%) featuring this mineralogy.

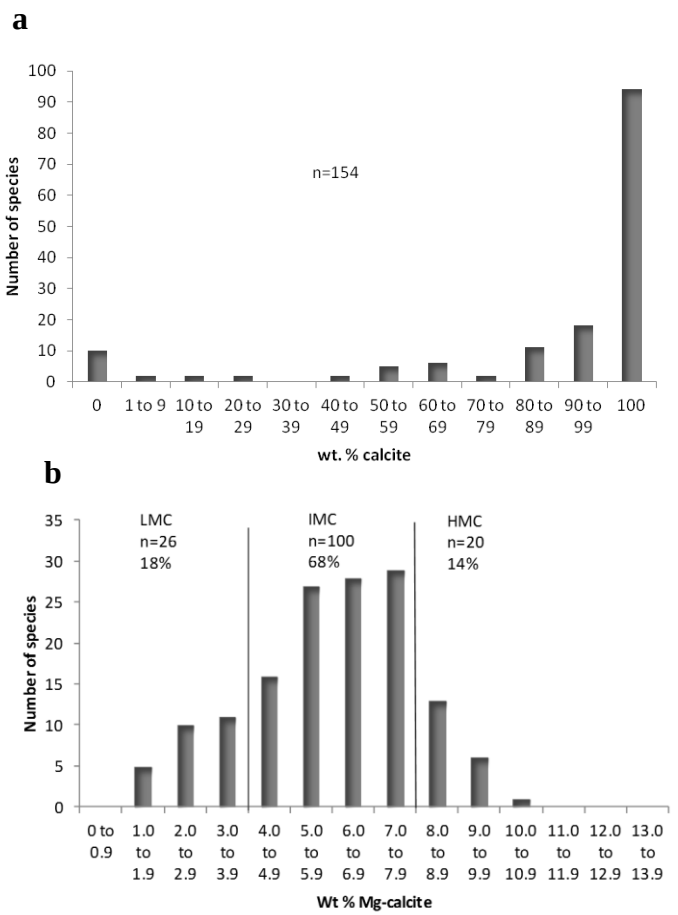


Figure 3.2: Frequency distributions for a) calcite content in 154 Scottish bryozoan species and b) Mg content in 146 calcite containing Scottish bryozoan species. LMC – low Mg-calcite; IMC – Intermediate Mg-calcite; HMC – High Mg-calcite. Wt. % Mg-calcite refers to wt% $MgCO_3$ in calcite.

All specimens were analyzed for wt% $MgCO_3$ in calcite and this ranged from 1.03 to 10.17, with a mean of 5.86. The majority (69%) of species featuring calcite were classed as intermediate Mg-calcite (4-8 wt% $MgCO_3$ in calcite). A further 18% formed low Mg-calcite (0-4 wt% $MgCO_3$ in calcite) and the remaining 12% of species featured high Mg-calcite (8-12 wt% $MgCO_3$ in calcite) (Figure 3.2b).

The phylogenetic position for 39 (11 cyclostomes and 28 cheilostomes) Scottish bryozoans were extracted from the bryozoan phylogeny published by Waeschenbach et al (2012) (Figure 3.3). There is no phylogenetic signal for the mineralogical trait of $MgCO_3$ in calcite (Blomberg's K = 0.17, p = 0.264) although a strong and significant phylogenetic signal relating to wt% calcite was found (Blomberg's K = 0.37, p = 0.022).

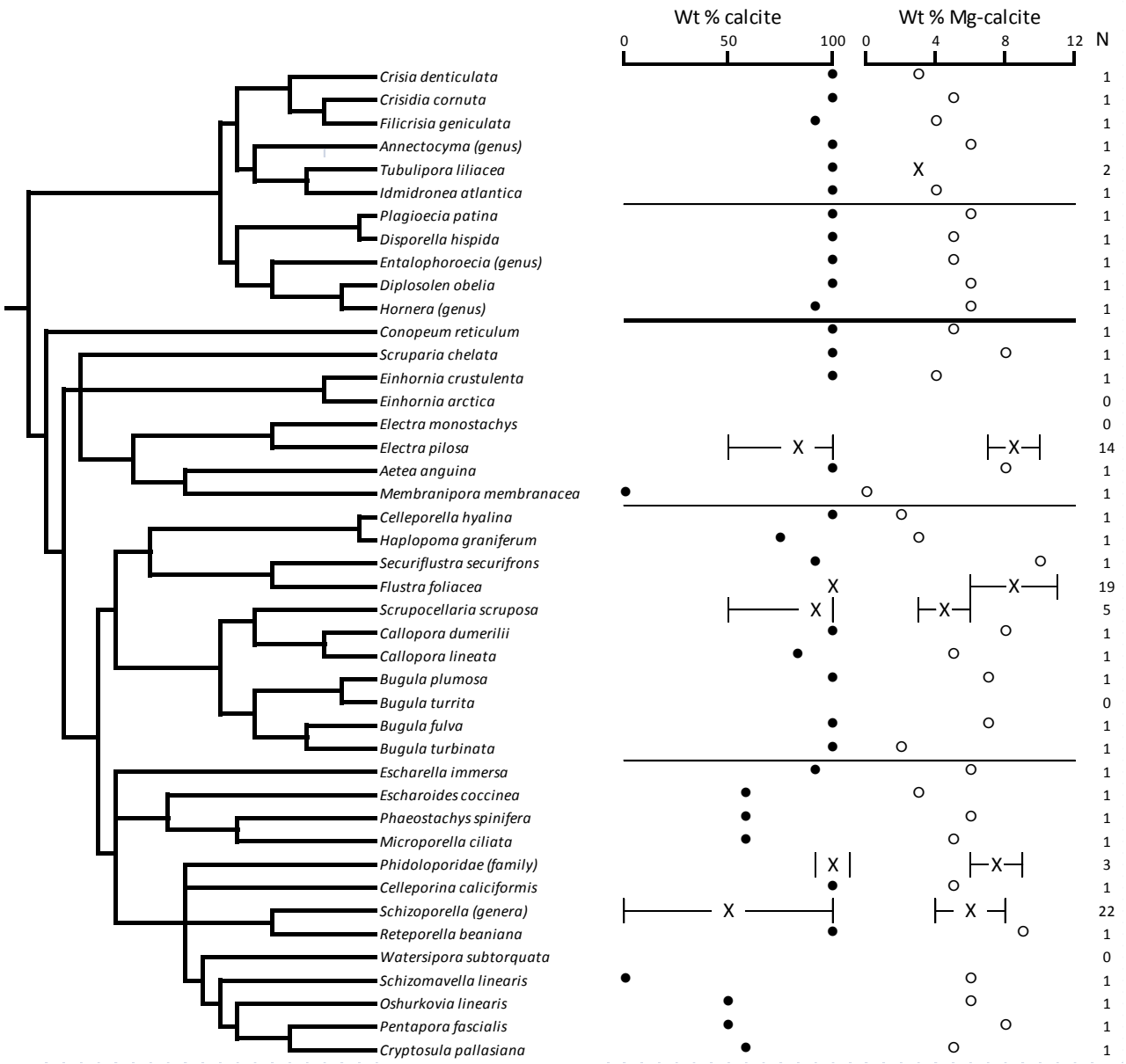


Figure 3.3: Phylogenetic distribution of skeletal carbonate mineralogy of Scottish bryozoans. Phylogenetic tree adapted from Waeschenbach et al. (2012). Crosses are means, with the range delineated by tails. Single measurements are represented by black (wt% $MgCO_3$ in calcite) or hollow (wt % calcite) circles. Wt. % Mg-calcite refers to wt% $MgCO_3$ in calcite.

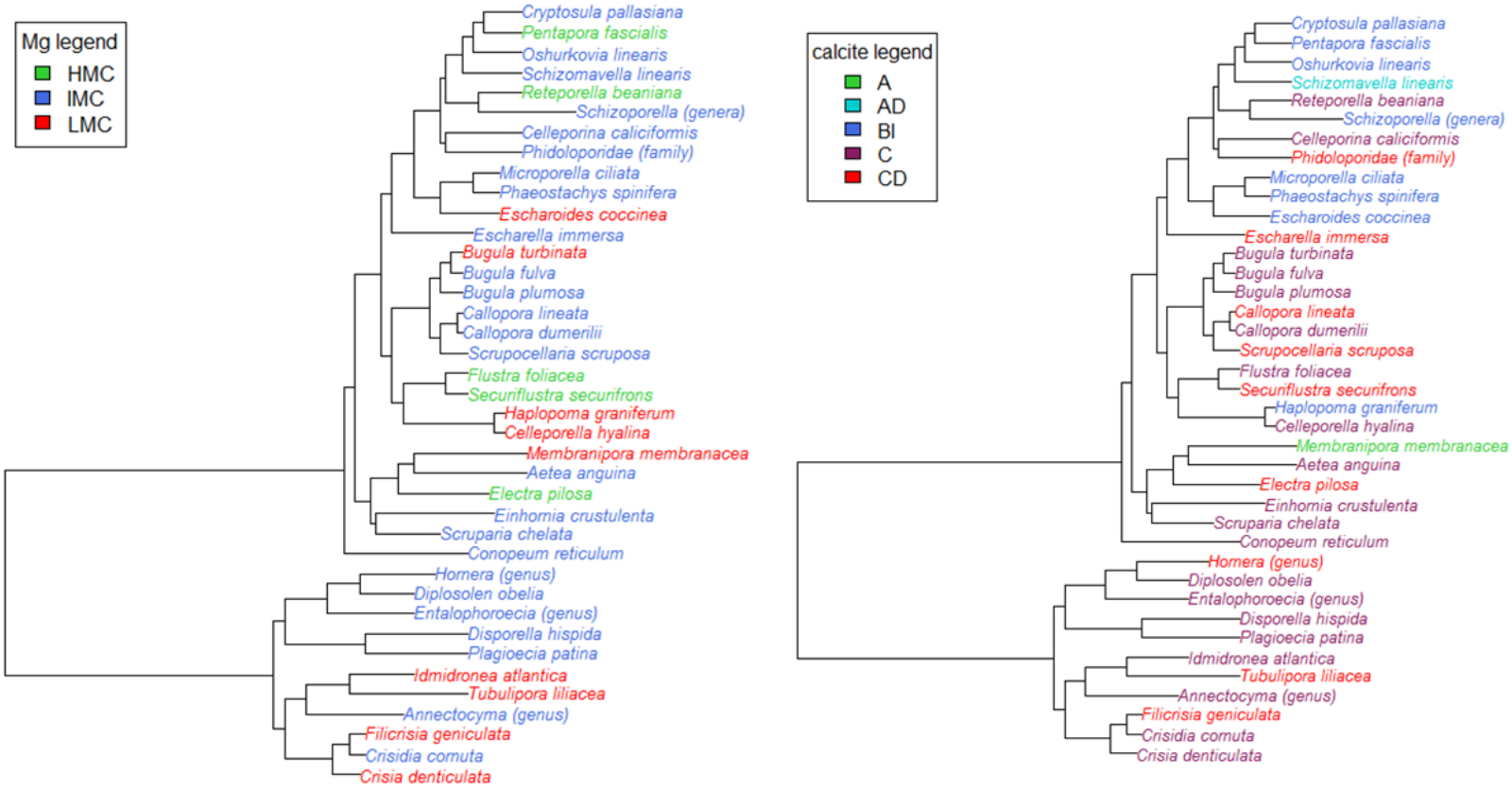


Figure 3.4: Phylogenetic trees modified from Waeschenbach et al (2012). Branch length indicates the number of substitutions per site. Left figure) species names are colour coded for wt% $MgCO_3$ in calcite: Green = HMC, blue = IMC and red = LMC. No significant pattern phylogenetic signal can be seen. Right figure) Species names are colour coded for wt% calcite content. Green = aragonite, turquoise= aragonite dominated, blue = bimineralic, red =calcite dominated and purple = calcite. A strong phylogenetic signal can be seen with relation to wt% calcite with more calcitic and calcitic dominated species in the cyclostome clade and a greater occurrence of aragonite containing species in the neocheilostomes. Mineralogical categorisation is detailed in Table 3.2.

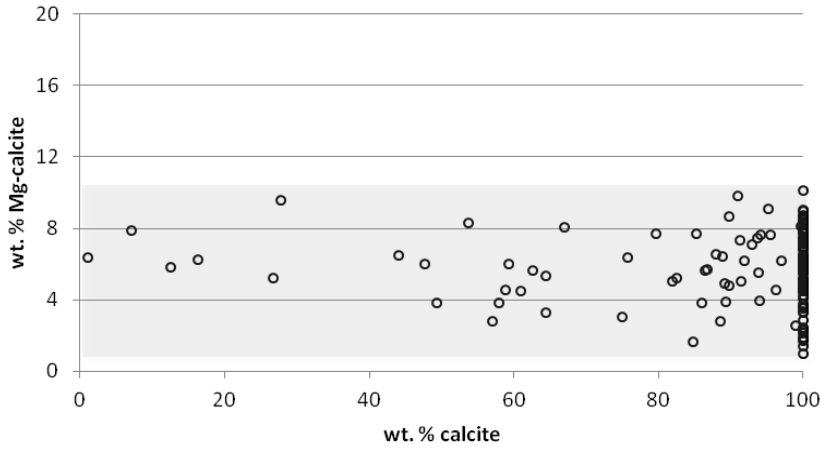


Figure 3.5: Skeletal carbonate mineralogy of 154 species of Scottish marine bryozoans. Shaded box indicates maximum mineralogical "space" occupied by the phylum in Scotland (42% of the total available mineralogical space)

The mineralogical "space" occupied by the phylum in Scotland is 914 $wt\%^2$, 42% of the possible area available for biomineralization and less than the 63% of mineralogical "space" occupied by the phylum globally (Smith et al. 2006) (Fig. 3.5).

ORDER CYCLOSTOMATA

Previous studies, including Smith et al. (2006) and Boardman & Cheetham (1987), have found this order to be almost entirely calcareous. Boardman & Cheetham (1987) went as far as to state "All stenolaemates have calcareous skeletons. All skeletons are calcitic except for one reportedly aragonitic species from the Triassic." .Cyclostomata is the only extant order of the five comprising the Class Stenolaemata and as such it is only Recent specimens from this order which have been analyzed in this Scottish study. Like Smith et al. (2006) data from this study indicates that Boardman & Cheetham's statement is mostly accurate although, like Smith et al.(2006) a few exceptions to this rule were found. 27 specimens of 25 species were analyzed with 20 (80%) found to be entirely calcareous. The remaining five species showed some aragonite with the measurements ranging from 82 to 99 wt% calcite. The mean wt% calcite for this class in Scotland is thus 98.5 (Fig. 3.6, Tab. 3.3). This mean value for the Order Cyclostomata is significantly higher than the mean for the Order Cheilostomata (mean=84.3, n=125) (Mann-Whitney U-test, p=0.016). Phylogenetic data were available for 11 cyclostome bryozoans (Figure 3.3) and analysis of this revealed that there is no phylogenetic signal within mineralogy for either wt% $MgCO_3$ in calcite (Blomberg's K = 0.75, p=0.200) or wt% calcite (Blomberg's K = 0.2, p=0.990).

Table 3.3: Skeletal carbonate mineralogy of higher taxa in the phylum Bryozoa in Scotland

Higher taxonomy of bryozoan mineralogy								Mean mineralogy		Biomineral space (% possible)
Phylum	**Classes**	**Order**	**Suborder**	**Families**	**Genera**	**Species**	**Specimens**	**Wt. % calcite**	**Wt. % Mg-**	
Bryozoa										
	Stenolaemata									
		Cyclostomata		12(12)	17(21)	25(31)	27	98.5	5.0	4%
			Articulata	1(1)	4(5)	7(8)	8	96.5	3.6	2%
			Tubuliporina	8(8)	9(11)	14(18)	15	99.5	5.2	2%
			Cancellata	2(2)	2(2)	2(2)	2	96.9	6.5	1%
			Rectangulata	1(1)	2(3)	2(3)	2	100.0	6.5	0%
	Gymnolaemata									0%
		Cheilostomata		36(38)	77(91)	129(167)	254	83.8	5.9	47%
			Inovicellina	1(1)	1(1)	3(3)	3	100.0	7.9	0%
			Scrupariina	1(1)	1(1)	2(2)	2	100.0	8.0	0%
			Malacostega	2(2)	4(4)	6(6)	18	92.0	6.8	6%
			Neocheilostomina	33(36)	71(85)	118(155)	231	83.2	5.9	47%

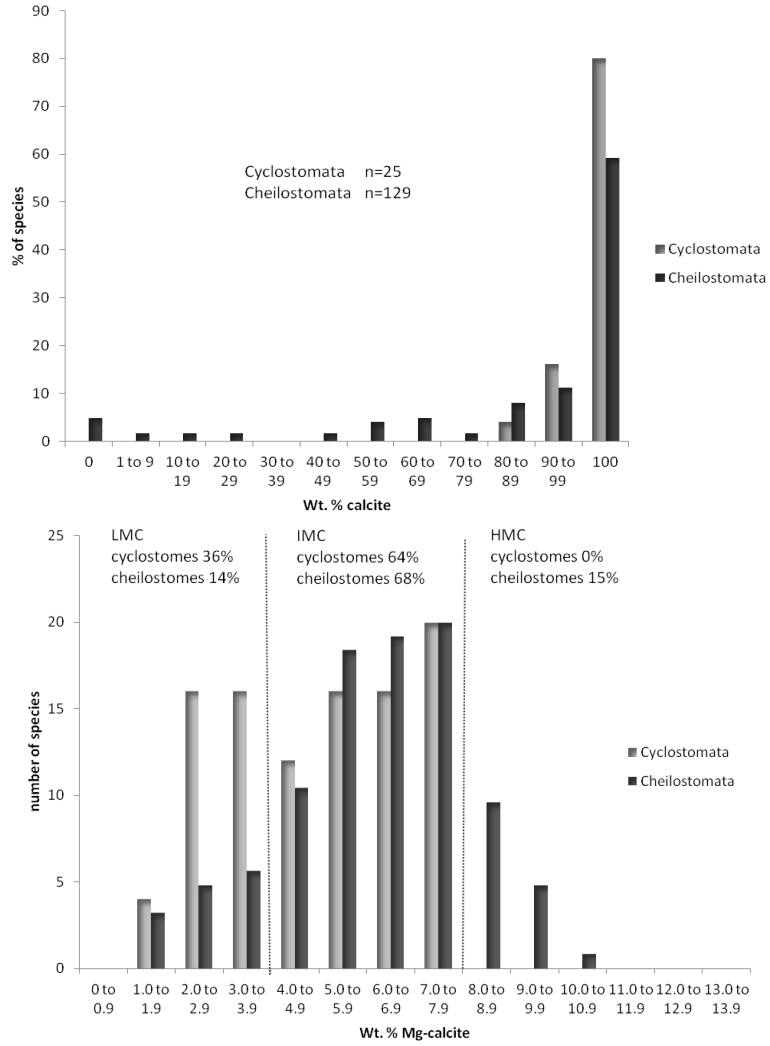


Figure 3.6: Frequency distributions for top) calcite content in Scottish cyclostomes (n=25) and cheilostomes (n=129) and bottom) wt% $MgCO_3$ in calcite containing Scottish cyclostomes and cheilostomes. LMC – low Mg-calcite; IMC – Intermediate Mg-calcite; HMC – High Mg-calcite.

The majority (64%) of species featured intermediate Mg-calcite with a further nine species exhibiting low Mg-calcite (36%). None were found to contain high Mg-calcite. The mean of 5.0 wt% $MgCO_3$ in calcite reflects the dominance of intermediate Mg-calcite in Scottish (Tab. 3.3). The mean wt% $MgCO_3$ in calcite for the Order Cyclostomata is significantly lower than the mean for the Order Cheilostomata (mean=5.94, n=125) (Mann-Whitney U-test, p=0.030). Cyclostomata take up only 4% of available biomineralogical space, less than a tenth of the space the phylum Bryozoa occupies in Scotland.

ORDER CHEILOSTOMATA

125 species of Scottish cheilostomatous Bryozoa were found to contain a wide range of mineralogical compositions. 57% (n=73) of species were found to be entirely calcareous with a further 39% (n=50) featuring bimineralic mixing in some degree and only 5% (n=6) found to be pure aragonite. The measured range for this group spanned the entire range from 0 to 100 wt% calcite. The mean wt% calcite for this Order in Scotland is thus 84.3 (Tab. 3.3). The majority (70%, n=85) of species featured intermediate Mg-calcite with the remaining species equally split between low Mg-calcite and high Mg-calcite (15%, n=18 for both IMC and HMC). The mean of 5.94 wt% $MgCO_3$ in calcite reflects the dominance of intermediate Mg-calcite in Scottish cheilostomatous species (Tab. 3.3). The Order Cheilostomata takes up 42% of the available biomineralogical space, and accounts for all the variability seen in the phylum Bryozoa in Scotland.

Phylogenetic analysis, on the 28 cheilostomatous species for which phylogeny data are available (Fig. 3.3), revealed no phylogenetic signal associated with wt% $MgCO_3$ in calcite (Blomberg's K = 0.38, p=0.250) (Fig.3.4a) but a statistically significant, strong phylogenetic signal associated with wt% calcite (Blomberg's K = 0.82, p= 0.020. As first observed by Smith et al. (2006) and confirmed here, *Neocheilostomata* is the most variable suborder in the Bryozoa with wt% calcite ranging between 0 and 100 and wt% $MgCO_3$ in calcite ranging between 1.0 to 10.2. Phylogenetic analysis confirms this pattern. The clade containing the *Neocheilostomata* (*Umbonulomorpha*, *Lepraliomorpha*, *Flustrina* and *Hippothoomorpha*) shows a highly significant, strong phylogenetic signal associated with wt% calcite (Blomberg's K = 0.85, p=0.005) (Figure 3.3)

Table 3.4: Mineralogical characteristics of 47 Bryozoa families from Scotland

Family	Genera	Species	Specimens	Overall mineralogy	Mean wt.% calcite	Mean wt.% Mg-calcite	Range wt.% calcite	Range wt.% Mg-calcite	Biomin space %	FAD stage	Age at top (Ma)	Total genera	Coverage of genera
Aeteidae	1	3	3	IMC	100.0	7.9	0.0	0.0	0.00%	Pliensbachian*	190	1	100%
Annectocymidae	1	1	1	IMC	100.0	6.4				Albian***	110	1	100%
Antroporidae	1	1	1	IMC	100.0	6.5				Maastrichtian**	65	1	100%
Beaniidae	1	1	1	IMC	100.0	7.5				Bartonian**	37	1	100%
Bitectiporidae	3	4	4	mix	45.3	6.6	99.0	3.1	14.14%	Ypresian**	49	3	100%
Bryocryptellidae	3	8	9	calcite	99.2	7.2	6.0	6.2	1.71%	Ypresian**	49	3	100%
Bugulidae	4	11	11	calc dom mix	85.0	4.6	50.8	5.2	12.19%	Recent**	0	4	100%
Calloporidae	7	12	12	calcite	92.6	5.4	56.0	5.9	15.08%	Albian**	99	8	88%
Candidae	4	7	13	mix	87.5	4.2	87.5	4.5	17.87%	Maastrichtian**	65	4	100%
Cellariidae	1	3	18	IMC dom mix	97.3	5.3	8.3	1.7	0.64%	Santonian**	83.5	2	50%
Celleporidae	7	10	24	IMC dom mix	98.6	6.2	11.3	4.5	2.32%	Priabonian**	33.7	7	100%
Chaperiidae	1	1	1	Arag dom	5.9	7.7				Maastrichtian**	65	1	100%
Chorizoporidae	1	1	1	IMC	100.0	5.5				Servallian**	11.2	1	100%
cribrilinidae	3	5	5	calcite	97.8	4.8	10.9	6.5	3.27%	Cenomanian**	93.5	4	75%
Crisiidae	4	7	8	calcite	96.5	3.6	10.3	2.7	1.28%	Maastrichtian**	65	5	80%
Cryptosulidae	1	1	1	mix	58.8	4.6				Tortonian**	7.1	1	100%
Diaperoeciidae	1	1	1	IMC	100.0	4.5				Hauterivian**	127	1	100%
Diastoporidae	1	1	1	IMC	100.0	5.8				Pliensbachian**	190	1	100%
Doryporellidae	1	1	1	IMC		7.2				Albian***	110	1	100%
Electridae	3	4	16	calc dom mix	89.2	6.6	24.3	4.6	5.11%	Tithonian*	163.5	4	75%
Escharinidae	3	5	5	calc dom mix	92.5	6.7	37.5	1.7	2.98%	Ypresian*	49	3	100%
Eucrateidae	1	1	1	calc dom mix	85.2	7.7				Recent**	0	1	100%
Exochenellidae	1	1	3	aragonite	0.0	0.0	0.0	0.0	0.00%	Ypresian*	49	1	100%
Exochellidae	1	2	2	calc dom mix	82.2	5.1	35.6	3.7	5.98%	Santonian*	83.5	1	100%
Flustridae	4	5	23	calc dom mix	83.7	8.7	72.2	3.6	11.77%	Recent**	0	6	67%
Haplopomidae	1	4	4	calc dom mix	91.6	4.6	25.1	5.7	6.54%	Lutetian**	90	1	100%
Hippoporidridae	0	0	0							Chattian*	23.03	1	0%
hippothoidae	2	2	2	mix	50.0	1.7	100.0	0.0	0.00%	Coniacian**	85.8	3	67%
Horneridae	1	1	1	calc dom mix	93.7	5.6				Barremian**	121	1	100%
Lacernidae	0	0	0							Priabonian**	33.7	1	0%
Lepraliellidae	1	1	1	calc dom mix	95.5	7.7				Santonian**	83.5	1	100%
Lichenoporidae	2	2	2	IMC	100.0	6.5	0.0	2.6	0.00%	Cenomanian**	93.5	3	67%
Membraniporidae	2	3	41	mix	65.1	7.1	100.0	4.1	18.64%	Priabonian**	33.7	2	100%
Microporellidae	2	2	2	mix	74.6	3.7	27.6	1.7	2.10%	Aquitanian**	20.5	2	100%
Microporidae	0	0	0									2	0%
Oncousoeciidae	2	3	3	IMC	100.0	7.0	0.0	1.6	0.00%	Sinemurian*	199.3	2	100%
Phidoloporidrae	1	3	3	calc dom mix	95.6	7.6	13.3	2.8	1.71%	Dunian**	61	3	33%
Plagioeciidae	1	1	1	calcite	100.0	6.2				Pliensbachian**	190	1	100%
Romancheinidae	4	10	10	calc dom mix	96.8	6.5	13.7	1.9	1.18%	Campanian**	71.3	6	67%
Schizoporellidae	1	3	22	arag dom mix	43.5	6.4	57.3	2.5	6.68%	Ypresian**	49	1	100%
Scrupariidae	1	2	2	calcite	100.0	8.0	0.0	0.1	0.00%	Maastrichtian*	70	1	100%
Setosellidae	1	1	1	aragonite	0.0	0.0				Thanetian**	54.8	1	100%
Smittinidae	5	7	7	mix	51.8	6.5	100.0	3.1	14.23%	Ypresian**	49	5	100%
Stigmatoechidae	1	1	1	IMC	100.0	7.4				Camponian*	83.6	1	100%
Stomachetosellidae	1	2	2	arag dom mix		7.7		2.5	0.00%	Maastrictian*	70	1	100%
Stomatoporidae	1	1	1	calc dom mix	92.8	7.2				Carnian*	235	2	50%
Terviidae	1	1	1	IMC	100.0	8.0				Ypresian**	49	1	100%
tessaradomidae	1	1	1	IMC	100.0	6.4				Danian**	61	1	100%
Tubuliporidae	2	5	6	calcite	99.8	2.8	1.1	3.6	0.19%	Campanian**	71.3	2	100%
Umbonulidae	1	1	1	mix	47.6	6.0				Lutetian**	41.3	1	100%

First appearance datum taken from the following sources * Taylor, 1993 ** Smith et al, 2006, *** Taylor et al 2009
range and mineralogical space has only been calculated where more than 1 specimen has been analyzed.

although no corresponding signal associated with wt% $MgCO_3$ in calcite (Blomberg's K = 0.37, p=0.335) (Fig. 3.4). In comparison the clade containing *Malacostega, Scrupariina* and *Inovicellata* shows no phylogenetic signal in either wt% calcite (Blomberg's K = 0.85, P = 0.710) or wt% $MgCO_3$ in calcite (Blomberg's K = 0.93, p=0.570)

FAMILIES

Specimens from 47 families within the phylum Bryozoa (Tab. 3.4) have been analyzed, 94% of the Recent families found within Scotland. Many of these families are only represented by one or two species although some include as many as 14 species. Although some families contain only one analyzed specimen and conclusions should, therefore, be approached with caution, the mean number of specimens analyzed per family is six, and the maximum is 41, allowing some generalizations to be made. Coverage of Scottish genera within analyzed families range from 33% to 100% with a mean of 81% of Scottish genera included here (Tab. 3.4)

Smith et al. (2006) surmised that bryozoan families fall into three general groups; those containing mostly aragonite, those containing mixed mineralogy and those containing mostly calcite. In general the data presented here concurs with this, although the distribution of Scottish species within these categories. (4% mostly aragonite, 32% mixed mineralogy, 64% mostly calcite) varies slightly from the Global data presented by Smith et al. (2006) (5% mostly aragonite, 20% mixed mineralogy, 75% mostly calcite). Most families, where more than one specimen has been analyzed, show some level of variation within their mineralogy. Some of the intermediate Mg-calcite families which exhibit little or no variation are *Aetidae* (n=3), *Licheniporidae* (n=2) and *Oncousoeciidae* (n=3). There are no families which consistently produce low or high Mg-calcite.

Families which are exclusively aragonitic (*Setosellidae* (n=1), *Exochenellidae* (n=3) and those which are aragonite-dominated (*Stomachetosellidae*, n=2; *Schizoporellidae*, n=22; *Chaperiidae*, n=1) are found to be a mixture of flustrinid and ascophoran cheilostomes. The most variable family studied from Scotland is the *Membraniporidae* (18.6% of potential mineralogical space, n=41).

There is no statistically significant difference between wt% $MgCO_3$ in calcite in families which appeared during aragonitic and calcitic seas. Families, which first appeared during

periods of aragonitic seas (approx 50Ma-recent and during the Triassic), do however, almost exclusively feature intermediate to high Mg-calcite (mean = 6.0, n=21) (Fig. 3.7). This fits with the sea water chemistry of the time where Mg/Ca ratios were at their highest. Families forming low Mg-calcite all evolved during times of calcitic seas where the Mg/Ca ratio in seawater was much lower (Figure 3.7).

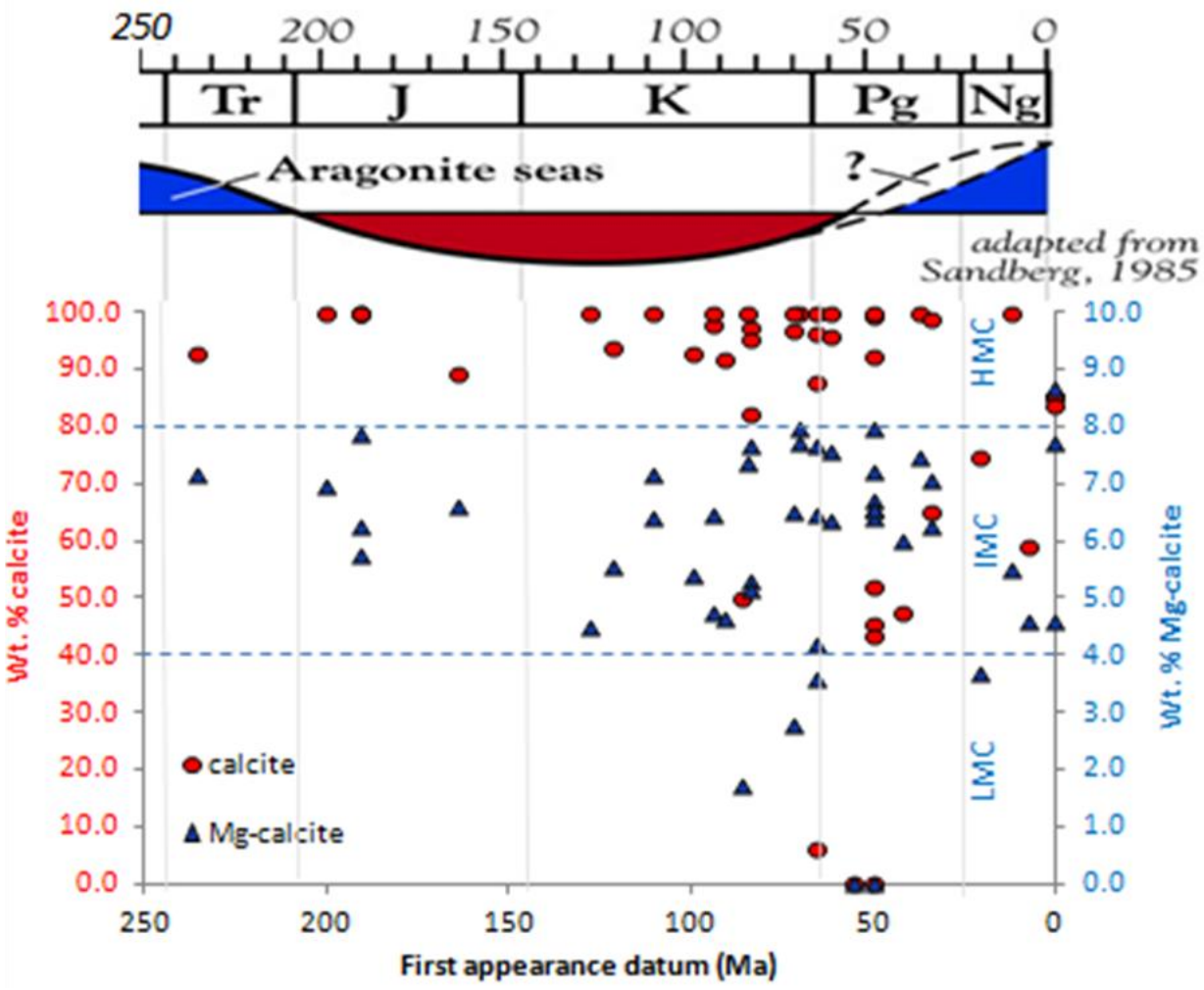


Figure 3.7: Figure showing the Wt. % calcite (red circles) and Wt. % Mg-calcite (blue triangles) against the First Appearance Date (FAD) for the family. Also figured is the geological timescale and seawater chemistry for the period of bryozoan evolution (adapted from Sandberg, 1985)(Sandberg 1985). Wt. % Mg-calcite refers to wt% $MgCO_3$ in calcite.

GENERA AND SPECIES

81% of known Scottish genera were analyzed in this study and carbonate mineralogy recorded for 91 genera shows that 49 % are purely calcite, 46% contain a mixture of polymorphs and 4% are pure aragonite. 67% of these genera, however, consist of only a single species and in this circumstance the mean for the genus should be approached with caution. Data for individual species, a more consistent measure and also one with biological significance, are more useful for further analysis.

Only one genus, *Cellaria*, consistently exhibited two phases of calcite within its skeleton. Both phases were present in all examined species (n=3) and specimens (n=18) in the approximate ratio of 1:1. The first phase is low or intermediate Mg-calcite with the second phases featuring intermediate or high Mg-calcite. There was some variation in the concentration of magnesium within these phases between species; *Cellaria fistulosa* (Linnaeus, 1758) (n=16) features a first phase of LMC with a mean of 1.9 wt% $MgCO_3$ in calcite (range 0.48-3.67). The second IMC phase showed a similar range of variation around a mean of 7.6 (range 5.94-9.86). Staining and previous publications (Schäfer & Bader 2008) show that the LMC phase is located predominantly in the central axis of the internode, and the highest wt% $MgCO_3$ in calcite is found around the chitinous joints which connect internodes. *C. sinuosa* (Hassall, 1840) (n=1) has the highest average wt% $MgCO_3$ in calcite of the species examined. *C. sinuosa* features first phase IMC (4.91 Wt. % Mg-calcite) in dominant proportions to the second phase HMC (9.8 wt% $MgCO_3$ in calcite). *C. salicornoides* Lamouroux, 1816 shows less of a marked difference between the phases with both phases IMC (4.16 and 6.65 wt% $MgCO_3$ in calcite).

For the remaining analysis in this Section and for Section 3.5.2 mineralogical data have been extended to include those summarized by Smith et al. (2006) and those completed by Taylor et al. (2009). OBIS distribution data have been added to by the authors own records and those of Smith et al. (2006) and Taylor et al. (2009) to get as complete a geographical species range as possible. *Electra pilosa* has been excluded from this analysis due to the unreliability of identification and distributional records. *E. pilosa*, historically thought to have a near global distribution, has been split into separate cryptic species in recent years such as *E. scuticifera* Nikulina, 2008; this has not yet been reflected in distributional data.

Anarthropora monodon (Busk, 1860) (n=3) is one of only two known species in the genus *Anarthropora*, the second being *A. voigti* Brown, 1958 an Australian Recent species. The data presented here are, to the author's knowledge, the first analysis of the genus and shows *A.monodon* to consistently present 100% aragonite. *Flustra foliacea* was found to be the most variable of all of the species analyzed with a wt% $MgCO_3$ in calcite range within Scotland of 4.4. Additional literature sources from other parts of Europe (Carver & Rucker 1969; Smith et al. 2006; Schopf & Manheim 1967) extend this range even further to 9.4. *Eucratea loricata* (Linnaeus, 1758) is found to be the next most variable species (wt% $MgCO_3$ in calcite range = 6.5). It should be noted, however, that this large range is due to two outlier LMC values from the Laptev Sea in Russia (Taylor et al. 2009) of 2.4 and 3.8 wt% $MgCO_3$ in calcite in this otherwise consistently IMC/HMC species. If these values are excluded, then the range becomes a conservative 2.7 (Mean = 7.1, min 6.2 max 8.9).

3.5.2 Distributional range of species

Statistically (Mann-Whitney U test, p=0.040) there is much less wt% $MgCO_3$ in calcite variation in cyclostomes than in cheilostomes, even in those with large geographical ranges of distribution, such as *Idmidronea atlantica* (Forbes, in Johnston, 1847) (latitudinal range 152.9 lat° (77.8°S to 75°N), wt% $MgCO_3$ in calcite range 1.3), although this large geographical range may be more indicative of taxonomic uncertainty of the species rather than true diversity of geographic distribution.

A sub-group of cheilostomes, which shows heightened variation in wt% $MgCO_3$ in calcite, are those which have become successful as alien/non-native species, extending their distributional range across the globe into non-native territory (mean species distributional range = 89.8 lat°, minimum range 24.3 lat° to maximum range 125.1 lat°; mean wt% $MgCO_3$ in calcite range = 5.2, range 3.4 to 7.2). This collection of species features statistically higher variation in wt% $MgCO_3$ in calcite than the other cheilostomates examined (Mann-Whitney U test, p=0.010).

The most mineralogically variable of these species is *Schizoporella unicornis* (Johnston in Wood, 1844) with an Mg-calcite range of 7.2 (0.6-7.8 wt% $MgCO_3$ in calcite) and a latitudinal range of 84 lat° (25.6S – 58.4N). This bimineralic species also features highly variable wt% calcite with a range of 55 wt% calcite (minimum 22-76 wt% calcite). The mineralogical plasticity of this species has also observed in previous publications (Clarke & Wheeler 1922; Lowenstam 1954; Schopf & Manheim 1967; Carver & Rucker 1969; Poluzzi & Sartori 1973; Smith et al. 2006). *Bugula neritina* (Linnaeus, 1758) has the widest geographical range in distribution covering 125.1 latitudinal degrees (70.1S to 55N) and a correspondingly high level of mineralogical variation with a range of 6.0 wt% $MgCO_3$ in calcite. An exceptional native Scottish species, which has become a successful invasive in the USA, is *Membranipora membranacea*. There have been reported 3 unusual records, two of 100% calcitic specimens (Ristedt 1977; Williams 1990) and a single bimineralic specimen (Taylor et al. 2009), however, as this species is otherwise reported as globally aragonitic, these should perhaps be approached with caution. There is no discernible pattern between the mean distribution (latitude) of a species and the mean wt% calcite or mean wt% $MgCO_3$ in calcite.

3.5.3 Latitudinal Patterns

MEDITERRANEAN TO ARCTIC

Mineralogical means for species from both this study and literature (Schopf & Manheim 1967; Carver & Rucker 1969; Smith et al. 2006) were combined with OBIS species distribution data from 35°N to 80°N (Intergovernmental Oceanographic Commission (IOC) of UNESCO 2010). The resulting dataset was analysed for significant patterns.

There is a statistically significant pattern of increasing HMC at lower latitudes (linear regression, R^2=70.4%, F= 16.66, p=0.005). IMC is present in a fairly constant proportion of specimens along the latitudinal gradient and shows no statistically significant relationship (Fig. 3.8). LMC does not show a statistically significant relationship to latitude.

There is a statistically significant pattern of increasing mean wt% calcite of specimens at higher latitudes (linear regression, R^2=65%, F=13.03, p=0.009). This is represented in Fig. 3.9. Although quantity of mixed mineralogy specimens remains fairly constant across the latitudes and 100% aragonite specimens seem to show no distributional patterns, the occurrence of 100% calcite specimens statistically increases at higher latitudes (linear regression, R^2= 50.4%, F=7.11, p=0.030) (Fig. 3.9).

A subset of 39 species, for which phylogenetic and latitudinal information was available, was tested using MCMCglmm to see the comparative and combined influence of these factors on mineralogical measurements. The best fitting model to explain the wt% calcite data was that which looked at the effects of latitude alone. Phylogeny has a negative impact on the fit of the model with the phylogeny + latitude model increasing the ΔDIC by 16 compared to the model for latitude alone. The best fitting model for wt% $MgCO_3$ in calcite was the combined model incorporating effects of both phylogeny and latitude. In this case the ΔDIC between the combined model and the next best fitting model, latitude alone, was small (4.4). Although a model using both of the factors was the best-fit, latitude was the most significant effect in this model accounting for over 88% of the total variance seen with phylogeny accounting for an additional 12% of the variability.

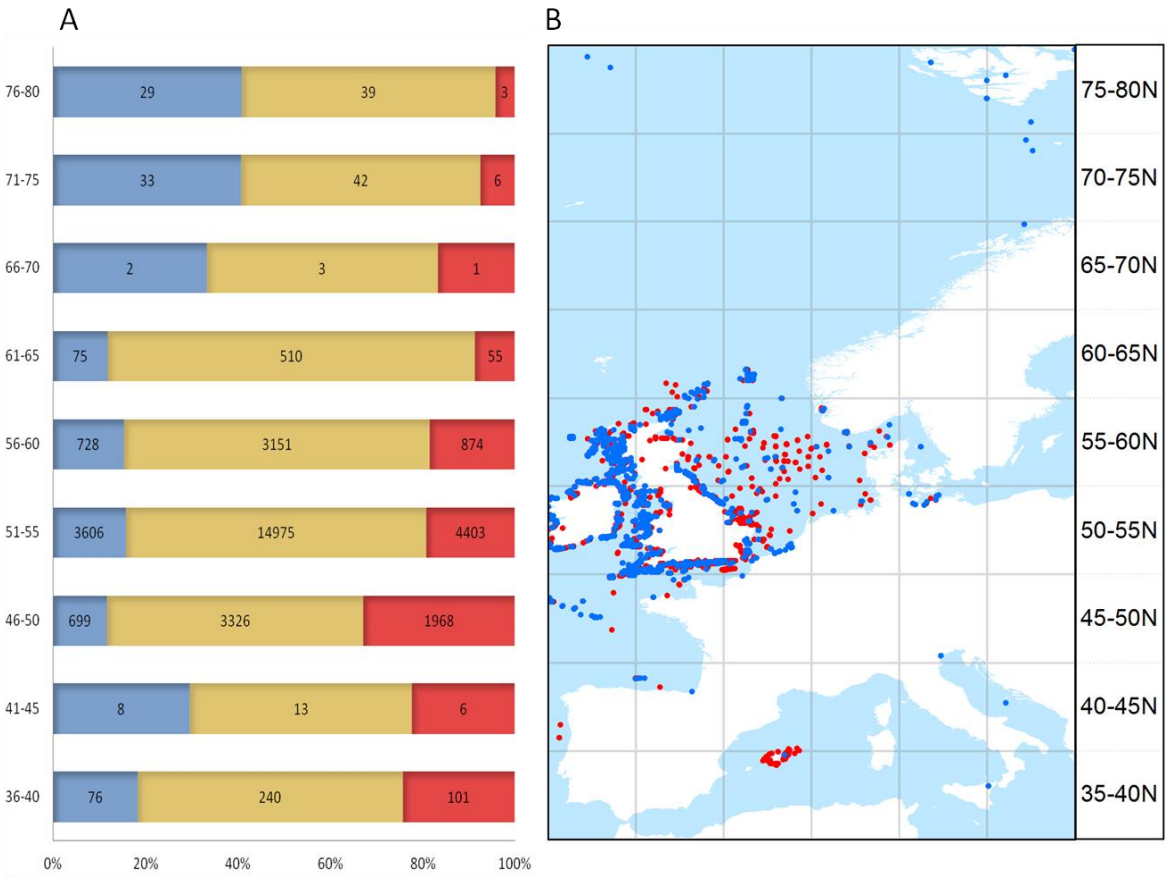


Figure 3.8: Map and barchart showing distribution of wt% $MgCO_3$ in calcite along the latitudinal gradients from 35°N-80°N. a) Barchart indicating % split of the Bryozoa featuring low Magnesium calcite (LMC -blue), intermediate Magnesium calcite (IMC -orange) and high Magnesium calcite (HMC -red) for each 5 degree latitudinal band. Volumes of distributional records are numerically displayed. b) GIS generated map showing distribution of LMC and HMC bryozoans. For the purposes of clarity IMC is excluded.

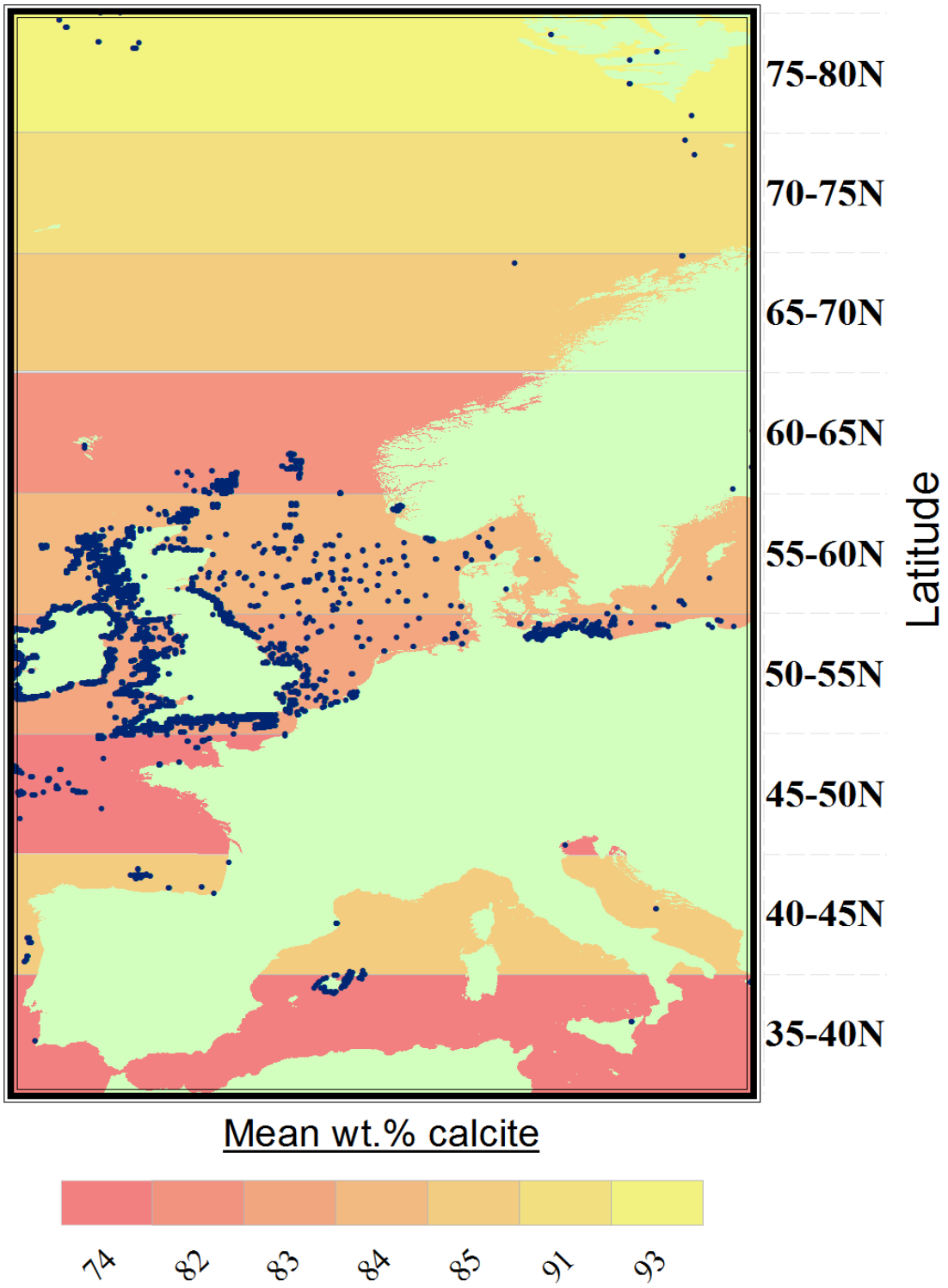


Figure 3.9: Map showing distribution records (dark blue points) from latitudes 35-80°N.

The map is divided horizontally into bands of 5 latitudinal degrees and each band is colourised to show the mean wt% calcite of the Bryozoa within that band. This shows the statistically significant trend of increasing wt% calcite with increasing latitude (p=0.009). The colour key is shown below the map.

3.5.4 Other temperate regions

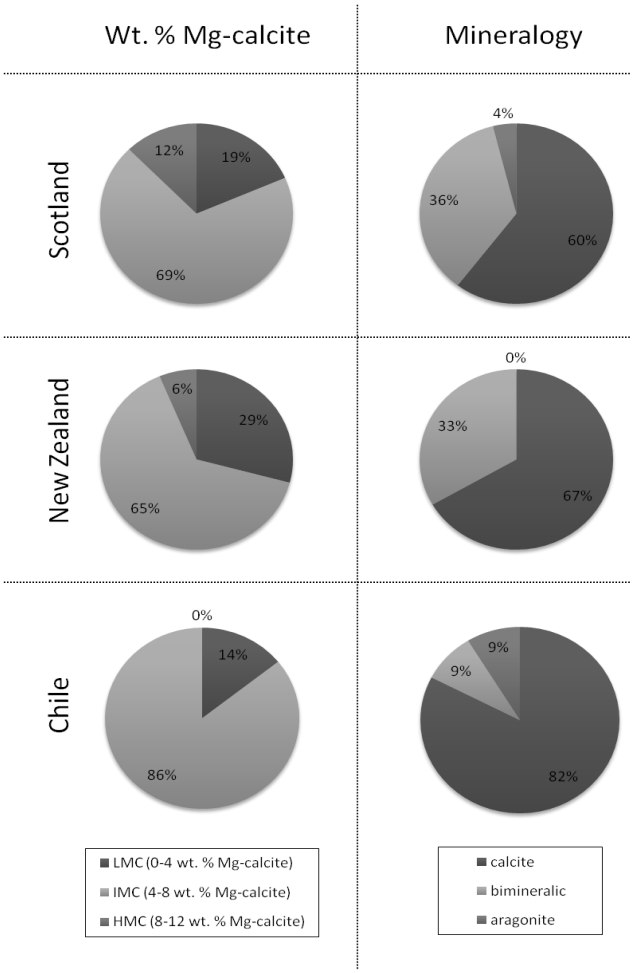


Figure 3.10: Comparative mineralogy of bryozoan species from Temperate regions. Scottish data authors own, New Zealand (Smith et al. 1998), Chile(Smith & Clark 2010)

NEW ZEALAND

49 species of New Zealand Bryozoa were investigated by Smith et al. (1998; 2006). This represents 5% of the known species of New Zealand Bryozoa (n=953) (Gordon 1984; Gordon et al. 2010). Analyzed species consisted of 32 (67%) that were calcitic, 16 (33%) which were a mixture of the polymorphs and no aragonitic species.

Of those species containing calcite, Smith et al. found 29% LMC, 65% IMC and 6% HMC. (Fig. 3.10). Mean wt% $MgCO_3$ in calcite was 5.1 (range 0-9.7 wt% $MgCO_3$ in calcite)

CHILE

Mineralogical analyses of 23 Bryozoa from Chile were collated and reviewed by Smith & Clark. (2010). This represents 9% of the described Chilean Bryozoa (n=267) (Moyano

1983). The Chilean collection comprised of 19 (82%) calcitic species and 2 (9%) each of aragonitic and those with mixed mineralogy. Calcite containing species were found to have a mean wt% $MgCO_3$ in calcite of 5.35 (range 2.1-7.7 wt% $MgCO_3$ in calcite) and be 14% LMC, 86% IMC and no HMC species.

There is a statistical difference in species wt% $MgCO_3$ in calcite between Scotland, New Zealand and Chile (ANOVA, F=3.12, p=0.046). *Post-hoc* Mann-Whitney analysis shows Scotland to be statistically different to New Zealand (p=0.030) but not Chile and no statistical difference between Chile and New Zealand.

3.5.5 Comparison to Global and Polar analyses

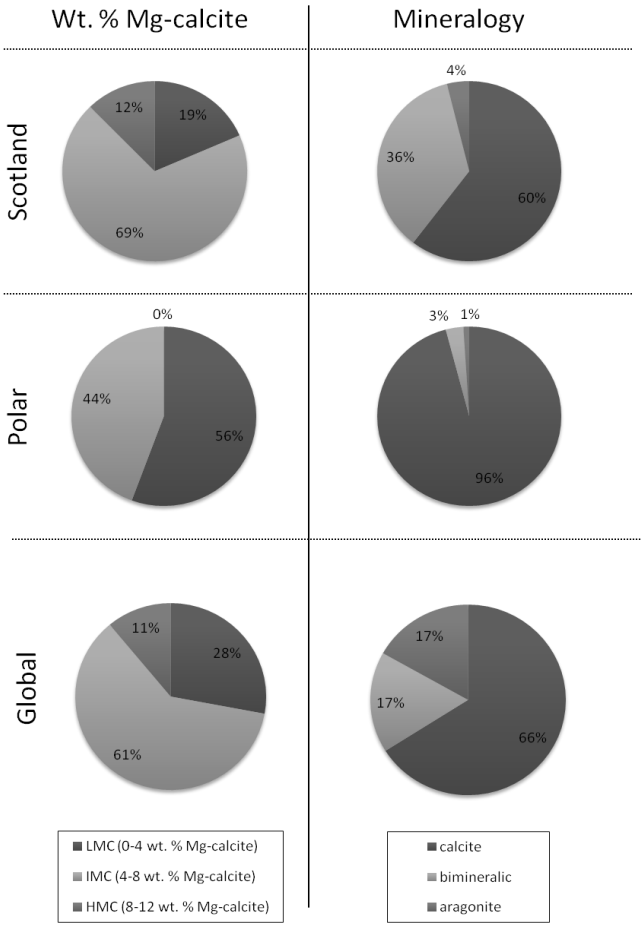


Figure 3.11: Comparative mineralogy of Bryozoa from Scotland, the Polar regions and Global. Scottish data authors own, Polar(Borisenko & Gontar 1991; Kuklinski & Taylor 2009) , Global (Smith et al. 2006)

Note: Polar and Scottish data is for individual species whilst Global data is for analysed specimens.

POLAR ANALYSES

Both Arctic and Antarctic species have been mineralogically examined. Borisenko & Gontar (1991) analyzed 21 Antarctic species and Kuklinski & Taylor (2009) added a further 76 Arctic species to the dataset.

Analyzed Polar species consisted of 93 (96%) that were calcitic, three (3%) which were a mixture of the polymorphs and just one aragonitic species (1%) (Fig. 3.11).

Of those Polar species containing calcite, 56% were found to contain LMC, 44% IMC and none HMC (Fig. 3.11). Mean wt% $MgCO_3$ in calcite was 3.5.

GLOBAL ANALYSES

The mineralogical data of 1183 specimens, covering a Global distribution, were summarized by Smith et al. (2006). Analyzed specimens consisted of 59% (n=694) that were calcitic, 15% (n=180) which were a mixture of the polymorphs and 15% (n=177) aragonitic (Fig. 3.11). Of those specimens containing calcite, Smith et al. (2006) found 28% LMC, 61% IMC and 11% HMC (Fig. 3.11). Mean wt% $MgCO_3$ in calcite was 5.0 (range 0-13.7 wt% $MgCO_3$ in calcite).

Wt% $MgCO_3$ in calcite in Scottish species (n=156) is statistically different (Mann-Whitney U-test, $p<0.001$) to those from its geographical neighbour, the Arctic (n=149) (Kuklinski & Taylor 2009).

3.6 Discussion

3.6.1 Bryozoa in Scotland

To date there has only been limited analysis conducted on the species which reside in Scottish waters (see Appendix A). In this study 115 new species, 57 new genera and 22 families are added to the record. This study is also of significance globally where the data augment the growing database of mineralogical analyses and offer first analyses for 26 genera and four families (see Appendix B).

3.6.2 Taxonomic, Temporal and Phylogenetic Patterns

Bryozoan skeletal mineralogy in Scotland was found to be very varied, occupying over two thirds of the mineralogical space occupied by the phylum globally. As may be expected from previous temperate studies (Smith et al. 1998; 2006; Smith & Clark 2010), the dominant mineralogy is IMC (68%) and this is reflected in the mean wt% $MgCO_3$ in calcite of 5.86. LMC (18%) and HMC (14%) make up the rest of the phylum. Calcite was the most common calcium carbonate polymorph (60% of species) although a large percentage of analysed specimens (36%) also featured some aragonite and this is reflected in the mean calcite of 84.4 wt%. Pure aragonite was found to be rare (4%).

The reports of previous publications led to the expectations that cyclostomatous species would feature lower mean Mg-calcite and less aragonite than their cheilostomatous counterparts. This is supported by the statistically significant data presented in this study (Section 3.5.1). Cyclostomes exhibit less than a tenth (4% mineralogical space) of the mineralogical variability exhibited by cheilostomes (42% mineralogical space) and literature has attributed this to the comparatively ancient evolutionary origins of cyclostomes (mean family FAD = 128Ma) compared to cheilostomes (mean family FAD = 61Ma). If the comparative phylogenetic diversity index (PD) is examined, a measure proposed by Faith (1992), between cyclostomes and cheilostomes then cyclostomes have a much lower PD value (2.5) than cheilostomes (P.D = 4.3). This indicates that cyclostomes are more clumped on the tree (Figure 3.3) and represent only a small part of the total phylogenetic diversity present in the phylum. This lower genetic diversity could account for the lower mineralogical variability of cyclostomes when compared to cheilostomatous species.

The recent publication of a bryozoan phylogeny also allows the observation that cyclostomes feature less genetic substitutions since their divergence from a common ancestor than their cheilostomatous counterparts, indicated by the comparative branch length (Waeschenbach et al. 2012). This indicates cyclostomes have a greater genetic similarity to the common ancestor than cheilostomes and may also explain the greater similarity of cyclostomatous species to the predicted ancestral mineralogy of low to intermediate Mg-calcite with aragonite being a derived trait (Rucker 1968; Boardman & Cheetham 1987; Taylor & Monks 1997; Taylor & Schindler 2004; Poluzzi & Sartori 1974; Smith et al. 2006) . In this study, however, far fewer cyclostomes were analysed than cheilostomes and it should be highlighted that there is a possibility this may be accentuating observed patterns of skeletal variability. In accordance with the publication by Smith et al. (2006) the most mineralogically variable of the cyclostomatous suborders is found to be *Tubuliporina* (2% mineralogical space). A recent phylogeny of cyclostomes published by Waeschenbach et al (2009) shows *T. liliacea* (Pallas, 1766) to have the greatest phylogenetic distance amongst the cyclostomes from the common ancestor, jointly with *Plagioceia patina* (Lamarck, 1816) and *Disporella hispida* (Fleming, 1828), and it is possible that this greater genetic divergence has allowed for the development of greater mineralogical plasticity amongst the rest of this suborder. It would be interesting to analyse multiple specimens of *D. hispida* and *P. patina* to understand if these also feature greater skeletal flexibility in their mineralogy than other cyclostomes. It is probable that, as Smith et al observe in their study (2006), the observed variability of *Tubuliporina* in this study is disproportionally magnified by to the higher number of analyzed species (n=15) when compared to the other Cyclostome suborders (mean n=4).

The most variable suborder within the Order Cheilostomata, as observed by Smith et al. (2006) and confirmed here, is found to be *Neocheilostomata,* accounting for all the mineralogical variability in the Phylum Bryozoa within Scotland. The phylogeny for the Bryozoa indicates 3 clades within Cheilostomata (Waeschenbach et al. 2012) (Figure 3.3). Two of these clades, containing *Umbonulomorpha*, and *Lepraliomorpha* and *Flustrina* and *Hippothoomorpha* respectively, share a common ancestor and as such are sister clades; together they represent the *Neocheilostomata*. This collective clade of *Neocheilostomata* shows a strong, statistically significant phylogenetic signal for wt% calcite (Blomberg's K = 0.85, p=0.005) which indicates that there is mineralogical similarity between these

closely related species. The majority of *Neocheilostomatous* species are bimineralic to some extent and this may indicate a bimineralic common ancestor. There is no such phylogenetic signal detected in *Neocheilostomata* for wt% $MgCO_3$ in calcite (Blomberg's K = 0.37, p=0.335).

Specimens from 94% of Recent families from Scotland were analysed. Data show a wide range of variability in mineralogy – ranging from those which consistently exhibit calcite in their skeletons to those which only feature aragonite or mixed mineralogy. The most variable family studied from Scotland is the Membraniporidae (18.64% of potential mineralogical space, n=41) which concurs with previous observations of the family (Smith et al. 2006). It is likely however that this is due to poor taxonomic grouping. Current taxonomy (Appeltans et al. 2012) groups the genera of *Conopeum* and *Membranipora* together in the family Membraniporidae. Recently published phylogenetic data on the species *C. reticulum* (Linnaeus, 1767) and *M. membranacea* show however, that these species are genetically distant from each other (Waeschenbach et al. 2012). The phylogenetic tree shows *C. reticulum* outside of the other observed cheilostomatous clades (Figure 3.3). It is proposed that it is the taxonomic grouping of these genetically dissimilar species into a single family which is causing an unexpectedly and incorrectly high observed variability in skeletal mineralogy. Smith et al. (2006) observe that the majority of families which feature aragonite first appeared after the mid-Cretaceous radiation of the Bryozoa. Scottish data seem to concur with this (Figure 3.7), however, Taylor et al. (2009) highlight that care should be taken in generalizing based on family first appearance datum. In their publication on the evolution of cheilostomes they state "The frequent switches in mineralogy within families, as demonstrated by the mixture of mineralogies found in many extant families, undermines this line of reasoning" (Taylor et al. 2009). An example of this may be the species *Aetea anguina* (Linnaeus, 1758) and *M. membranacea*. Although these species are currently taxonomically organised within different families and infraorders (Appeltans et al. 2012), phylogenetic analysis reveals that they are sister species sharing a common ancestor and highly conserved genetic material (Waeschenbach et al. 2012). Their mineralogy, however, is clearly different with one featuring an entirely calcitic skeleton and the other aragonitic (Figure 3.3). This may also go some way to explain the three exceptional families of *Stomatoporidae*, *Electridae* and *Horneridae* which are reported to be amongst the oldest examined families in Scotland and all appear to contain some

aragonite. The literature generally links familial patterns in mineralogy to evolutionary origins of the family, usually represented by First Appearance Date (FAD) in the palaeontological record. It is reported that the ancestral mineralogy of bryozoans is low to intermediate Mg-calcite (Smith & Key 2004; Smith et al. 2006; Taylor et al. 2009) with aragonite and wider skeletal variability only appearing after the Cretaceous radiation of bryozoans (Smith et al. 2006; Taylor et al. 2009). It is therefore expected that older families will present lower wt% $MgCO_3$ in calcite and little or no aragonite. The data obtained for Scottish cyclostomes concur with this as all examined families (mean FAD = 128Ma) feature LMC/IMC and only trace aragonite was detected in a few specimens. The data presented in this thesis provide no evidence, however, that this applies to cheilostomes. FAD driven reasoning assumes that Recent specimens exhibit the same mineralogy today which their families evolved with. Taylor et al.(2009) highlight a number of examples of species, genera and families which are known to have "switched" their mineralogy between their first appearance date and the present day, observations which undermine the FAD based line of reasoning (Taylor et al. 2009). Other authors present the theory that familial patterns relate less to the evolutionary age of the family alone, but more to the chemistry of the ocean at the time of evolution (Taylor 2007) The data presented in this study concur with this as all families exhibiting LMC appeared during the times of calcitic seas when Mg/Ca ratios were lowest. Additionally families which first appeared during times of aragonitic seas, when Mg/Ca ratios were higher, almost exclusively feature intermediate to HMC. An ongoing debate surrounds the prediction from literature that aragonite biomineralization would have been favoured during times of aragonitic seas only. The data presented do not support this as the polymorph appears during times of both calcitic and aragonitic seas, and instead seems to align closer to the alternative theory of independent parallel evolution of aragonite within clades, which was proposed by Taylor et al. (2009) and is supported by the observation of aragonite across all the calcifying bryozoan phylogenetic clades identified by Waeschenbach et al. (2012) (Figure 3.3).

As has been highlighted by recent phylogenetic studies (Waeschenbach et al. 2009; Waeschenbach et al. 2012) , there is a high degree of taxonomic uncertainty surrounding many bryozoan families which means that great care should be taken when applying generalizations at the family level. It should also be considered that many families are represented by low numbers of specimens in this dataset and, due to limited metadata in the

case of some museum specimens; it has not been possible to consider variability which may be caused by collection location, depth, season and developmental stage of individuals – all factors which are known to influence mineralogy.

3.6.3 Regional comparisons

The dominance of IMC in bryozoan skeletons in temperate waters has been reported in previous publications investigating global mineralogical patterns in Bryozoa (Smith et al. 2006) and in region studies of New Zealand (Smith et al. 1998) and Chilean bryozoans (Smith & Clark 2010). All the Bryozoa from temperate regions reviewed here feature similar mineralogies with just a few notable exceptions. All are dominantly calcitic, while New Zealand and Scotland feature more mixed mineralogies than Chile. All regions feature some pure aragonitic species; in all regions calcite is predominantly IMC. All regions feature LMC to some extent, but only New Zealand and Scotland feature HMC. In general Scotland has more variety in wt% $MgCO_3$ in calcite than New Zealand and Chile, although, this may be accounted for by the greater number of species presented in this study (Scotland n=154, New Zealand n=48, Chile n=23) and the greater proportion of the bryozoan species analysed (Scotland = 79%, New Zealand = 5%, Chile = 9%). Scotland has a higher mean wt% $MgCO_3$ in calcite (5.9) than Chile (5.35) and New Zealand (5.1). The wide range of mineralogical variety and the solid presence of aragonite, LMC and HMC in the Scottish bryozoan fauna may also be a combined reflection of the inclusion of Polar/Boreal and Lusitanian species in the Scottish fauna, due to Scotland's open geographical situation, and the diverse range of habitats and seasonal conditions which it offers.

Chile features less bimineralic species than the other temperate regions and no HMC. Chile also covers the greatest latitudinal range of over 40 degrees (17-57°S) and sea temperature is highly variable from its sub-Antarctic Southern regions to its near tropical North. On a single arbitrary day, Chile has been observed to feature an 18°C difference in sea surface temperature between the North and the South (data for 7th February 2013 (Meteo365.com 2013)) with the North exhibiting temperatures up to the mid-20°C's. As such it would be expected that Chile, with its wide variety of habitats and environmental conditions, would exhibit more variable mineralogies that other temperate regions, not less. The most likely explanation for this anomaly is the source for the specimens analysed in the mineralogical

study by Smith & Clark (2010). All sampling sites were located at greater than 40°S with 15/19 of these in waters 48°S or more. This southern region of Chile is highly influenced by the cold Humboldt Current, originating in Antarctica, and the trends reported appear to reflect this. If a wider sampling were to be taken, covering the full latitudinal range of Chile, it may be hypothesized that the reported mineralogical variability would be far greater.

Polar species feature lower mean wt% $MgCO_3$ in calcite and less aragonite than Scottish species. Aragonite occurred in just 4% of analysed species. Both Arctic and Antarctic bryozoans species feature slow growth rates (Barnes & Dick 2000; Barnes & Arnold 2001; Barnes et al. 2007) caused by low nutrient levels, low temperature and increased seasonality. Links between mineralogy and growth rate have been shown by Burton and Walter (1985) with slower growing animals generally depositing less wt% $MgCO_3$ in calcite. In addition the low temperatures and intrinsically low saturation of carbonate ions in Polar waters means that deposition of calcite is favoured over aragonite. Antarctica was effectively cut off from the rest of the world 25 million years ago by the formation of the Circum-Antarctic current (Comiso 2010). Despite the slow growth rates, long generation times and subsequent long time required to entrain genetic change (Peck 2011), this long isolation has allowed Antarctica to develop species with a high degree of endemism and specialisation. It seems likely that this specialisation extends to mineralogical adaptation to this challenging environment and may explain the low mean of 1.6 wt% $MgCO_3$ in calcite seen in Antarctic species. In comparison the Arctic features relatively few endemic species but consists predominantly of species with Pacific or Atlantic origin which entered Arctic waters following the last glacial maximum approximately 25 thousand years ago (Dunton 1992). Arctic species exhibit a mean of 4.025 wt% $MgCO_3$ in calcite, showing some adaptation to the Polar environment (Kuklinski & Taylor 2008), although this mean is closer to that exhibited in Scotland than the mean for Antarctica, and possibly reflects the relatively young bryozoan fauna in the Arctic.

3.6.4 Latitudinal and environmental patterns

This study uses a database of 53,423 distribution records of Scottish bryozoans. When used in combination with the mineralogical data for 156 Scottish species, as already documented in this Chapter, this enables modelling of bryozoan mineralogical distribution from the Mediterranean to the Arctic. The data presented in this study show that, at higher latitudes, there are statistically less HMC species, more LMC species, more 100% calcite species and a decreasing mean wt% aragonite in bimineralic species when compared to lower latitudes. A subset of 39 species, for which phylogenetic information was available, was used to populate MCMCglmm models investigating the comparative and combined influence of latitude and phylogeny on mineralogical characteristics. This analysis reinforces the patterns exhibited the distribution study. The variability of wt% $MgCO_3$ in calcite between species was found to be best explained by the full model including both factors; latitude and phylogeny. Latitude showed the strongest relationship to wt% $MgCO_3$ in calcite, accounting for over 77% of the variability seen. The results for wt% calcite were even more decisive with the best-fit model being that which included the variable of latitude alone.

Mean mineralogy has been applied to all specimen records of a species to create a dataset which can be used for the purposes of looking at the mineralogical patterns associated with latitude. The data presented in this Chapter do not, however, take into account any inter-species variation caused by factors such as depth, temperature, season, astogeny or breeding cycle. Please see Chapters 4, 5 and 7 in this thesis for detailed analysis into the effects of these factors on mineralogy. As can be seen in Figures 3.8 & 3.9, data points are not distributed evenly over the latitudinal gradient but are most numerous around the UK and Ireland. There are two possible explanations for this; the first is that that this is attributable to sampling bias. The second possibility, and the one which the data from Section 3.6.2 reflects, is that, as we are looking at the distribution of Scottish dwelling species only, this is the accurate and natural distribution of these species.

The observation that wt% $MgCO_3$ in calcite and wt% aragonite varies with environmental conditions has been reported in scientific publications for nearly a century (Clarke & Wheeler 1922). Studies specifically relating to the mineralogy of bryozoans with relation to latitude are also numerous (e.g. Lowenstam 1954; Chave 1954; Carver & Rucker 1969;

Lombardi et al. 2008; Kuklinski & Taylor 2008; Taylor et al. 2009) and within these there have been a multitude of proposed explanations for this pattern with many of these theories standing the test of time and still inciting debate today.

Sea water temperature follows a general pattern of decreasing with increasing latitude and it is probable that temperature, and physiological processes linked to temperature, are amongst the strongest sources of the observed latitudinal patterns in mineralogy. The link between temperature and wt% $MgCO_3$ in calcite in invertebrates was first observed tentatively by Clarke and Wheeler (1922), since then it has gained in popularity with Lowenstam (1954) and Chave (1954) both observing the same relationship in the Bryozoa. More recent studies into the mineralogy of Arctic bryozoans by Kuklinski and Taylor (2008, 2009), reviewing global bryozoan mineralogy (Taylor et al 2009) and a more directed study on the occurrence of aragonite in *Pentapora* (Lombardi 2008) all reinforce this pattern. The most commonly cited explanation for this pattern is that in warmer waters there is more energy available for animals to deposit aragonite and $MgCO_3$, while these mineralogical forms may be too thermodynamically demanding in cooler waters (Kuklinski & Taylor 2009). Not all mineralogical studies concur with this explanation however with Smith et al. (1998) finding no such pattern in New Zealand bryozoans. Alternative explanations link the increased wt% $MgCO_3$ in calcite and wt% aragonite less directly to the increased growth rates observed at higher temperatures (Cohen & Branch 1992). Growth rates in bryozoans have been observed to be faster at lower latitudes, decreasing as we move up the latitudinal gradient (Barnes et al. 2007; Smith 2007; Smith & Lawton 2010). This occurs for a variety of reasons but is usually attributable to the higher nutrient levels (Bone & James 1993), longer growing seasons and increased temperature/energy (Bone & James 1993) at lower latitudes (Smith & Lawton 2010). Faster growth rates can result in increased wt% $MgCO_3$ in calcite (Burton & Walter 1985) and increased wt% aragonite, due to factors such as a higher rate of secondary calcification and astogeny (Smith & Girvan 2010).

Salinity follows a general pattern of decreasing in higher latitudes (NASA 2012). Clarke and Wheeler (1922) first speculated that salinity may also be influencing wt% $MgCO_3$ in calcite with the view that in more saline, "concentrated" waters, the increased Mg^{2+} ions were more likely to be incorporated into calcite. This sea water chemistry theory continues

today, if in a rather more sophisticated manner, with more recent publications linking the concentration of inorganic ions (e.g. Watabe 1974; Dodd & Stanton 1981; Nudelman et al. 2006), Mg/Ca ratios in seawater (e.g. Ries 2005; Fabre & Lathuiliere 2007; Taylor et al. 2009; Martínez-Botí et al. 2011; Bohaty et al. 2012), salinity (e.g. Eisma 1966; Nooijer et al. 2009; Bohaty et al. 2012), sulphate (Burton & Walter 1985), aragonite compensation depth (ACD) and carbonate chemistry (Berger 1978) to both wt% aragonite and wt% $MgCO_3$ in calcite. Experiments have also shown high levels of Mg^{2+} in seawater to inhibit the deposition of calcite while not affecting the formation of aragonite (Berner 1975) and it is this mechanism which is thought to be the key factor determining the mineralogical state of our seas (Taylor 2007).

3.6.6 Patterns related to distributional range

For the 41 species where multiple analyses have been conducted, it has been possible to compile a database of distributional data and thus identify the latitudinal range the species occupy. To the author's knowledge distributional range has not been previously assessed against mineralogical diversity.

To minimize identification errors, only records from scientifically reliable sources have been included in this study. There are, however, a number of species that are notoriously difficult to identify, even for highly experienced taxonomists. In the phylum Bryozoa there is also the added complication of taxonomic uncertainty. The Order Cyclostomata, for example, is widely regarded as in need of taxonomic review (Waeschenbach et al. 2009), preferably based on phylogenetic data. It is likely that the group consists of many more species than are currently recognized and this may go some way to explain the incredibly wide reported distributional range for Scottish cyclostomatous species (mean species distributional range = 75.6 latitudinal degrees). It may also be a factor in the lack of adherence of this group to patterns exhibited by the more taxonomically stable cheilostomes. It is also only with the increased use of phylogenetic techniques that it is being found that many species, which have previously been considered taxonomically stable, actually consist of cryptic species. One such example is *E. pilosa*, historically thought to have a near Global distribution, which has been split into separate cryptic species (e.g. *E. scuticifera*) in recent years (Nikulina 2008) and, therefore, brings into doubt the recorded distributional range for the species of 114 latitudinal degrees. This particular

known example has been excluded from the analysis for this reason but it should be considered that there may be further, as yet undetected, cryptic species yet to be identified.

A sub-group of cheilostomes that feature both a wide geographical range and a corresponding wide range in mineralogy are those that have become successful as non-native (alien) species, either in the UK or abroad. With the exception of the genus *Schizoporella*, the majority of these non-native species (e.g. *Cryptosula pallasiana* (Moll, 1803), *B. neritina, M. membranacea*) feature morphologically simple skeletons with limited or no avicularia, spines and secondary calcification and a preference for internal brooding of embryos. It has been reported that this type of morphologically simple species have skeletal chemistry which is strongly influenced by seawater chemistry and have relatively weak control over their own calcification (Stanley & Hardie 1998; Schafer & Bader 2008). As they inhabit a wide latitudinal range, and the wide variety in seawater this encompasses, this may therefore offer an explanation for the corresponding wide range of wt% $MgCO_3$ in calcite incorporation exhibited. A more likely explanation for the variability seen in non-native species, however, is mis-identification and cryptic species. An example is *S. unicornis* which is non-native in a number of countries and has been widely reported in bryozoan mineralogical publications for its mineralogical plasticity (Clarke & Wheeler 1922; Lowenstam 1954; Schopf & Manheim 1967; Carver & Rucker 1969; Poluzzi & Sartori 1973; Smith et al. 2006) with authors using and recommending it as an ideal species for environmental correlations with mineralogy (Lowenstam 1954) and potential palaeoenvironmental interpretation (Smith et al. 2006). Both recent publications and work for this thesis (Chapter 6), however, has highlighted that many records and museum specimens previously identified as *S. unicornis* are actually misidentified *S. japonica, S. errata* (Waters, 1878), *S. dunkeri* (Reuss, 1848) or other *Schizoporella* species (Hayward & Ryland 1995; Tompsett et al. 2009; Clarke Murray et al. 2011). Tompsett et al's (2009) re-description of the species observes that out of 25 examined papers where the species is named, only four (16%) present correct identifications (Tompsett et al. 2009). Similar studies have been recently conducted on invasive species of *Watersipora* (Ryland 2009; Mackie et al. 2012) and *Bugula* (Ryland et al. 2011) which also identified taxonomic mis-identifications and cryptic speciation. With this taxonomic doubt cast on past distribution and mineralogical records of many non-native species, caution should be exercised in interpreting patterns relating mineralogy to distributional range.

3.6.7 Gap analysis and recommendations

Overall the species coverage of this study has been very thorough, leaving only the rare and challenging species still to analyse. Many of the species in this study, however, have only had one specimen analysed. It is only by increasing the available data to cover multiple specimens that many patterns may emerge and it is recommended that further mineralogical investigations on UK bryozoans continue in the future. There also remain a number of areas where knowledge is still limited or incomplete. The Phylum Bryozoa is still subject to a high degree of taxonomic uncertainty in some orders, families and genera. The integration of the phylogenetic information which is currently available has improved the accuracy of some of the taxonomy of bryozoans and allowed for the investigation into phylogenetic signals in mineralogy. At the time of this study, however, phylogenetic information was only available for 39 Scottish species, and a full phylogenetic review of the Bryozoa is needed to verify the reliability of observed taxonomic and evolutionary patterns. It is recommended that as more phylogenetic information becomes available taxonomic trends are revisited and the interactions between phylogeny, latitude and mineralogical traits are reinvestigated using MCMC (Markov chain Monte Carlo) Multi-response Generalized Linear Mixed Modelling (Hadfield 2009; Hadfield & Nakagawa 2010).

This study also highlights that there are many factors at work, which may play a role in mineralogy, and these all need further investigation. These include biological factors such as breeding cycles, astogeny and physiological “fitness”; environmental factors such as temperature, salinity, nutrient level, carbonate chemistry and habitat depth, and mechanical properties associated with the localisation of minerals. All of these factors will be investigated in more depth in subsequent Chapters of this thesis.

3.7 Summary and Conclusions

Analysis of the mineralogical composition of Scottish Bryozoa shows that the group reflects both reported patterns in evolutionary/genetically "pre-programmed" mineralogy superimposed by a high level of variation driven by a combination of environment and biological/physiological factors. In such a variable region as Scotland, open to biological and environmental influx from all directions, it is perhaps no surprise that bryozoans exhibit this in the wide range of mineralogy they present.

- Cyclostomes feature lower mean Mg-calcite and less aragonite than cheilostomes. This concurs with previous studies and can be explained by the greater evolutional age of the order combined with the phylogenetic evidence of less genetic distance from the shared calcitic ancestor than cheilostomes.
- Groups of species with wider latitudinal distribution have a greater range of Wt. % Mg-calcite. This suggests a high degree of environmental control in the case of morphologically simple species, and sophisticated physiological control in complex ones. Observations relating to non-native species raise the question whether it is wide distribution which is driving species to become more variable, or if it is physiological and morphological plasticity of a species which is enabling it to expand its range.
- Wt% $MgCO_3$ in calcite and wt% aragonite are reduced at higher latitudes and in Polar regions. This can be attributed to biological factors driven by changing environmental conditions, such as temperature, along the latitudinal gradient (e.g. growth rate and physiological fitness).
- Temperate regions feature similar mineralogies with differences primarily identified as artefacts caused by sampling bias and varying species coverage between studies.

Despite the data presented in this Chapter, more study is needed for a better understanding of the influence of genetic/ evolutionary, environmental and biological factors in bryozoan mineralogy.

Chapter 4: Investigating Mineralogical Effects of Seasonal and Spatial Differences in Environmental Conditions on British Bryozoan Skeletons

4.1 Abstract

Bryozoans exhibit a highly variable geochemistry within their calcium carbonate skeletons (Smith et al. 2006). Previous studies have predominantly attributed this variability to differences in seawater temperature influencing the relative extent of incorporation of magnesium into the calcite lattice. Prior to this study, however, the effects of depth, site and seasonality on $MgCO_3$ deposition in calcite had not been examined in detail.

The aim of the study was to conduct a multi-site, multi-year study to investigate the effects of factors related to site, depth and seasonality on the skeletal mineralogy of temperate bryozoan species. The study was conducted using arrays of settlement panels located at 6m and 12m at sites in the East, West and North of Scotland between 2010 and 2013. Each array of 15 settlement panels was co-located with temperature and conductivity loggers. Arrays and loggers were serviced by SCUBA diving approximately bimonthly. At each service a triplicate of panels were replaced and water samples were collected for additional environmental analysis. The bryozoan communities on panels were recorded and samples of common species were extracted for XRD analysis. Community, environmental and mineralogical data were interrogated to investigate patterns related to depth, site and season.

No statistically significant differences in environmental measurements, community structure or $MgCO_3$ in skeletal calcite were attributable to differences in depth. Significant differences were detected between sites and with seasonal changes in seawater temperature. Data suggest that seawater temperature at the time of deposition is the primary causative factor in determining $MgCO_3$ content in bryozoan calcite for the study species. This concurs with previous studies which have shown increasing $MgCO_3$ deposition in calcite at warmer temperatures. Although the underlying mechanisms of wt% $MgCO_3$ in calcite variability as yet remain unclear, this study emphasizes the importance of multi-year studies, sample replication and accurate measurement of environmental conditions as well as consideration of site and species specific biological factors.

4.2 Introduction

4.2.1 Scotland's coastline

The waters around Scotland are characterized by complex bathymetry and oceanography (Figure 4.1; Figure 4.2 & Figure 4.3) but can be considered to consist of two major bodies: the Atlantic margin (Atlantic Frontier Environmental Network 2001) and the North Sea. The Atlantic Margin extends along the West of Scotland and East to Orkney and Shetland and is predominantly influenced by the Continental Slope Current and the warm North Atlantic Current, originating in the Western Atlantic from the "Gulf Stream"(Atlantic Frontier Environmental Network 2001). Lesser influences include colder Arctic water from Iceland and Norway (Hiscock 1998). In contrast, the North Sea is comparatively enclosed, open to less exchange with other water bodies and is more subject to influence from weather conditions such as wind direction, air temperature and fresh-water run-off (Hiscock 1998).

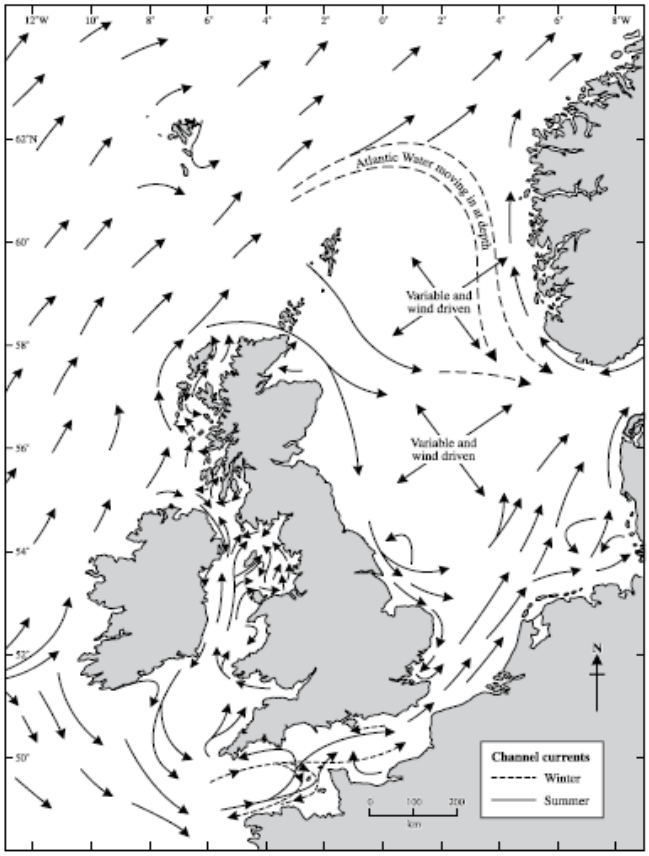


Figure 4.1: The direction of near-surface residual currents around the British Isles. Reproduced from Hiscock (1998)

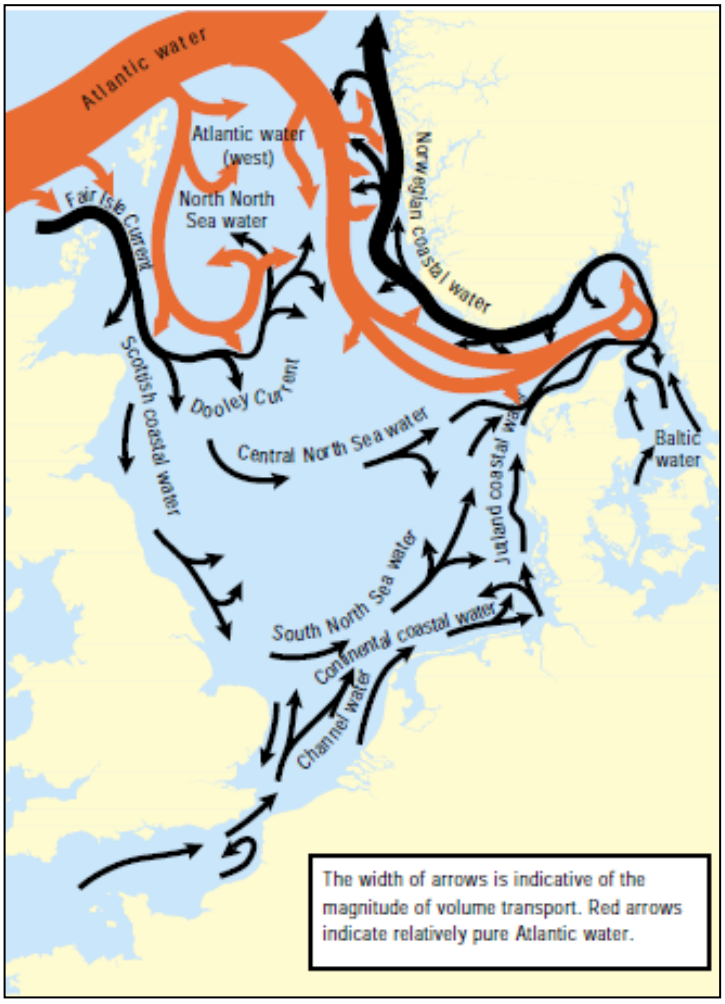


Figure 4.2: General circulation in the North Sea. Reproduced from Hiscock(1998)

The Atlantic Margin is strongly influenced by the North Atlantic Oscillation (NAO) and the closely related Arctic Oscillation (AO), especially during the winter months when atmospheric patterns are at their strongest. The NAO is the difference in atmospheric pressure between the northern and southern parts of the Atlantic and can result in inter-annual climate anomalies including temperature and salinity fluctuations, changes in water mass distribution and variation in wind strength and wave height (Hurrell 1995). In addition to affecting the temperature, salinity and variability of the water bodies, the currents also act as transport systems of fauna from different regions. The UK is situated between Arctic, Boreal and Lusitanian (Southern) biogeographical zones (Hiscock 1998). The West coast of Scotland is considered Lusitanian-Boreal, and the East coast and North of Scotland (including Orkney) is considered Boreal (Hiscock 1998; Atlantic Frontier Environmental Network 2001) (Figure 4.4).

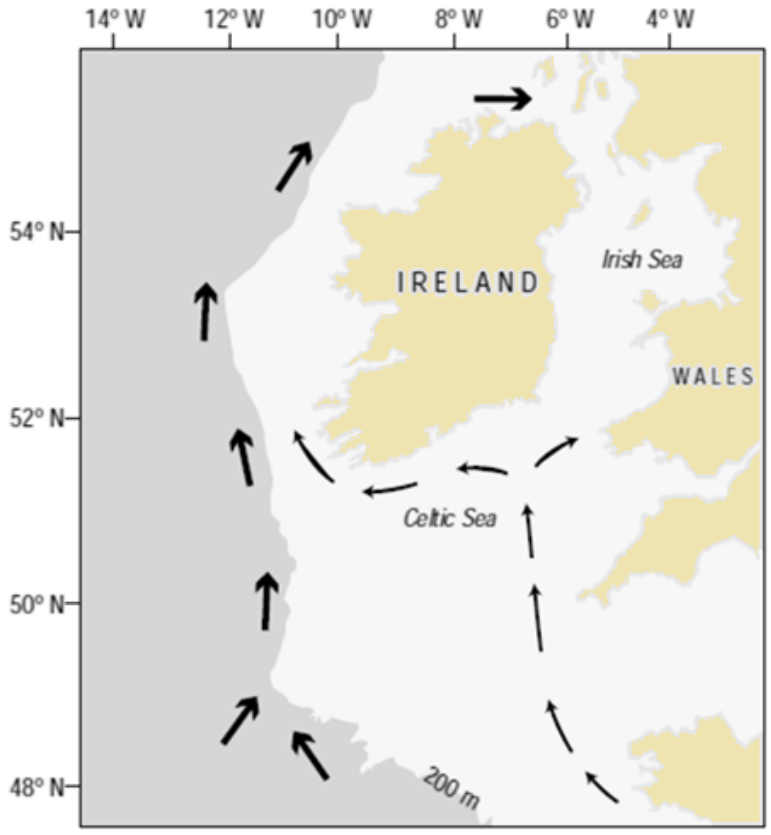


Figure 4.3: Schematic diagram of general circulation in the Irish Sea Shelf. Reproduced from Hiscock (1998)

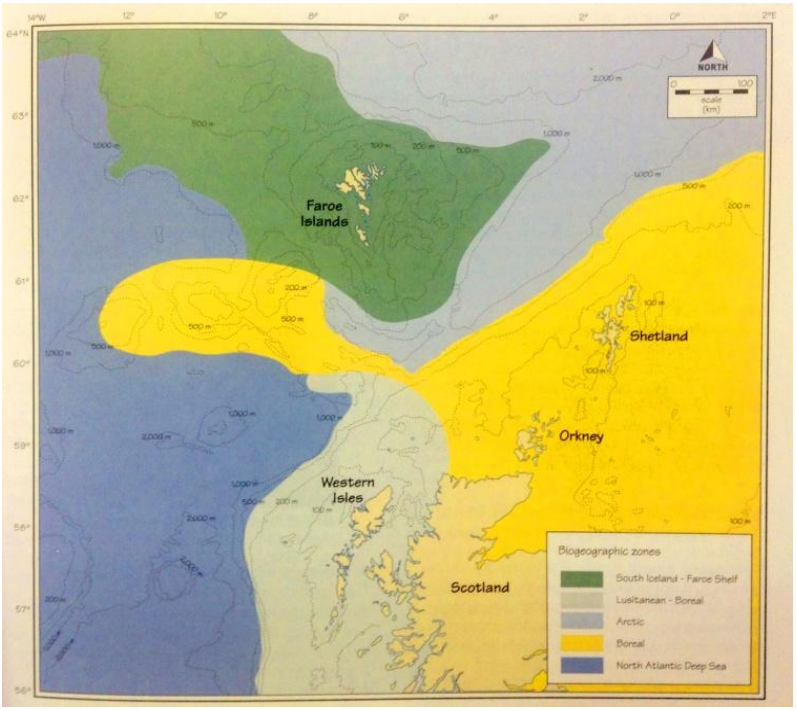


Figure 4.4: Schematic diagram of biogeographical zones around Scotland. Reproduced from Atlantic Frontier Environmental Network (2001)

4.2.2 Settlement panel studies

The first records of the use of settlement panels for analysis of faunal settlement come from the United States, in commissioned reports investigating the fouling of ships. The first of these studies was conducted by Gardner (1922) on immersed tiles in Beaufort, North Carolina. The tiles were simple steel panels, painted with different coloured paints and submerged 6 ft below the surface for a summer to investigate patterns of fouling. This study was considered of value from both an "economic and purely scientific viewpoint" (Visscher 1927) and subsequently followed by a flurry of annual experiments in Beaufort and Woods Hole (USA) by Gardner (1923,1934,1935) and Perry & Bray (1923) to investigate the effects of surface glaze, colour, light and anti-fouling paints on settlement. Seasonal differences in bryozoan settlement were first observed from Woods Hole by Visscher (1927) and subsequent settlement studies on bryozoans continued in the USA using panels through to the latter half of the 20th century (e.g Pomerat & Reiner 1942; McDougall 1943; Pomerat & Weiss 1946; Maturo 1959). Subsequent settlement panel studies from the late part of the 20th century through to the present day have helped to establish recruitment and succession patterns in many parts of the world, e.g. Australia (Holmes et al. 1997), Japan (Nandakumar et al. 1993), Hong Kong (Lee & Trott 1973), Antarctica (Bowden et al. 2006) and the Arctic (Barnes & Kuklinski 2005).

The first settlement panel experiment conducted in the United Kingdom involved panels submerged vertically approximately 1m below the surface at harbours in Northern England and Scotland (Meadows 1969). A longer term study with a similar set-up was used in the Menai Straits, Wales by Fry (1975), although only four species of bryozoan were recorded from this study. Settlement panels suspended from piers or floats have been criticised for their isolation from predators and benthic sources of larvae (McKinney & Jackson 1989), with benthic arrays preferred for community studies. It was not until the early 1980s that the first benthic settlement panels were deployed in the UK. In 1981, in Plymouth Sound, Rubin (1985) deployed plastic benthic settlement panels horizontally attached to an old metal bedframe in 8m of water, these were visited and serviced regularly using SCUBA in order to investigate competition between serpulids and bryozoans. Between 1981 – 1984 settlement panels were positioned both intertidally and subtidally in Argyll, Scotland (Todd & Turner 1986; 1988) with the subtidal panels positioned at 1.5m depth and serviced by wading. Turner and Todd (1993) deployed settlement panels intertidally in St Andrews

Bay. Between 1997 and 2002, a multi-site, multi-depth experiment was conducted in Loch Hyne, Ireland using intertidal and benthic 6m and 12m settlement panels (Maughan 2001; Watson & Barnes 2004); this successful experimental design was taken as a base for the design of the experiment contained in this Chapter.

Settlement panels offer advantages over natural substrata as they are constant in surface area, colour, size and shape, their location and orientation can be controlled and they are easy to handle and observe in a non-destructive manner (Richmond & Seed 1991). The consistency of size, shape and position of settlement panels can also be seen as a disadvantage however, with some authors observing that settlement panel communities may not reflect those on nearby natural substrata (McKinney & Jackson 1989). This mis-match with local conditions is most likely to occur if settlement panel arrays are not positioned carefully near a larval source; resulting in turning them into "ecological islands" (Richmond & Seed 1991).

4.3 Aims and Objectives

4.3.1 Summary of purpose of study

Examine bryozoan colonies deposited on settlement panels during multi-year experiments at three sites and two depths. Analyse the ecology and skeletal mineralogy of the bryozoan communities to elucidate any effects of factors related to site, depth and seasonality.

4.3.2 Objectives of study

1. Examine how bryozoan communities vary with site, depth and season.
2. Investigate the effect of factors related to site and depth on the skeletal mineralogy of four bryozoan species.
3. Investigate the effects of environmental conditions and seasonality on the skeletal mineralogy of five bryozoan species.

4.4 Materials and Methods

4.4.1 Sample collection

This study is based on the mineralogical examination of 184 specimens of six bryozoan species collected from settlement panels colonised at 6 and 12m in three sites in Scotland (Figure 4.5) between December 2010 and February 2013.

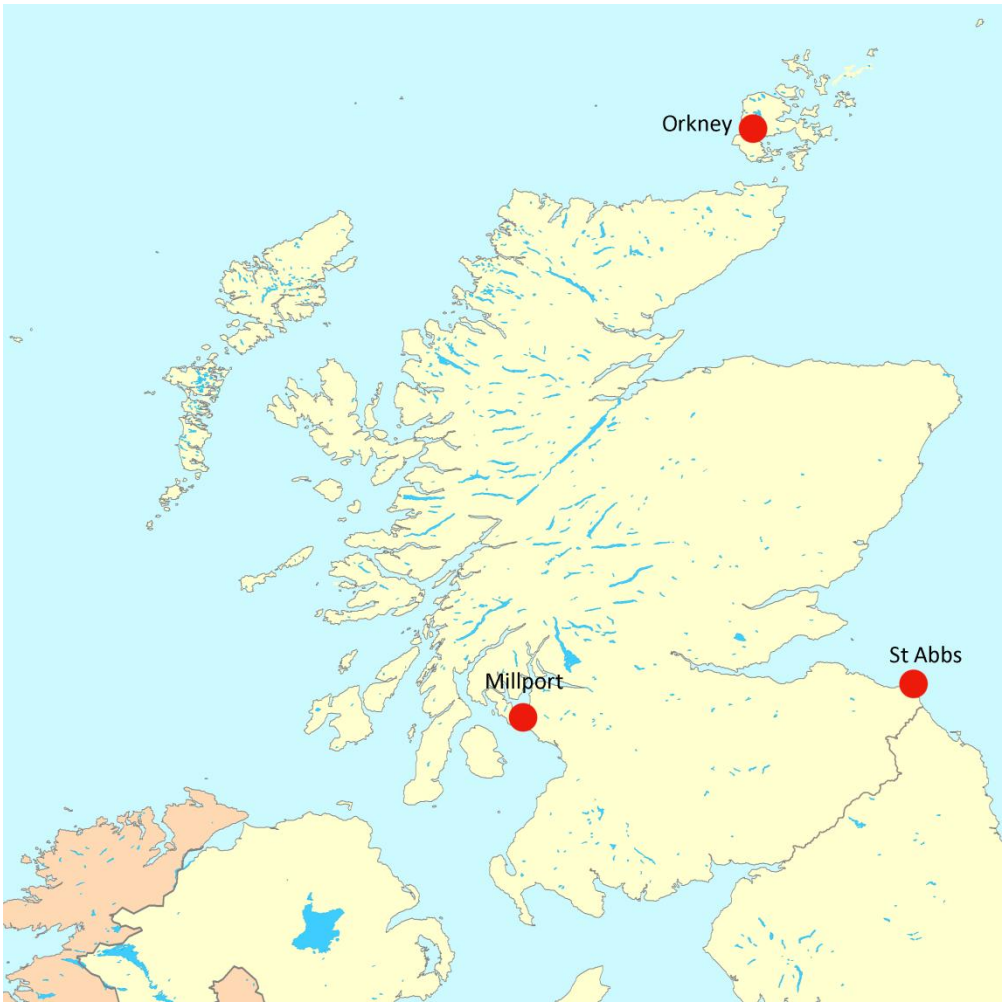


Figure 4.5: Map of Scotland showing panel sites.(indicated with red circles).Modified from a map by Gaba (2010)

SITE DESCRIPTIONS:

At each site two panel arrays were deployed during winter 2010/2011 at approximately 6m and 12m depth. Arrays were situated within swimming distance (<100m) of each other on rocky/ boulder substrate. Each panel array consisted of a steel frame supporting 5 pairs of polyurethane struts, each holding three opaque black acrylic 15cm x 15cm panels (Fig. 4.6). Panels were thus suspended 7cm above the seabed allowing bryozoan settlement on the

underside of the panel array. The steel frame was weighted to the seabed by positioning boulders on the steel "wings" (Figure 4.6). Arrays were visited regularly by SCUBA diving when selected panel sets (3 x panels) were removed and replaced with fresh struts and panels for colonisation. During the experiment steel wing-nuts attaching the struts to the steel frame were replaced with cable ties to enable ease of access underwater. A temperature logger was situated on each steel frame and a conductivity logger in close vicinity; see later Section for further details.

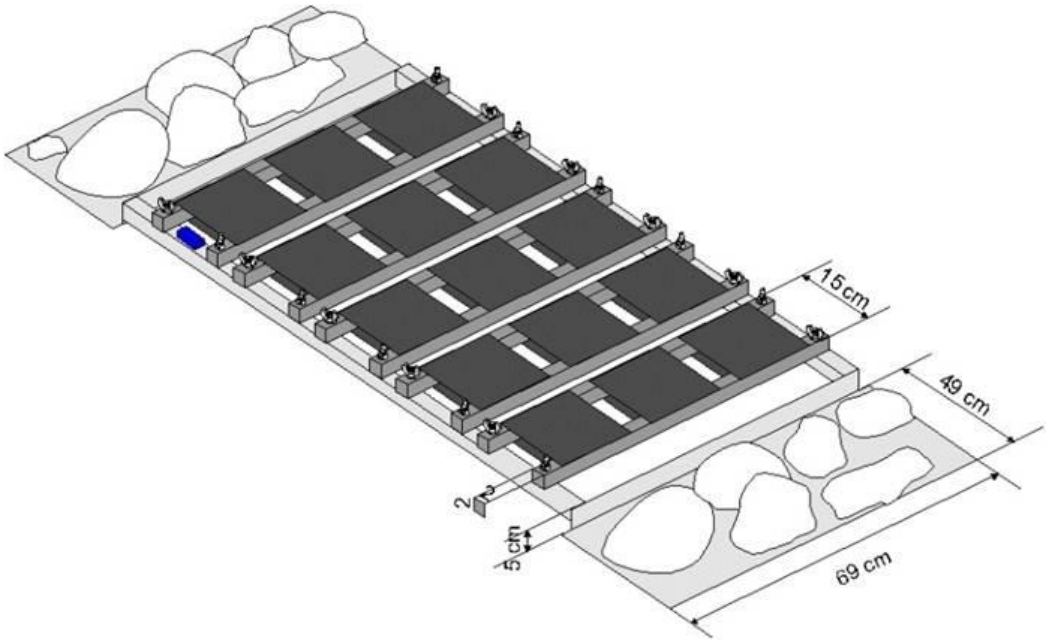


Figure 4.6: Design of settlement panel array. Light grey indicates components constructed of marine grade steel. Dark grey squares indicate individual polyurethane settlement panels. Blue box indicates position of temperature logger.

ORKNEY

Orkney is an archipelago of islands situated to the North of mainland Scotland (Figure 4.5). Scapa Flow is situated in the centre of the Orkney islands and is a semi-enclosed marine basin covering an area of approximately 130km^2 (Joyce 2004) where water depths predominantly range between 10-70m. Tides and currents circulate through Hoy Sound, in the northwest and Hoxa Sound in the South which open to the Atlantic Ocean and Pentland Firth respectively (UK Hydrographic Office 1986). The settlement panels were deployed in Swanbister Bay, a small shallow bay in the North of Scapa Flow. The bay was selected for its ease of accessibility, suitable substrate and depth and relative isolation from the main recreational dive sites of Scapa Flow. Swanbister Bay reaches a maximum depth of 20m approximately 800m from shore (Seazone Solutions Ltd. 2011). Anthropogenic use of the

bay is minimal with fishing activity limited to sporadic creeling and only occasional recreational diving. Situated to the West of the bay is a salmon fish farm although the main bay is protected from influx of fish farm effluent by Toy Ness (Figure 4.7). The panels were deployed by boat and the 12m settlement array (58º 55' 27.3N, 003º 06' 54.8W) was positioned on the edge of the rocky zone with silt and sand dominating the substrate at depths greater than 14m. The 6m settlement array (58º 55' 30.0N, 003º 06' 57.9W) was positioned 92m from the 12m panel at the beginning of the infralittoral zone in an area of substantial algal cover during the spring and summer months. The flora of the bay is highly dynamic and in summer/autumn the bay acts as a trap for algal debris with depths of 30cm + noted during some panel visits.

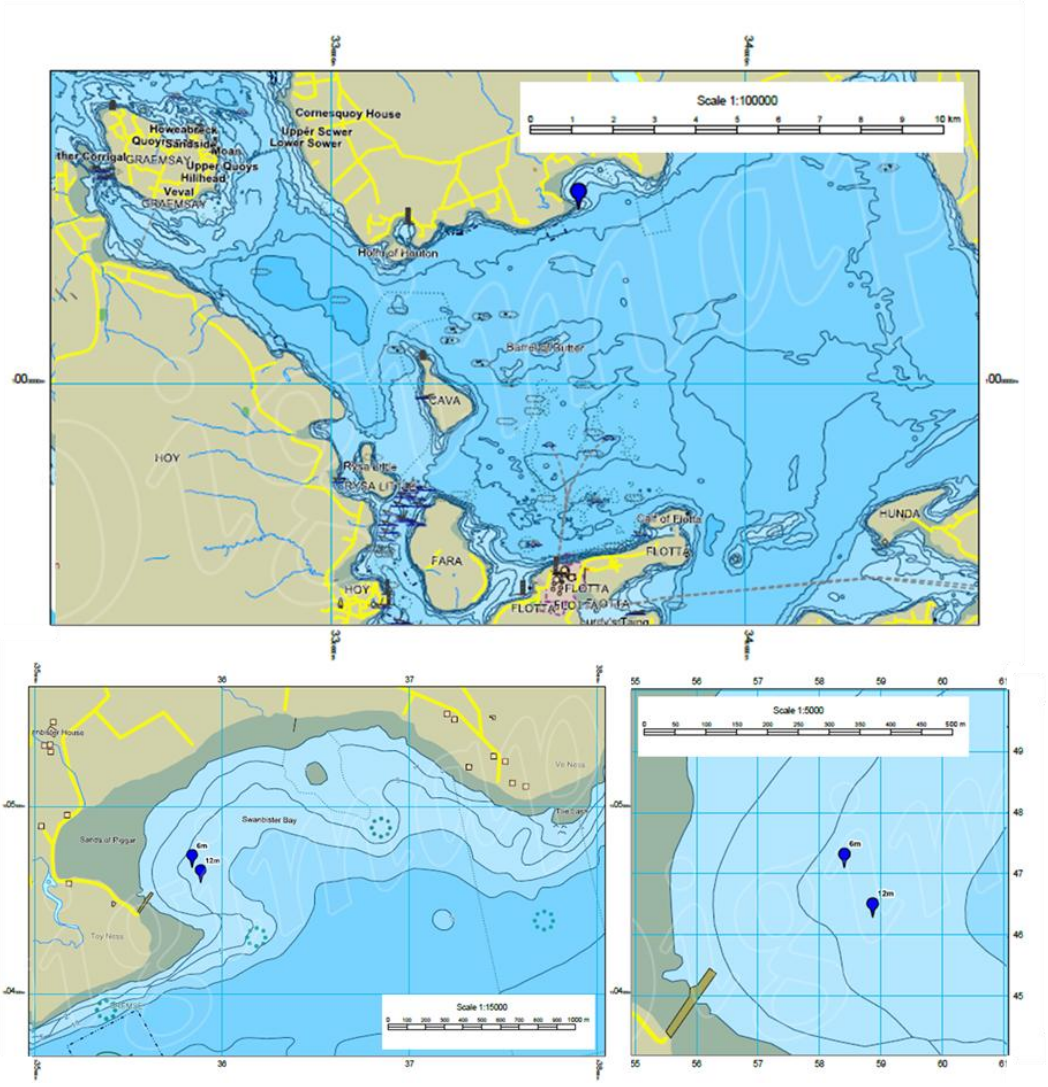


Figure 4.7: Maps of the Orkney settlement panel sites. Top: Scapa Flow. Blue marker indicates panel location. Bottom-left: Swanbister bay with markers showing location of panels; Bottom-right: close up of panel site.

During early visits it was occasionally impossible to relocate the panel arrays due to the disorientating algal debris and poor visibility. This resulted in some gaps between panel changes of up to 8 months. The issue was eventually eliminated by a combination of sub-surface markers and physically linking the panels with line bolted into the bedrock.

Benthic communities in the bay are dominated by rocky bottom encrusting fauna including bryozoans, hydroids, calcifying worms (predominantly *Pomatoceros triqueter*), saddle oysters (*Anomia ephippium*), ascidians and sponges. Macrofauna include urchins (*Echinus esculentus*), starfish (*Asterias rubens*) and squat lobsters (*Galathea squamifera).* A wide range of algae is present although dominated by filamentous red algae and extensive patches of encrusting coralline algae.

St Abbs

St Abbs is situated on the East coast of Scotland near the border with England (Figure 4.5). St Abbs North Sea coastal waters fall within the St Abbs and Eyemouth voluntary marine reserve (VMR), an area of 1030 hectares of Berwickshire coast extending to 50m depth from Eyemouth in the South to Petticowick in the North (St Abbs & Eyemouth VMR 2013). The VMR was introduced in 1981 as a result of the increased popularity of the area as a SCUBA diving destination. The VMR aims to balance the needs of the many users of the region, including fishermen, and recreational users, while protecting the marine life.

In addition to the VMR the area around St Abbs Head is also a Static Gear Reserve (SGR) up to 1 mile offshore (St Abbs & Eyemouth VMR 2013). Established under the Inshore Fisheries Act 1984 (UK Parliament 1984) the use of mobile fishing gear (trawling) is prohibited within the SGR in order to protect damage to static gear, such as lobster and crab pots, and to reduce risk to marine life.

The settlement panels were deployed in Petticowick, a small shallow bay in the North of the St Abbs VMR. The bay was selected for its suitable substrate and depth and relative isolation from the main recreational dive sites of St Abbs. Petticowick is surrounded by cliffs, stacks and seacaves and reaches a maximum depth of 14m approximately 200m from shore. Petticowick is sheltered from the worst weather conditions for much of the year but during the winter months, when the wind is often North-Easterly, it can be subject to severe swell with heights of 5m not uncommon. Anthropogenic use of the bay includes lobster and

crab creeling, line fishing from shore and recreational SCUBA diving. A disused stone jetty is positioned in Petticowick although erosion to shore access means that this was inaccessible during the experimental period. The wreck of the Odense, a steamship sunk by a U-boat in 1917 (RCAHMS 2013), is found at the western end of Petticowick (Figure 4.8 Mid-left) although its very poor condition means it is less regularly visited by SCUBA divers than other wrecks in the area. No anthropogenic debris was seen in the vicinity of the

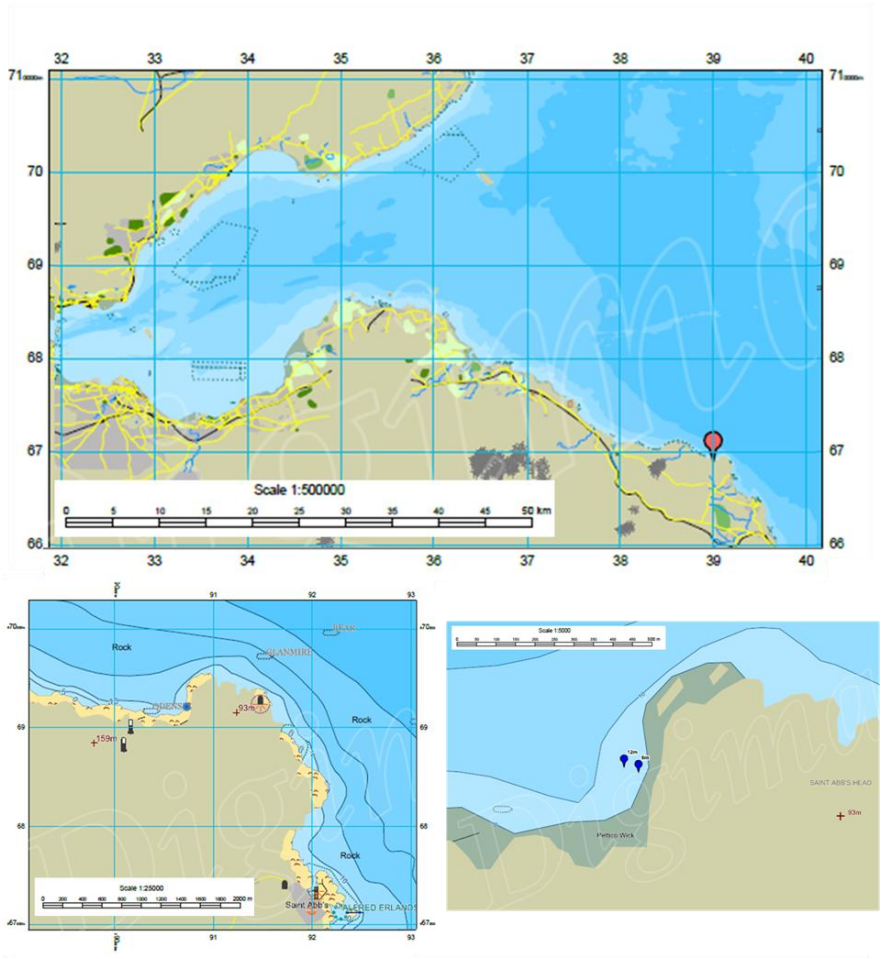


Figure 4.8: Maps of the St Abbs settlement panel sites. Top: South East Scotland between Berwickshire and Fife, marker indicates panel location. Mid-left: St Abbs and Eyemouth voluntary marine reserve, Petticowick indicated with blue marker; Mid-right: close up of panel sites in Petticowick

panel arrays during SCUBA visits to the site. The site was chosen for its suitable depth, substrate and being "off the beaten track" of the common SCUBA dive sites. In order to prevent tampering with the experiments by curious SCUBA divers, awareness posters (Figure 4.9) were displayed on all dive boats and local notice boards and the experiment was undertaken in cooperation with the St Abbs and Eyemouth VMR ranger. The panels

were deployed by boat and the 12m settlement array (55º 54’ 99.2N, 002º 09’ 00.5W) was positioned on the edge of the rocky zone with silt and sand dominating the substrate at depths greater than13m.

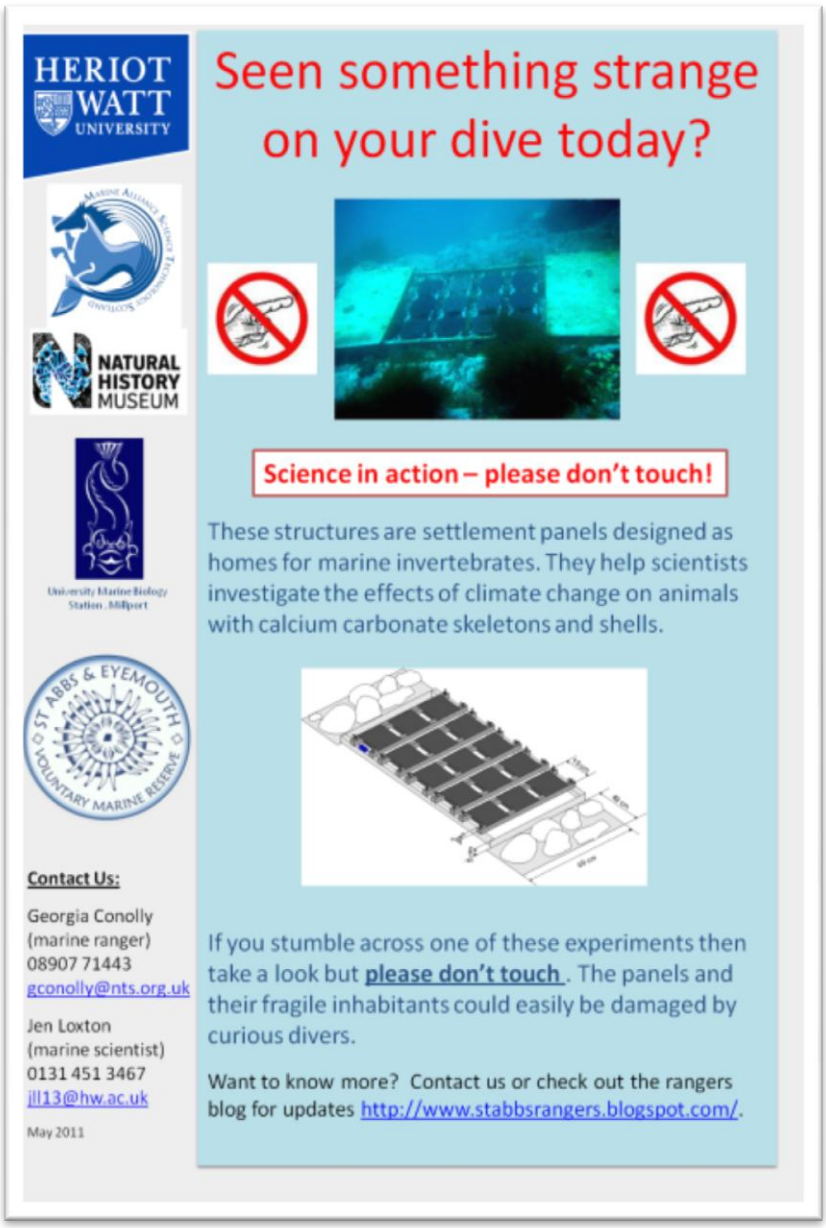


Figure 4.9: Awareness poster advising against tampering with settlement panels.

The 6m settlement array (55º 54’ 99.2N, 002º 09’ 02.6W) was positioned 42m from the 12m panel at the lower depth limit of the kelp zone. Low visibility during winter months meant it was necessary to connect the two panels using a benthic line to allow underwater navigation between the sites. During the experimental period, creeling between the 6m and 12m panels snapped the connecting lines on a number of occasions. The issue was

eventually eliminated by the use of a "breadcrumb trail" of tiles connecting the panels. Benthic communities in Petticowick are dominated by rocky bottom encrusting fauna including bryozoans, hydroids, calcifying worms (predominantly *Pomatoceros triqueter*), saddle oysters (*Anomia ephippium*) and ascidians. Macrofauna include urchins (*Echinus esculentus*), starfish (*Asterias rubens*), crabs (*Cancer pagurus)* and lobsters *(Homarus gammarus).* A wide range of macroalgae is present.

MILLPORT

The Isle of Cumbrae is located in the Clyde estuary on the West coast of Scotland between the upper and lower Firth of Clyde (Figure 4.5). The climate of the Firth of Clyde is influenced by the Atlantic Ocean and the North Atlantic Current (Hiscock 1998), resulting in a milder climate than much of Scotland. Cumbrae is sheltered from the main estuary by the Isle of Arran to the South and from the Atlantic Ocean by Kintyre to the West (Seazone Solutions Ltd. 2011). The waters East of Cumbrae are an area of intense anthropogenic activity including yachting, fishing (both trawling and creeling) and shipping (Firth of Clyde Forum 2010). The Hunterston shipping route in the centre of the channel is heavily used for commercial and naval vessels. The mainland, 2.5km across the water from Cumbrae, is home to large yachting marinas, coal and nuclear power stations and wind farms. Marine Research has been conducted in Millport since the arrival of a research vessel, The Arc, in 1885 with Millport Marine Station documenting and researching the regions marine life from 1897 to the present day (Moore & Gibson 2007). Clashfarland Point was chosen for its easy shore access, sheltered position and suitable substrate and depth. The Point has been used for previous experiments by Millport Marine Station although none were running concurrently with this study. At this location the Clyde estuary is approximately 1.8km wide and 45m deep in the centre of the channel (Figure 4.10 Bottom-left). The seabed rapidly slopes reaching a depth of 30m within 100m of the shore. Clashfarland Point is a popular SCUBA dive site on Cumbrae and arrays were clearly labelled underwater to prevent tampering by curious divers. The panels were deployed by boat and the 12m settlement array (55º 45' 56.7N, 004º 53' 33.5W) was positioned on the edge of the rocky zone with silt and mud dominating the substrate at depths greater than 13m. The 6m settlement array (55º 45' 56.7N, 004º 53' 85.3W) was positioned 40m from the 12m panel in the infralittoral zone.

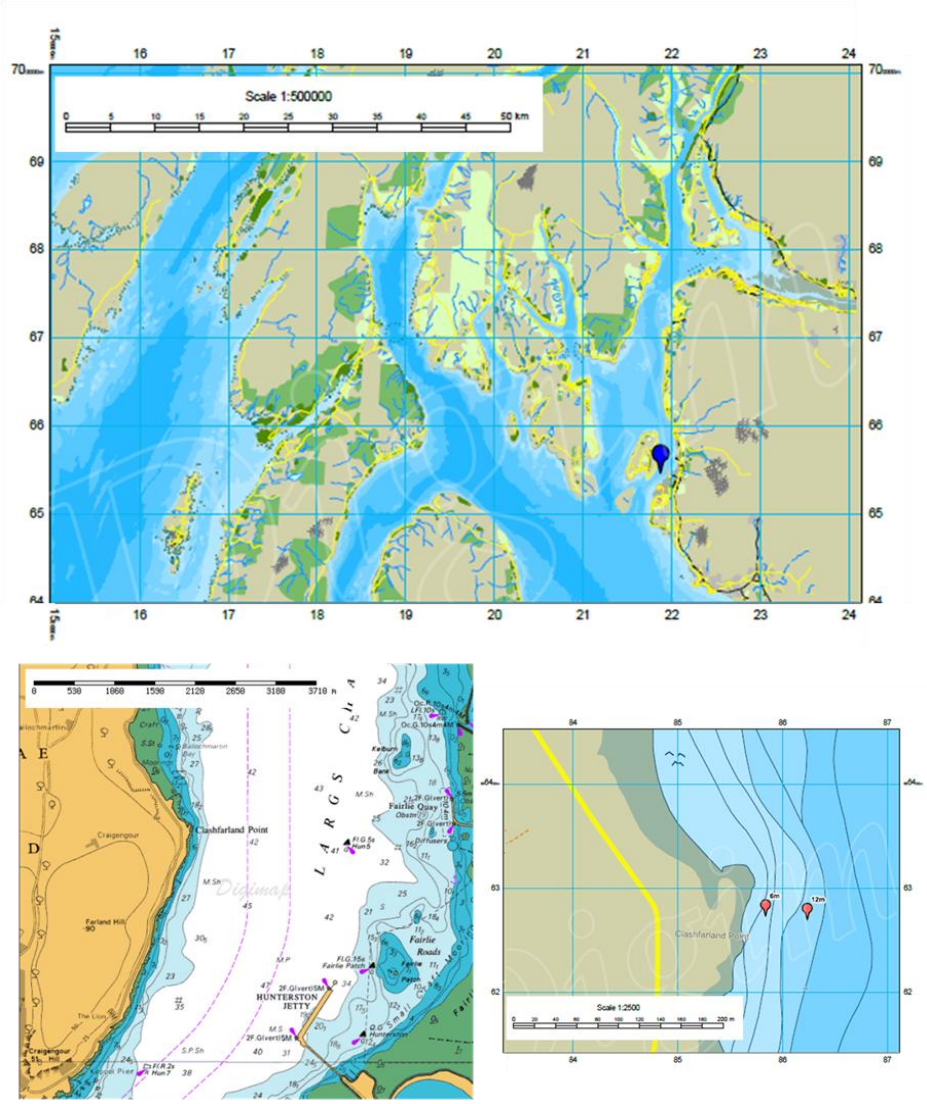


Figure 4.10: Maps of the Millport settlement panel sites. Top: West coast of Scotland showing Isle of Cumbrae in the Clyde estuary, marker indicates panel location. Bottom-left: Largs Channel with Clashfarland point marked. Bottom-right: Clashfarland Point on the East coast of Cumbrae, panel sites indicated with markers.

Benthic communities on Clashfarland Point are dominated by rocky bottom encrusting fauna including bryozoans, hydroids, calcifying worms (predominantly *Circeis spirillum* and *Pomatoceros triqueter*), barnacles (*Elminius modestus* and *Balanus balanus*), saddle oysters (*Anomia ephippium*) and ascidians. Macrofauna include soft corals (*Alcyonium digitatum*), urchins (*Echinus esculentus*), starfish (*Asterias rubens* and *Marthasterias glacialis*), crabs (*Cancer pagurus)* and lobsters *(Homarus gammarus).* A wide range of algae is present although dominated by kelps.

METHOD OF SPECIMEN COLLECTION

All settlement panel arrays were regularly visited using SCUBA diving. Millport and St Abbs were visited approximately every 1-2 months and Orkney was visited approximately every 2-3 months. Limitations in weather and diver availability necessitated that the visit schedule was flexible. SCUBA diving was conducted following HSE scientific and archaeological diving guidelines (Health and Safety Executive 1998).

During each visit panel arrays were checked for stability and re-secured where necessary, a set of panels was removed and replaced (3 x panels), environmental loggers were checked for functionality, cleaned and changed as required and water samples were collected. Following panel removal each panel was marked on the reverse side with identifying codes, rinsed in fresh water and air dried.

ENVIRONMENTAL MEASUREMENTS

Conductivity was measured *in situ* hourly using an HOBO U24-002 salt water conductivity logger and converted to Practical Salinity Units, as defined on the Practical Salinity Scale of 1978 (Intergovernmental Oceanographic Commission 2010) using the Gibbs function of seawater. Temperature was measured *in situ* hourly using a HOBO UA002-64 pendant temperature logger. Loggers were checked for functionality during every panel visit and changed every 2-4 months. The loggers were downloaded and the data processed using HOBOware Pro (Onset Computer Corporation 2011).

A 500ml water sample (A) was collected within 30cm of the panel array in a sterile plastic bottle during each panel visit between May 2012 and Feb 2013. Following collection the water sample was filtered through glass microfiber filter paper and frozen until analysis.

Water sample A was processed to quantify nitrate, silicate and phosphate using Hach Lange protocols, reagents and a Hach Lange DR2000 spectrophotometer.

A second 500ml water sample (B) was collected at each panel site visit between May 2012 and February 2013 in a sterile borosilicate glass bottle. These samples were fixed using mercuric chloride at a 0.02% volume ratio (Dumousseaud et al. 2011) and analysed by the National Oceanographic Centre (NOC) Carbonate Chemistry Facility for total Dissolved Inorganic Carbon (DIC), Total alkalinity (TA), pH, pCO_2 and aragonite and calcite saturation states. Methods used to determine these factors are described in the Carbonate Chemistry Facility practical guide (Dumousseaud et al. 2011).

4.4.2 Panel processing

Only one side of the panels (the underside) was analyzed (225 cm^2) as the mounting struts obstructed the upper side surface and thus the surface was not fully exposed for colonization. All bryozoan colonies were identified under a light microscope using field studies council monographs (Hayward & Ryland 1985; 1998; 1999). Five species present at both Millport and St Abbs through multiple months were chosen for seasonal analysis. Orkney panels were not used for seasonal analysis as they were not replaced with as high regularity as the St Abbs and Millport panels. Four species present at two or more sites were chosen to investigate differences between site and depth.

Individual colonies of each species were selected and samples were extracted from the growing edge of the colonies. A minimum of 5 zooids was extracted for each sample (~2mm). In the case of some small colonies it was necessary to sample the whole colony. As far as possible care was taken to ensure that no substrate (e.g. coralline algae) or epibionts were included within the sample as they could potentially contaminate results through their added mineralogies.

4.4.3 Analysis techniques

XRD

Mineralogical analyses were conducted following the protocols detailed in Sections 2.3.3. and 3.4.3 to determine wt% $MgCO_3$ in calcite and wt% aragonite.

4.4.4 Data sources, processing and presentation

STATISTICS AND DATA ANALYSIS

Hourly temperature and salinity were interrogated to determine the daily mean temperature and salinity (24 hour), maximum and minimum daily temperatures. Temperature and salinity data used in Section 4.5.3 was the mean daily measurement for the period of panel submersion and settlement.

All mineralogical measurements underwent weighted average transformation in the context of beta regression following the methodology described in Section 3.4.4. p-values for all analyses were calculated; this is a measure of the strength of the evidence and is based on the traditional probability of error of 0.05. Only when the p-value is less than 0.05 is the

evidence considered statistically significant. Statistics and data analysis were conducted using MINITAB release 14 software (Minitab Inc. 2004) and the R programming environment (R Core Team 2013).

Bray Curtis similarity analysis of communities was conducted on species presence and absence data using Primer software (PRIMER-E Ltd 2006); results were visualised using a 2D MDS plot.

4.5 Results

4.5.1 Environmental variation

Temperature variation was shown to be highly seasonal at all sites (Fig. 4.10), with temperatures greater than the annual mean between approximately June-November at all sites. Temperature is highly consistent between 6m and 12m with no statistical difference for any of the sites (ANOVA) (Fig. 4.11). At all sites winter 2011/2012 was shown to be warmer that both the winter of 2012/2013 (all) and 2010/2011 (Orkney only).

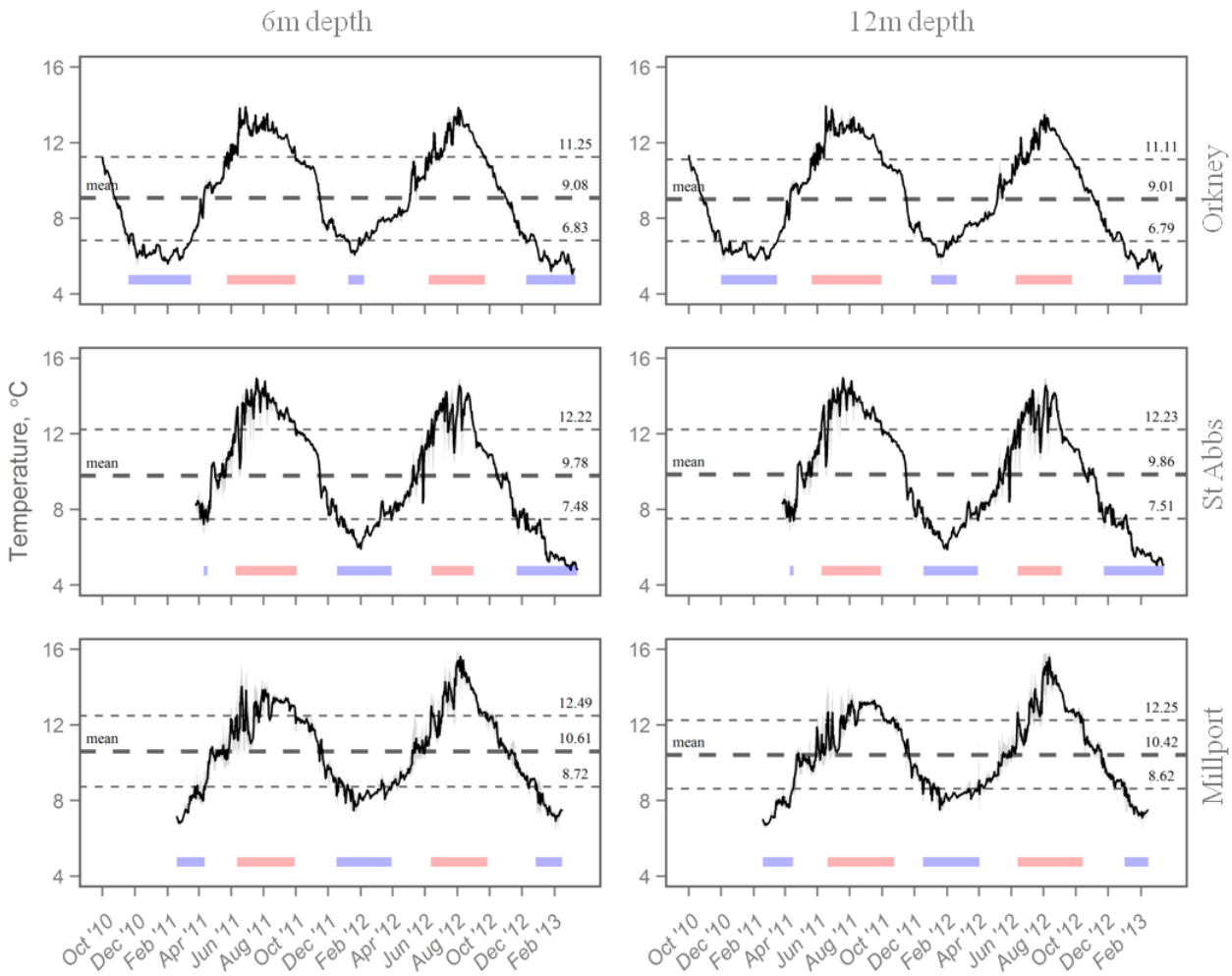


Figure 4.11: Seasonal variation in mean daily temperature at 6m and 12m for Orkney, St Abbs and Millport. Black line: mean daily temperature, ºC; Grey line: daily temperature range (min – max), ºC; Straight black dashed line: site mean temperature; Straight grey dashed line: upper and lower quartile for site; Blue bar: time period when temperature is below the lower quartile; pink bar: time period when temperature is above the upper quartile.

Figure 4.11 shows that between March 2011 – March 2013 Orkney was found to be the coldest and most stable of the three sites; mean temperature = 9.52ºC (6m) and 9.41ºC (12m); temperature range = 8.85ºC (6m) and 8.75ºC (12m). St Abbs is the most variable site; temperature range = 10.14ºC (6m) and 9.92ºC (12m). Millport is the warmest site; mean temperature = 10.61ºC (6m) and 10.42ºC (12m). ANOVA shows the temperature of the sites, Orkney, St Abbs and Millport, to be statistically significant from each other; ANOVA: $p<0.001$, $F = 69.92$; Tukey *posthoc* = $p<0.001$ between all sites (Figure 4.12).

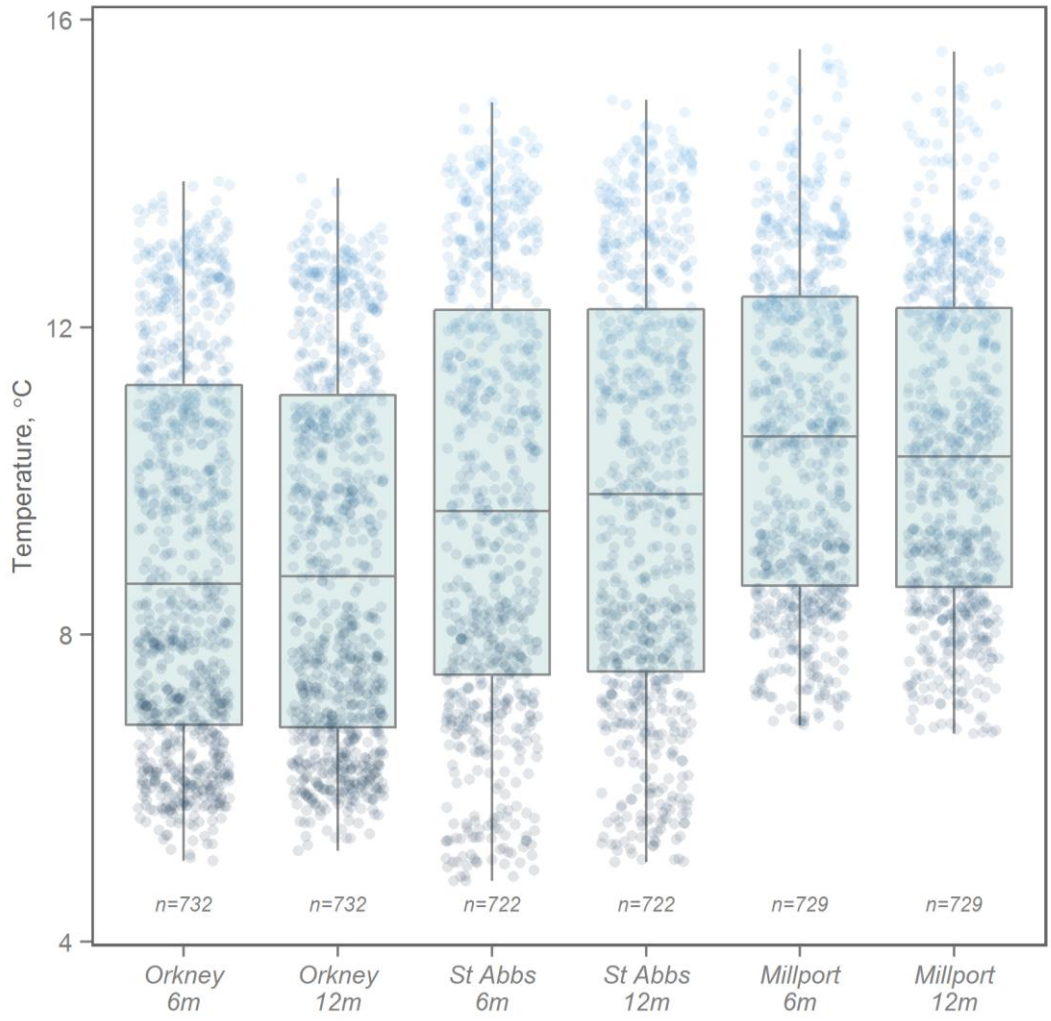


Figure 4.12: Boxplot showing mean daily temperature variation (ºC) at each site between March 2011 and March 2013. Horizontal line indicates mean, box indicates interquartile range, tails indicate total range. Points show individual measurements.

Additional environmental factors were measured between April 2012 and April 2013 at all sites (Appendix C). At three sites, Orkney 6m, St Abbs 6m and Millport 12m, an extended

period of low salinity was mistakenly recorded during the measurement period, an artifice caused by fouling by the serpulid, *Pomatoceros triqueter* affecting sensor operation.

4.5.2 Colonisation patterns

A fauna of 23 bryozoan species was found on panels (Table 4.1). Seven species, *Microporella ciliata* (Pallas, 1766), *Electra pilosa*, *Tubulipora* sp., *Chorizopora brogniarti* (Audouin, 1826), *Celleporella hyalina* (Linnaeus, 1767), *Fenestrulina malusii* (Audouin, 1826) and *Membranipora membranacea*, showed no preference for settling during a specific season but settled year round (Tab. 4.1). Nine species, *Escharoides coccinea* (Abildgaard, 1806), *Callopora craticula* (Alder, 1856), *Disporella hispida*, *Tegella unicornis* (Fleming, 1828), *Amphiblestrum flemingii* (Busk, 1854), *Scrupocellaria scruposa* (Linnaeus, 1758), *Callopora dumerilii* (Audouin, 1826), *Aetea sica* (Couch, 1844) and *Scruparia ambigua* (d'Orbigny, 1841) settled predominantly during the warmer months (March-Oct). Seven species settled predominantly during the winter months; *Escharella immersa* (Fleming, 1828), *Smittinidae indet.* , *Celleporina caliciformis* (Lamouroux 1816), *Membranipora nitida* (Johnston, 1838), *Cribrilina* sp., *Conopeum reticulum* and *Crisia eburnea*. Orkney sites were more species rich than the other sites. St Abbs showed the greatest differentiation between 12m and 6m bryozoan communities (Figure 4.13).

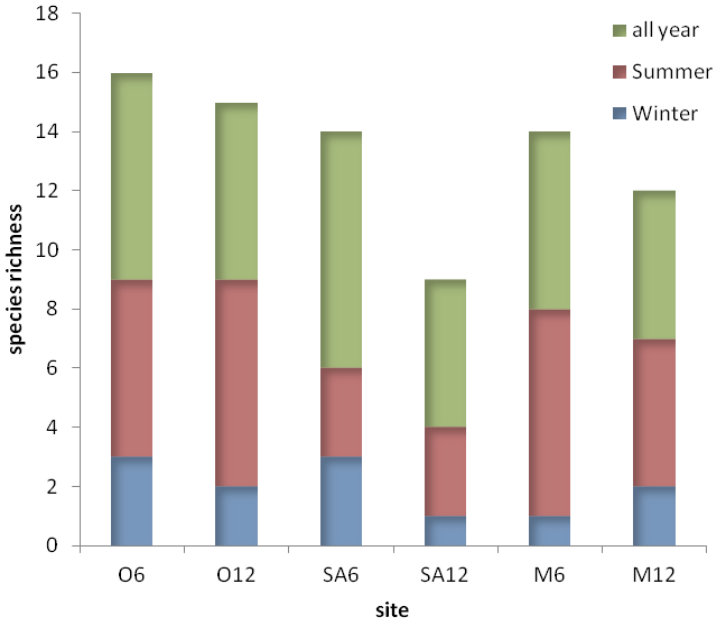


Figure 4.13: Stacked bar chart showing the total species richness at each site. Blue bar indicates winter settlers, red bar indicates summer settlers and green bar represents species with no seasonal settlement preference.

Table 4.1: Presence and absence of species at each site during the total experiment is indicated with black circles (left). Lines show seasonality of settlement across all sites. Solid lines show settlement during a specific month, as determined from single month settlement panels. Dotted lines represent settlement on panels which were settled over a period longer than one month.

species	6m			12m		
	Orkney	St Abbs	Millport	Orkney	St Abbs	Millport
Microporella ciliata	●	●	●	●	●	●
Escharella coccinea	●	●		●	●	
Electra pilosa	●	●	●	●	●	●
Tubulipora sp.	●	●	●	●	●	●
Chorizopora brogniarti	●	●		●	●	
Celleporella hyalina	●	●	●	●	●	●
Callopora craticulata	●	●	●	●	●	●
Escharella immersa	●	●		●		●
Disporella hispida	●	●	●	●	●	●
smittinidae indet.		●				
Celleporella caliciformis		●			●	
Membraniporella nitida	●			●		
Fenestrulina malusii	●		●	●		●
Tegella unicornis				●		●
Amphiblestrum flemingii	●		●	●		
Membranipora membranacea	●		●			
Scrupocellaria scruposa			●	●		●
Callopora dumerilii	●		●	●		●
Cribrilina sp.	●					
Aetea sica	●		●			
Conopeum reticulum			●			
Scruparia ambigua			●			
Crisia eburnea						●

Amongst the sites Millport featured the lowest proportion of winter settling species and St Abbs featured the lowest proportion of summer settling species (Fig. 4.12).

The majority (74%) of species occur at more than one site. Orkney and Millport have the most species in common (11 species) while Millport and St Abbs have the least species in common (6 species). Species with no seasonal preference are more widespread than seasonal species and all of the year-round settlers occur in more than one site (Figure 4.14).

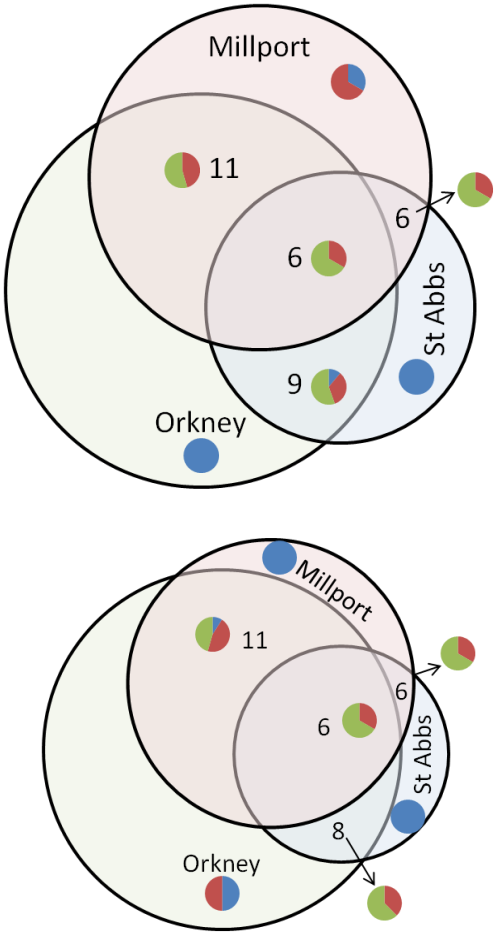


Figure 4.14: Venn diagrams of species distribution with inset pie charts of species preference. Top:6m depth, Bottom: 12m depth. Size of circles indicates comparative species richness, overlapping segments and numbers indicate number of shared species between sites. Pie charts show split of species preferences, green indicates no seasonal settlement preference, blue indicates winter preference, red indicated summer preference.

Species occuring at all three sites are the year-round settlers, *M. ciliata*, *E. pilosa*, *Tubulipora* and *C. hyalina*, and the summer settlers, *C. craticula* and *D. hispida.*

Bray Curtis similarity analysis of species presence and absence data was conducted to analyse the similarity of the site communities (Table 4.2). The most similar sites to each

other were the 6m and 12m sites at the same location as can be seen in the MDS plot shown in Figure 4.15. St Abbs had the greatest similarity between 6m and 12m communities. Between locations Orkney and Millport showed the most similar communities, followed by Orkney and St Abbs. Millport and St Abbs showed the least similar bryozoan communities.

Table 4.2: Bray Curtis similarity of bryozoan communities between sites. Analysis conducted in Primer on presence/absence data from 2010-2013.

	Orkney 6m	St Abbs 6m	Millport 6m	Orkney 12m	St Abbs 12m	Millport 12m
Orkney 6m						
St Abbs 6m	64.00					
Millport 6m	71.43	43.48				
Orkney 12m	82.76	66.67	66.67			
St Abbs 12m	60.87	88.89	47.62	63.64		
Millport 12m	61.54	57.14	66.67	80.00	52.63	

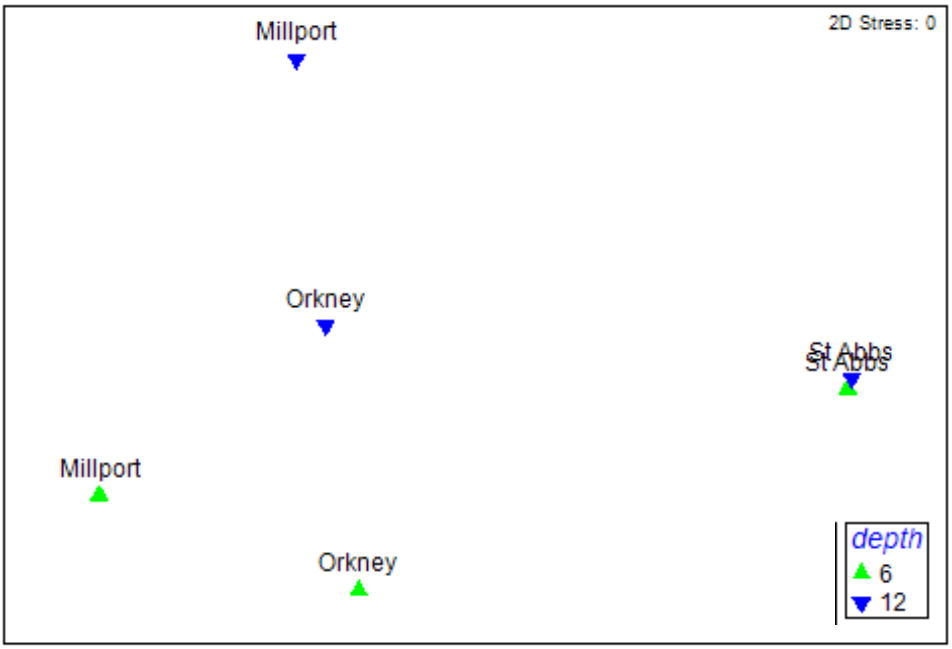


Figure 4.15: MDS plot of Bray Curtis similarity of bryozoan communities between sites. Produced in Primer using presence/absence data collected on dates between 2010 and 2013.

4.5.3 Effects of depth and site

Specimens of four species, *C. craticula, C. hyalina, E. pilosa and Tubulipora* sp., were extracted from panels from the different sites and mineralogically analysed. Panels were colonised during winter 2010 - 2012 (Millport 4/11/2011 – 19/4/2012, Orkney 14/10/2011 – 30/4/2012, St Abbs 1/11/2011 – 25/4/2012).

The wt% $MgCO_3$ in calcite of the four species was found to be statistically distinct (ANOVA, $p < 0.001$, $F = 91.58$) (Figure 4.16). Across species the sites were found to be mineralogically distinct (ANOVA, $p < 0.001$, $F = 19.47$). Tukey posthoc analysis confirms this distinctness between Orkney and Millport and St Abbs and Millport, however St Abbs and Orkney are statistically indistinct.

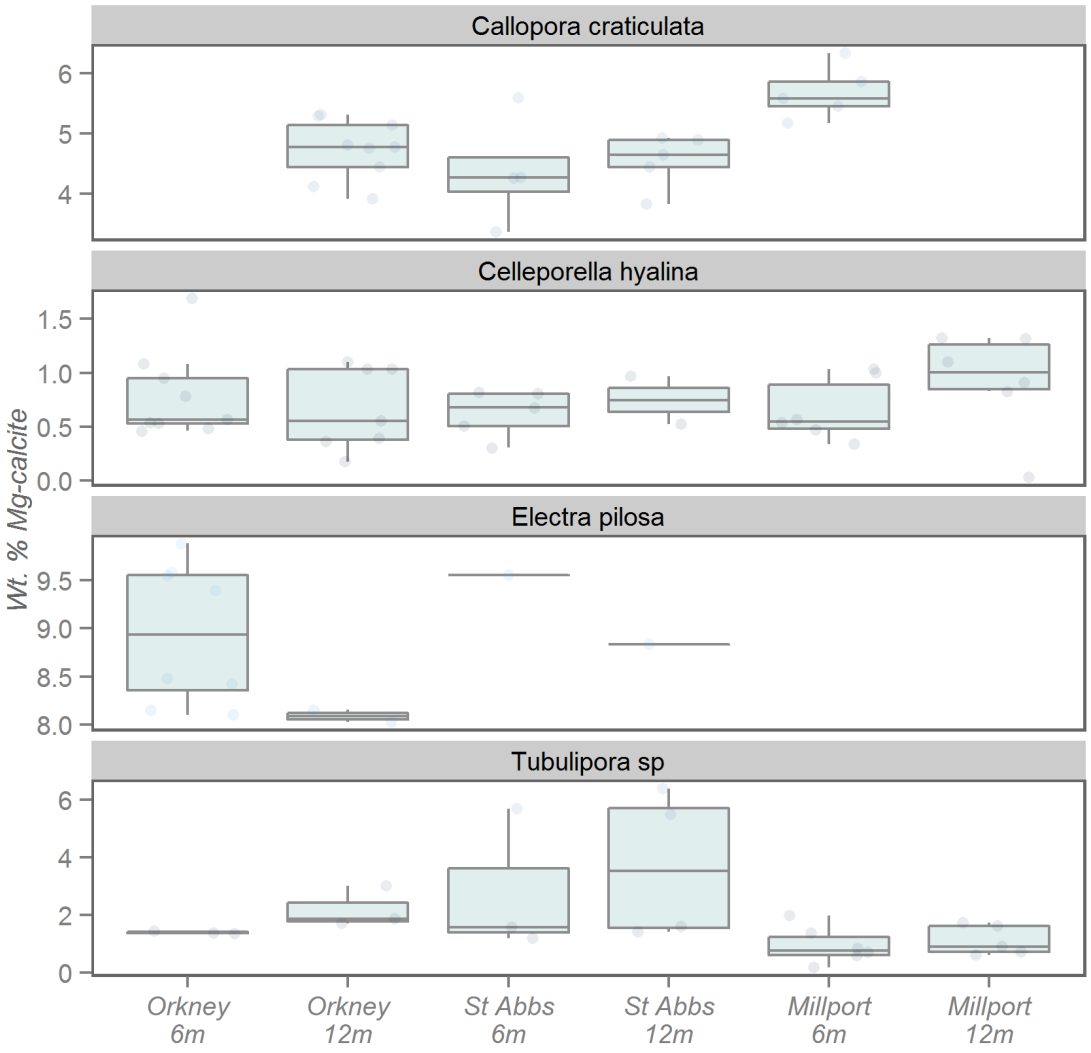


Figure 4.16: Boxplots showing wt% $MgCO_3$ in calcite variation at each site for each species during winter 2011/2012. Horizontal line indicates mean, box indicates interquartile range, tails indicate total range. Points show individual measurements. Wt. % Mg-calcite refers to wt% $MgCO_3$ in calcite.

There was a weak trend and marginally significant linear relationship between the mean site temperature and the wt% $MgCO_3$ in calcite for two of the four species (Linear model: *C. craticula*, p=0.028, R^2=20.99%; *Tubulipora* sp., p=0.010, R^2=26.85%) (Figure 4.17): *C. craticula* showed a trend of increasing wt% $MgCO_3$ in calcite with temperature while

Tubulipora sp. showed a trend of decreasing wt% $MgCO_3$ in calcite with temperature (Figure 4.17). There was no statistically significant difference in wt% $MgCO_3$ in calcite related to depth for any of the study species.

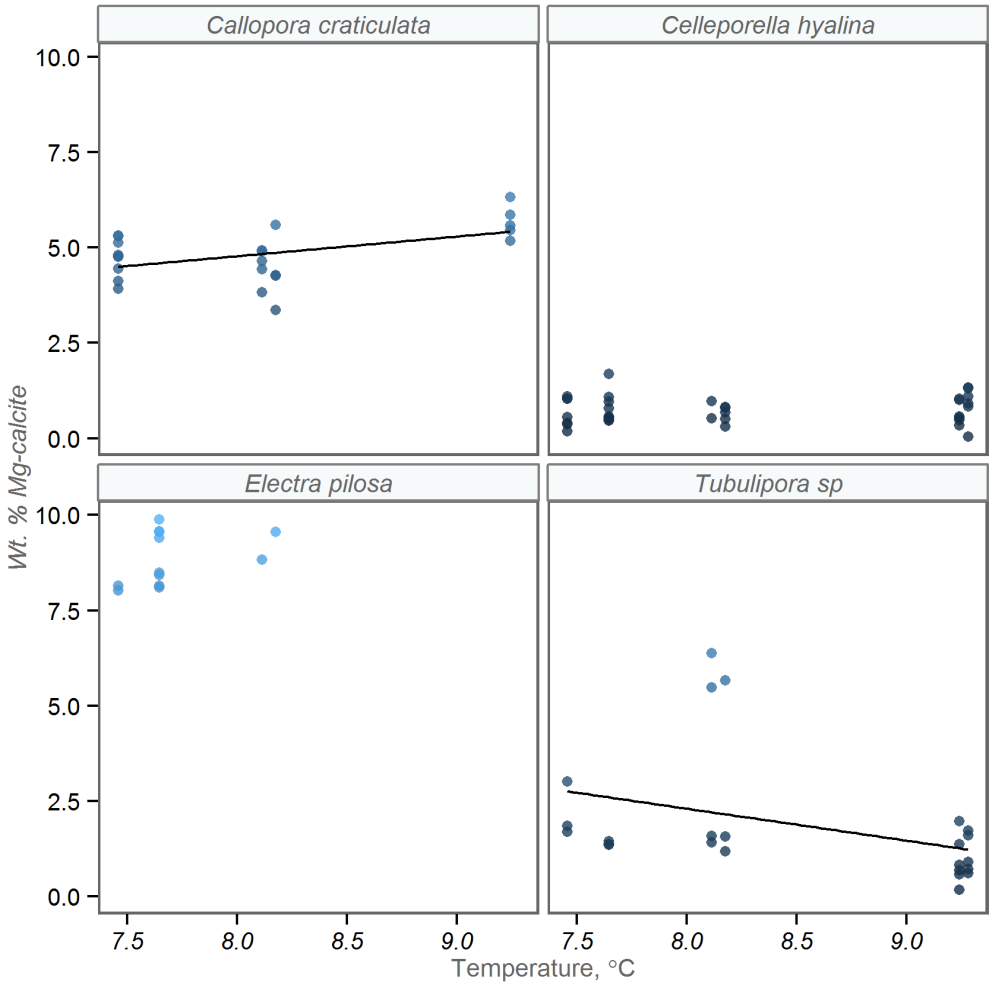


Figure 4.17: Graph showing each species linear relationships between wt% $MgCO_3$ in calcite and mean daily temperature (ºC) for the period of panel submersion. Wt. % Mg-calcite refers to wt% $MgCO_3$ in calcite.

There was a weak, marginally significant difference between the site and wt% $MgCO_3$ in calcite for two of the four species (ANOVA; *C. craticula*, p=0.011, F=5.75; *Tubulipora* sp., p=0.010, R^2=26.85%). *Posthoc* Tukey analysis on both *C. craticula* and *Tubulipora* sp. data show only St Abbs and Millport to be statistically distinct (p=0.020 and p=0.011 consecutively) from the other sites. A cluster of outlier values is observed in *Tubulipora* sp. at 11ºC; these are samples from St Abbs (1/11/12 – 25/4/12) and wt% $MgCO_3$ in calcite in the region of 5, much higher than the mean.

4.5.4 Seasonal variation in mineralogy

Samples from five species, *E. pilosa, C. hyalina, F. malusii*, *Tubulipora* sp. and *M. ciliata*, were extracted from plates settled at Millport between 08/03/2011 and 07/02/2013.

*Table 4.3: Results of ANOVA for each species: log wt% $MgCO_3$ in calcite against environmental factors. *indicates significance, · indicates approaching significance. Crosses indicate no statistical significance*

log Wt.% Mg-calcite *			*E.pilosa*	*C.hyalina*	*F.malusii*	*Tubulipora*	*M.ciliata*
8/3/11 - 7/2/13	linear model	date	×	×	p = 0.0041* R^2 = 61.83	×	p = 0.0213* R^2 = 39.18
		depth (m)	×	×	×	×	×
		mean daily temperature (ºC)	p = 0.0596· R^2 = 8.255	p = 0.0316* R^2 = 11.03	p = 0.0166* R^2 = 39.18	p = 0.0713· R^2 = 12.92	p = 0.0003* R^2 = 77.68
4/11/11 - 7/2/13	one-way GLM ANOVA	salinity (psu)	×	×	×	p = 0.029* F-value = 5.672	×
		pH	×	×	×	×	×
		HCO_3 (µmol/kg seawater)	×	×	×	×	×
		pCO_2 (µatm)	×	×	×	×	×
		nitrate (mg/l)	×	×	N/A	N/A	×
		phosphate (mg/l)	×	×	×	×	×
		silicate (mg/l)	×	×	×	×	p = 0.013* F-value = 10.94
		Ω calcite	×	×	×	×	×
		Ω aragonite	×	×	×	×	×
		max daily temperature (ºC)	×	×	×	×	×
		min daily temperature (ºC)	×	×	×	×	×
		DIC (µmol/kg seawater)	×	×	×	p = 0.0284* F-value = 5.737	×
		TA (µmol/kg seawater)	×	×	×	×	×
* indicates significance. · inidicates approaching significance							

Over the full time period wt% $MgCO_3$ in calcite was shown to be statistically different between species (ANOVA log wt% $MgCO_3$ in calcite x species: $p < 0.001$, $F = 25.43$); Tukey posthoc analysis shows this to be the case in 6/10 species combinations ($p < 0.001$) with the exceptions of *F. malusii* with *E. pilosa* and *C. hyalina* and *M. ciliata* with *E. pilosa*

and *Tubulipora* sp. All species showed either a significant or approaching significant relationship between log wt% $MgCO_3$ in calcite and mean temperature for panel submersion period (see Table 4.3). In all cases, wt% $MgCO_3$ in calcite increased with increasing temperature (Figure 4.18).

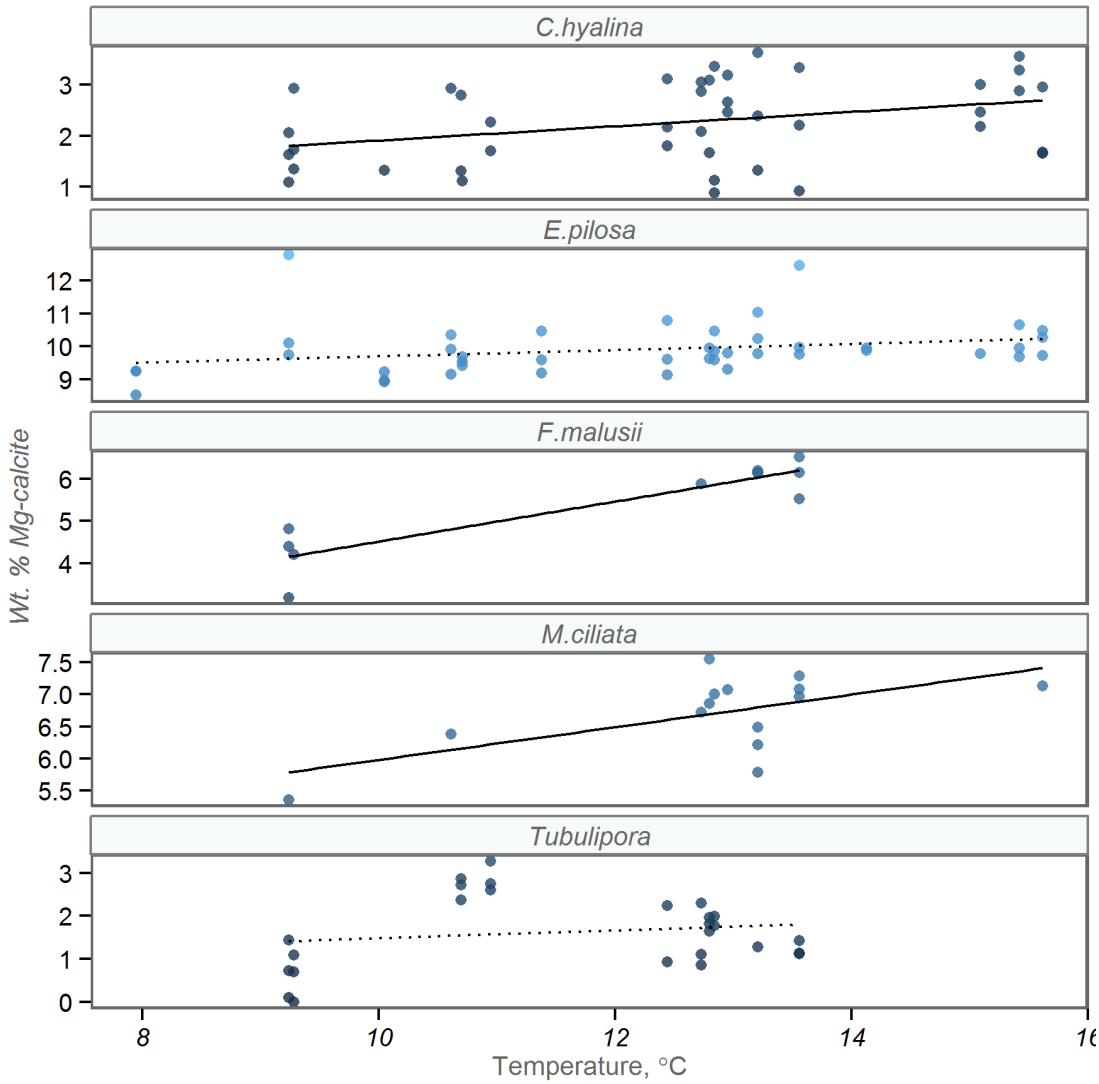


Figure 4.18: Graph showing each species linear relationships between wt% $MgCO_3$ in calcite and mean daily temperature (ºC) for the period of panel submersion as recorded between 8/3/2011 – 7/2/2013. Both 6m and 12m data for Millport are presented. Solid line indicates significant linear relationship, dotted line indicates approaching significance (see Table 4.3). Wt. % Mg-calcite refers to wt% $MgCO_3$ in calcite.

A cluster of outlier values is observed in *Tubulipora* sp. at approximate 11ºC, these are samples from Millport (27/11/12 – 7/2/13) and have wt% $MgCO_3$ in calcite in the region of 3, higher than the mean observed for *Tubulipora* sp. and mineralogically distinct from the outliers observed in Section 4.5.3

4.6 Discussion

4.6.1 Environmental conditions

There are only a few temperature time-series available with which to compare results as government agency time series are limited in regularity and measurement period (Table 4.4), usually due to budgetary constraints.

Table 4.4: Table showing available government agency data sets of seawater temperature in the vicinity of study sites.

Site		Time period	Position
Scapa Flow	Scotlandgov	Jan 2010 – Aug 2011 (daily)	Surface (float)
Clyde estuary (4 sites)	SEPA	2008-2010 (monthly)	Varying depths
Millport	Scotlandgov	2007 – Dec 2011 (daily)	Surface (float)
Scapa	SEPA	2010-2011 (monthly)	Varying depths
Dunbar/Eyemouth (4 sites)	SEPA	2008-2009	Varying depths

Three time series are available, however, which were collected during the experimental period; surface temperature at Scapa Flow and Millport (Scottish Government unpublished data) and monthly measurements in Scapa Flow (SEPA unpublished data). These are plotted alongside collected data in Figure 4.19 & Figure 4.20. There are no comparable time series available with salinity data. Surface temperature measured by the Scottish Government and SEPA are generally slightly warmer than the data from this study in the summer and cooler in the winter; this can be attributed to the greater effect of air temperature at the surface compared to benthic measurements. In general the study data closely aligns with government agency collected data at both Orkney and Millport sites. The close alignment between the measured temperature series increases confidence in the study data and the recorded trends. The range of seawater temperatures observed at the three sites are similar to the range recorded in Scottish waters by the Scottish Government (2011). The Orkney study site ranged between 5 - 14ºC, slightly cooler than the recorded range for the Scottish Continental Shelf (6-14ºC); The St Abbs site ranged between 5 - 15ºC, slightly cooler in winter and slightly warmer in summer than the range for the Northern North Sea (6-14ºC).

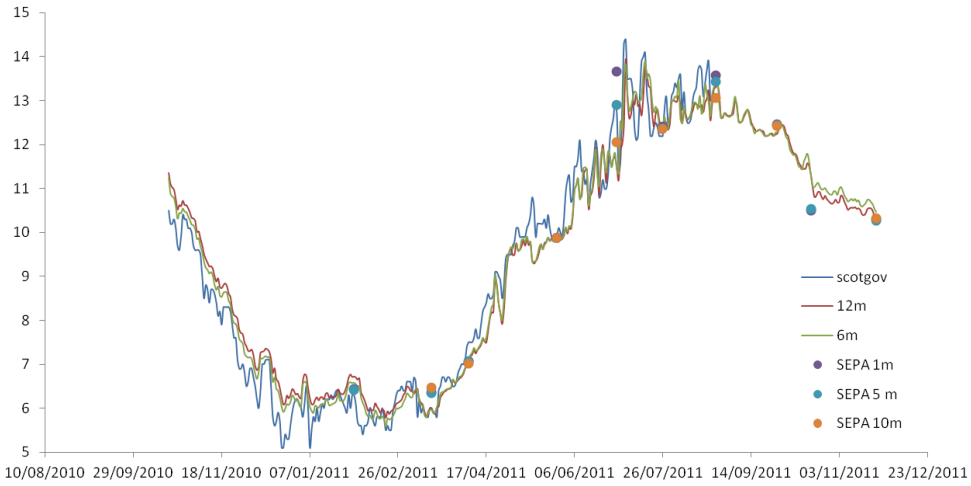


Figure 4.19: Daily mean temperature between 2010 and 2011 in Orkney from the 6m and 12m sites in Swanbister Bay compared to Scottish Government data from a surface buoy in Scapa Flow (unpublished data). Points are shown for spot measurements of water temperature at 1m, 5m and 10m depth by SEPA in Scapa Flow (unpublished data).

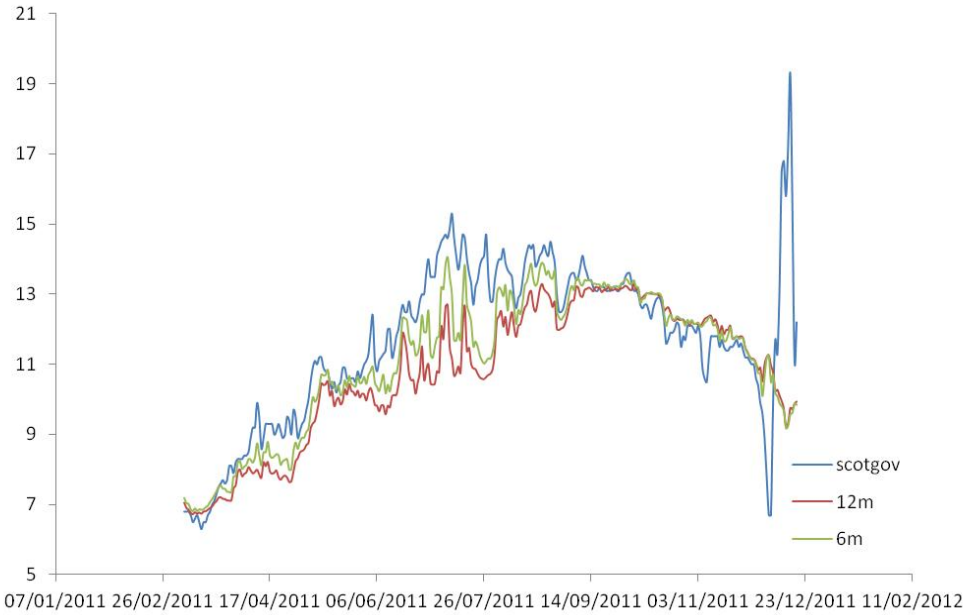


Figure 4.20: Graph showing daily mean temperature between 2011 and 2012 on the Isle of Cumbrae from the 6m and 12m sites in Clashfarland Point compared to Scottish Government data from a surface buoy. The erratic measurements recorded in November 2011 by the Scottish Government are likely caused by accidentally logged data following removal of the sensor from the water.

The Millport site ranged between 7 – 15.5ºC, in the middle of the recorded range for the Irish and Clyde Sea (4-18ºC). With only four areas encompassing the whole of the Scottish

coastline the areas covered by the Marine Scotland areas are wide, as such it is to be expected that there is some variation in study data from these general ranges. Millport and St Abbs are influenced by two very different water bodies; the North Atlantic current and associated influence of the gulf stream (Hurrell 1995; Hiscock 1998) causing Millport to be warmer than the other sites. The North Sea is documented as being more variable than the Atlantic margin as it is more susceptible to the influence of air temperature (Blackford & Gilbert 2007); this explains the wider range of temperatures recorded at St Abbs than the other sites (Figure 4.12). Orkney is situated at the meeting point between the North Sea waters and Atlantic water and is also under additional influence from Arctic waters (Hiscock 1998); as such it is the coolest of the sites.

The winter of 2011/2012 was warmer than the preceding and following winters at all sites, and this is especially marked at Millport and Orkney (Figure 4.11). In 2011/2012 the North Atlantic Oscillation (NAO) was raised (+2.08) compared to 2010/2011 (-0.91) and 2012/2013 (-0.58) (Osburn 2013). The NAO is responsible for much of the inter-annual variability in seawater temperature (Hurrell 1995) and positive phases of NAO have been shown to result in periods of warmer seawater temperature (Hurrell 1995); it is likely that this is the source of the warmer than usual winters, and summer in Millport, during 2011/2012 (Figure 4.11).

The collected salinity data were susceptible to error caused by fouling at all three sites. Despite this the data do show that all sites can be considered full salinity (30-35psu). St Abbs shows the most daily variability in salinity and the greatest difference between 6m and 12m. Petticowick is a bay surrounded by hills and cliffs which reduce to sea level at the bay. As a consequence it is likely that rain water run-off is channeled into the bay and is the source of the regular fluctuations in salinity. This would be more marked at 6m than 12m as the 6m site is less than 20m from the shore. At the shoreline closest to the 6m site there is a sea cave, and it is possible that the formation of this feature has been influenced by rainwater run-off in addition to wave action (Gillieson 2009) and may be an indication of a run-off channel in the vicinity of the 6m site.

Unusually high carbonate measurements in May 2012 in Orkney cannot be explained by any meteorological or oceanographic occurrence and may be a consequence of incomplete nutrient measurements at this sampling point; this may have caused inaccuracies in subsequent carbonate calculations and this data point should be treated with caution. Minor

disturbance in carbonate measures and pH in Orkney during early 2013 can most likely be attributed to dredging taking place in nearby Stromness harbour as part of an ongoing dock construction. Dredging has been shown to increase nutrient concentration and decrease pH in the short term (Ohimain et al. 2008) although no studies investigate the impact of dredging on carbonate chemistry.

Overall there were no discernible patterns in nutrients or carbonate chemistry, and this may because the monthly sampling method was not frequent enough to detect any site, depth or temporal trends.

4.6.2 Bryozoan community

The greatest species richness was observed in Orkney (6m, n=16) followed by Millport (6m, n=14) and St Abbs (6m, n=11). This is in line with observations by the Scottish bryozoans diversity study by Rouse et al. (2013) who found highest bryozoan diversity in Orkney, followed by the West coast, and attributed this to the high habitat heterogeneity found in these regions. High biodiversity is also attributed to the strong tidal currents and sheltered areas offered by Orkney (Bennett & Covey 1998). Hiscock (1998) observed that island habitats in the UK, such as Millport and Orkney, were found to favour benthic biodiversity. In contrast St Abbs is a relatively homogeneous site and this may be reflected in the species diversity at this location. In this study, at all sites more species were recorded at 6m than at 12m. Over their five year study in Ireland, Watson & Barnes (2004) also observed a statistically significant difference in species richness associated with depth with 6m panels featuring the highest diversity (~20 species). This may be a consequence of 6m panels being open to colonization from some shallow intertidal species as well as subtidal, while 12m sites are accessible to subtidal species only. It also may be a consequence of the often more complex environment at 6m than 12m. At all study sites the 6m panel sites were in algal rich areas with kelp, red and brown seaweeds. In contrast the 12m sites featured less seaweeds and no kelp. This more limited choice of colonization substrate at 12m may restrict the species due to competition pressures.

Species presence and absence data were analysed to investigate community similarity between depths and sites. The most similar communities were seen between 6m and 12m depths at the same sites and this is most likely to be a reflection of the physical closeness (40-92m) of the 6m and 12m panel arrays within each location. Between regions the most

similar communities were found between Orkney and Millport followed by Orkney and St Abbs; the least similar bryozoan communities were St Abbs and Millport. These results are in line with the ecological descriptions of species distribution between these regions as published by Hiscock (1998). Orkney and Millport are both described as Lusitanian/Boreal, with both sub-arctic and Iberian influences, and it is therefore unsurprising that they share similar communities. In contrast St Abbs is described as Boreal only, with a sub-arctic influence, and this may explain the differences between the bryozoan communities between this site and Millport.

Across all the sites there appears to be a seasonal influence on species richness (Figure 4.21). The coolest period of the year occurs approximately December to April at all sites (Figure 4.11) and there is a mean of 5.2 species (across all sites) settling on the panels during this time. The warmest months occur between approximately June and October. During this warmer period there is a wider range of species settling with a mean species richness of 9.4 (across all sites). This indicates that the majority of the bryozoan species around Scotland are breeding in the Spring/Summer period, a pattern which has been previously reported by both Denitto et al. (2007) and Maughan (2000) in temperate waters, and which coincides larval distribution with the period of increased food availability (Coma et al. 2000). The majority of the species observed in this study reproduce during summer months and this may be an indication that they are primarily of Boreal/Lusitanian origin. Boreal/Lusitanian species will have evolved an ecological preference for the warmer conditions dominant in their distributional range. These species are at the northerly end of their distributional range in Scotland and so it may be that they are physiologically challenged during the colder winter months and therefore have become obligate summer breeders. In contrast Boreal species are at the more southerly part of their range in Scotland and are likely to be more adapted to colder conditions. Settlement data show that the least winter settling species are found in Millport, which may be a reflection of the Lusitanian influences in this region (Hiscock 1998).

The North Atlantic current, which runs along the West Coast of Scotland and around the North to Orkney (Hiscock 1998), is the most likely explanation for the high level of similarity between bryozoan species and communities in Orkney and Millport. Both Millport and Orkney are island habitats, which have been show to favour biodiversity by

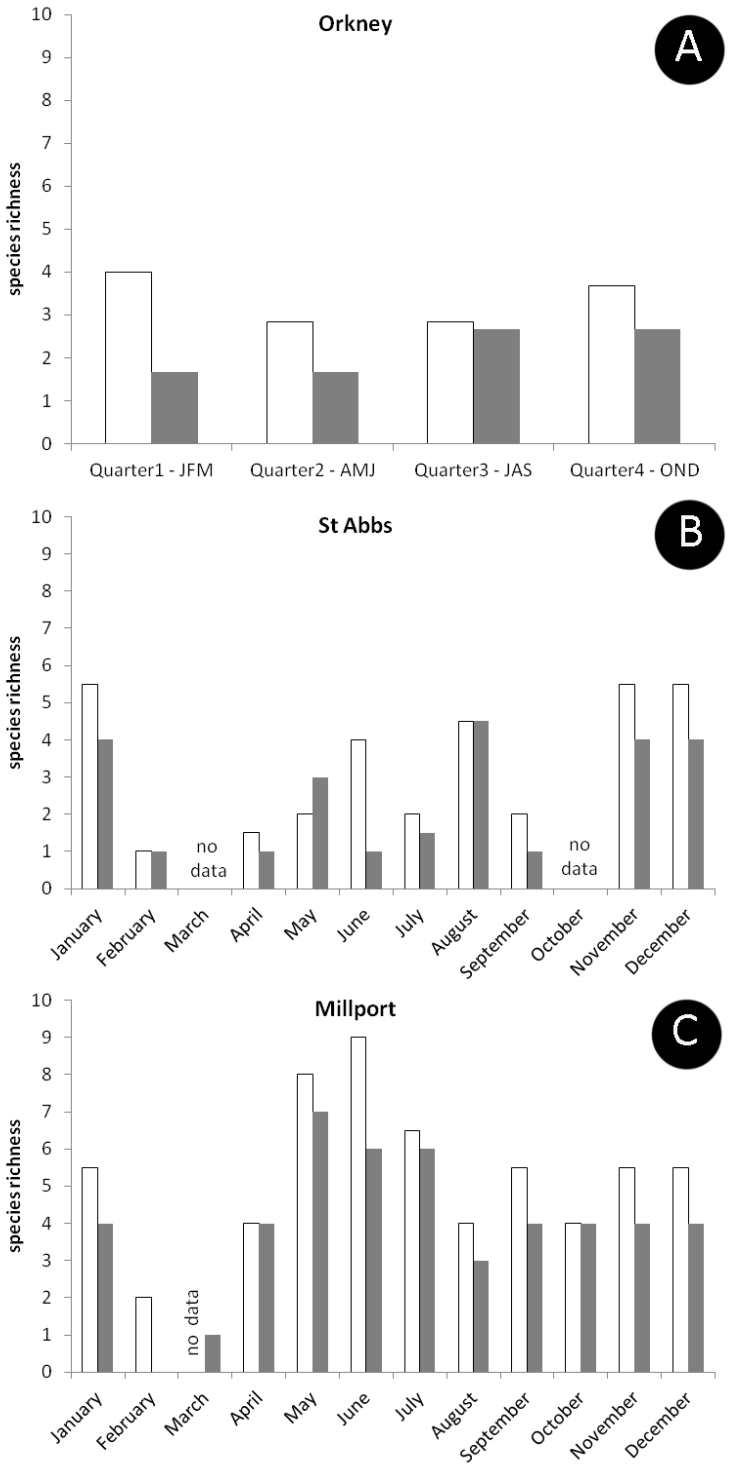


Figure 4.21: Bar charts showing season variation in total number of species observed for each site. Hollow bars indicate 6m depth, grey bars indicate 12m depth. A) Orkney. Due to the reduced frequency of panel changes compared to the other sites data are presented for each quarter (Quarter 1 – 4), months included in each quarter are indicated by letters B) St Abbs, monthly data C) Millport monthly data. Data were collected between 2010-2013.

Hiscock (1998) and may offer similar niches to each other, attracting similar species. In contrast currents around the Northern North Sea predominantly direct flow (and larval movement) in a Southern direction (Figure 4.2) (Hiscock 1998). This may explain why, although there are shared species between Orkney and St Abbs sites, the only species which are shared between St Abbs and Millport are the cosmopolitan species which are found all around Scottish coasts and at all panel sites.

A selection of species which appeared at one site only during a limited period are shown in Figure 4.22. *Scruparia ambigua* was found in Millport only and this is in keeping with its predominantly Southern distribution (Hansson 1999) and the predicted transportation routes of Lusitanian species along the West coast of the UK in the North Atlantic Current (Hiscock 1998; Atlantic Frontier Environmental Network 2001).

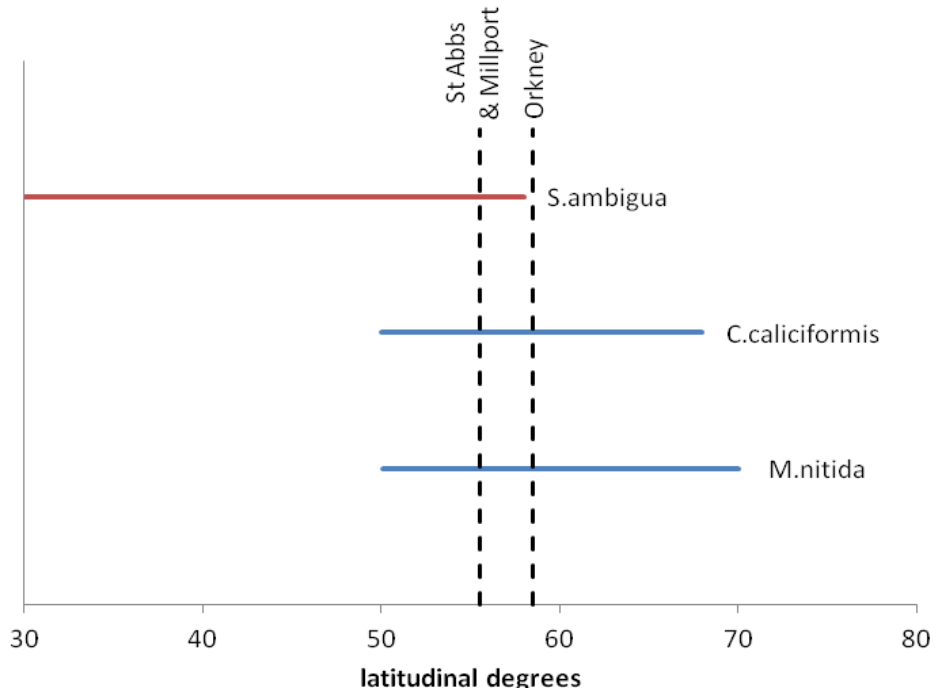


Figure 4.22: Latitudinal distribution of a selection of species found on settlement panels. Blue lines indicate species with a winter settling preference, Red lines indicate species with a summer preference for settlement. Dashed lines show location of study sites.

Its preference for warmer, Lusitanian waters would explain its summer breeding pattern in Scotland. In contrast two species, *C. caliciformis* and *M. nitida* were found in St Abbs and Orkney respectively and were found to settle only during the coldest months of the year.

Both of these species have a predominantly Northern distribution (Hansson 1999) occurring as far north as Northern Norway. These species are only found in Northern parts of Scotland (and the Outer Hebrides) and in the North Sea, where Boreal influences are stronger than Lusitanian (Hiscock 1998; Atlantic Frontier Environmental Network 2001). The families of these species both evolved relatively recently, 33.7 Ma ago (Smith et al. 2006). Since their appearance it is probable they have become adapted to living and reproducing in the colder waters of the limited geographical range they inhabit; this is reflected in their predominant settling during winter in Scotland. *C. caliciformis* in particular is nearing its most Southern extent in St Abbs and may be physiologically stressed and less able to reproduce during the warmer summer months (Peck 2011).

4.6.3 Site and Depth comparisons

There was no statistically significant difference in wt% $MgCO_3$ in calcite related to depth for any of the four study species. There was also no statistical difference in temperature between 6m and 12m and it is possible that this mineralogical similarity may be a reflection of the temperature homogeneity between the two depths. No previous mineralogical studies have been published which analyse samples from different depths while limiting season and spatial proximity of sampling sites and this study therefore offers important evidence that bryozoans in the infralittoral/circalittoral zone are not impacted by small scale depth variation.

Across the four study species wt% $MgCO_3$ in calcite was found to be mineralogically distinct between sites. Specifically Orkney and Millport and St Abbs and Millport were different to each other and this can be explained by the difference in temperature between these sites. During the panel settlement period (Winter 2011/2012) Orkney was the coldest site (mean at 12m = 8.02ºC) and Millport is the warmest (mean at 12m = 9.28ºC). Similarly St Abbs is also colder than Millport with a mean of 8.11ºC (12m depth). wt% $MgCO_3$ in calcite from specimens settled at St Abbs and Orkney was found to be statistically indistinct and this may reflect the closeness in temperature regimes between these two regions. The temperature difference between Millport and the other sites was also accentuated during the winter 2011/2012 compared to 2010/2011 and 2012/2013 (Fig. 4.10). This is explained by the influence of factors related to the NAO which, as discussed in Section 4.6.1, was raised during the winter of 2011/2012 (Osburn 2013) and resulted in

warmer than normal seawater temperatures in Millport during this period. Smith et al. (1998) observed warmer seawater temperature caused by the El Niño-Southern Oscillation (ENSO) influenced skeletal growth rate in New Zealand bryozoans and it is possible that warmer temperatures caused by the NAO may have similarly increased the rate of skeletal deposition and Mg incorporation into calcite at West coast sites in winter 2011/2012. The mineralogical divergence between West and East coast sites may therefore have been accentuated by the impact of the NAO. The general influence of seawater temperature on wt% $MgCO_3$ in calcite between sites seen in this study reinforces previous studies (Lombardi et al. 2008; Kuklinski & Taylor 2009) which recognize temperature as the key control in wt% $MgCO_3$ in calcite content in bryozoans.

At the species level the only statistically significant differences between sites were found for *Callopora craticula* and *Tubulipora* sp. which were mineralogically distinct between St Abbs and Millport. These species also both showed statistically significant relationships between mean site temperature and, again, this is therefore the more likely explanation for the mineralogical divergence between sites. There is, however, no consistency in the wt% $MgCO_3$ in calcite response to seawater temperature between species. Neither *E. pilosa* nor *Celleporella hyalina* show any relationship between seawater temperature and wt% $MgCO_3$ in calcite; *Callopora craticula* exhibits a positive relationship between seawater temperature and wt% $MgCO_3$ in calcite while *Tubulipora* sp. features an inverse relationship of decreasing wt% $MgCO_3$ in calcite with increasing seawater temperature. Overall the relationships between temperature and wt% $MgCO_3$ in calcite are unclear from this specific experiment. This is due to a combination of limited samples and the long period of time the panels were in the sea and colonizing for during winter 2011-2012 (~6 months). This means that a wide range of temperatures were experienced by the animals during this period (Orkney 12m = 5.55ºC – 11.62ºC; St Abbs 12m = 5.76ºC – 12.30ºC; Millport 12m = 7.38ºC – 12.59ºC) and it is not possible to accurately determine when the individual colonies settled and the water temperature at the time of skeletal deposition. Statistical analysis undertaken in this Section was conducted on the mean temperature for the period however this introduces a possible source of error and may explain why only weak or no relationships were detected. The results of this experiment will be complemented and expanded on by a more thorough study of the relationship between wt% $MgCO_3$ in calcite in temperature in Section 4.6.4.

4.6.4 Seasonality of mineralogy

Examination of the seasonal variation in wt% $MgCO_3$ in calcite of five species from Millport showed that all species approached or achieved a statistically significant positive relationship with seawater temperature at the time of deposition. Additional relationships were detected in a few cases between wt% $MgCO_3$ in calcite and date of deposition (*F. malusii* and *M. ciliata*), wt% $MgCO_3$ in calcite and salinity (*Tubulipora* sp.), wt% $MgCO_3$ in calcite and silicate concentration (*M. ciliata*) and wt% $MgCO_3$ in calcite and dissolved inorganic carbon (DIC) (*Tubulipora* sp.). No significant relationships were detected for any species between wt% $MgCO_3$ in calcite and pH, carbonate content (HCO_3), partial pressure of CO_2, nitrate or phosphate concentrations, saturation level of calcite or aragonite, total alkalinity, maximum or minimum daily temperature.

The mechanism of Mg substitution in calcite under different temperatures has been the focus of inorganic precipitation studies. These studies demonstrate that the partition coefficient, D_{Mg}, a measure of the difference in solubility between Mg in seawater and calcite, increases with temperature resulting in preferential incorporation of magnesium into the calcite lattice under warmer temperatures (Chilanger 1962; Katz 1973; Burton & Walter 1985; Mucci 1987; Oomori et al. 1987). Thermodynamic control of biogenic wt% $MgCO_3$ in calcite deposition has been calibrated in controlled culture experiments in some phyla such as Foraminifera (Nornberg et al. 1996; Lea et al. 1999) and coccoliths (Ra et al. 2010) , however this has not yet been conducted for bryozoans. The relationships detected in this study between wt% $MgCO_3$ in calcite and seawater temperature concur with previous publications on bryozoan mineralogy (e.g. Lombardi et al. 2008; Kuklinski & Taylor 2009) however within the phylum Bryozoa, there is still debate about the relative influence of environmental and biological controls on $MgCO_3$ incorporation in calcite. Data presented in the previous Chapter of this thesis, Chapter 3, provide evidence of a vital effect and some biological control in the skeletal mineralogy of bryozoans.

Data presented in this Chapter indicate that, of the environmental factors measured in this experiment, differences in seawater temperature between sites and seasons are the dominant cause of wt% $MgCO_3$ in calcite variation. Laboratory controlled calibrations of Foraminifera wt% $MgCO_3$ in calcite concluded that drastic changes in salinity can influence $MgCO_3$ incorporation into calcite to an equal or greater extent than changes in temperature (Nornberg et al. 1996). In the bryozoan species studied, however, no such

pattern was consistently detected with only a single weak relationship detected for *Tubulipora*. This may be due to the consistency of salinity throughout the year at the Millport study site which may not have featured a high enough variability to trigger a mineralogical response. In some cases statistically significant relationships with sample date, salinity, silicate and DIC were observed for one or two species, however, these are not necessarily a cause of wt% $MgCO_3$ in calcite variation. All of these factors follow a general correlation with seawater temperature and fluctuate with the seasons. The inconsistency of these patterns between species suggests that in the few rare cases where relationships are shown, we are observing coincidental correlations with wt% $MgCO_3$ in calcite variability rather than causative factors. It is therefore proposed that in this experiment seawater temperature at the time of deposition is the primary causative factor in determining Mg content in bryozoan calcite. It is not possible to determine from this experiment, however, whether the mineralogical variability is thermodynamically driven or is due to temperature driven biological factors known to affect mineralogy such as increased feeding (Bone & James 1997), metabolism (Stanley & Hardie 1998), growth rates (Barnes et al. 2007; Smith 2007; Kuklinski & Taylor 2009) or reproduction. It should be noted that all of the study species from this experiment featured a similar pattern of wt% $MgCO_3$ in calcite increasing with seawater temperature. This may be influenced by the ecological similarity of these species. All the study species are widely distributed, occurring at all study sites (with the exception of *F. malusii* which occurred at 2/3 of the study sites) and are in the central range of their distribution in Scotland; all of the species also settle throughout the year. Future experiments using bryozoans which are at their Northern or Southernmost extent of their distributional range and/or feature limited breeding cycles, such as the species *S.ambigua, C.caliciformis* or *M.nitida* (Fig. 4.21) may show differential mineralogical responses to temperature driven by ecological/physiological specializations of the species.

The experiments in both Section 4.5.3 and 4.5.4 highlight unusual tight clusters of measurements for *Tubulipora* sp. which are statistical outliers (> 2 standard deviations) from the group mean of 1.25 wt% $MgCO_3$ in calcite (+/- 0.57, n=40). A cluster, *Tubulipora 2*, (n=3) from St Abbs (1/11/12-25/4/2013) had a mean wt% $MgCO_3$ in calcite of 5.84 (+/- 0.47), while a cluster, *Tubulipora 3*, (n=6) from Millport (27/11/2012 – 7/2/2013) had a mean wt% $MgCO_3$ in calcite of 2.76 (+/- 0.30). *Tubulipora* is extremely difficult to identify to the species level and it is possible that these outlying clusters indicate

different species to the dominant species (*Tubulipora 1*). Although the dominant species (*Tubulipora 1*) settled through much of the year, the presence of these clusters occurs only during the winter and may indicate additional *Tubulipora* species which are winter breeders.

Microscopic analysis of the species does appear to indicate three different morphologies and supports the possibility of distinct species (Figure 4.23).

The mineralogy of these clusters, when compared to data from Chapter 3, offers possible identities for these additional *Tubulipora* species. *Tubulipora 2*, from St Abbs, consists of IMC (5.84 Wt. %), similar to *T. penicillata* (O. Fabricius, 1780) (4.66 wt% $MgCO_3$ in calcite), a species with a Boreal distribution (Intergovernmental Oceanographic Commission (IOC) of UNESCO 2010) and a preference for colder waters. This would be consistent with its settlement in winter at St Abbs only, with its known Boreal influences (Hiscock 1998). Tubulipora 3, from Millport, consists of LMC (2.76 Wt. %), which may indicate species such as *T. phalangea* Couch, 1840 (1.92 Wt. %) or *T. liliacea* (2.60 Wt. %). Electron microscopy would assist in confirming the identity of these potentially different species.

The observation of mineralogically distinct clusters of samples, potentially indicating different *Tubulipora* species, does raise interesting possibilities for the use of mineralogy to help differentiate between morphologically similar and taxonomically unstable species in the future.

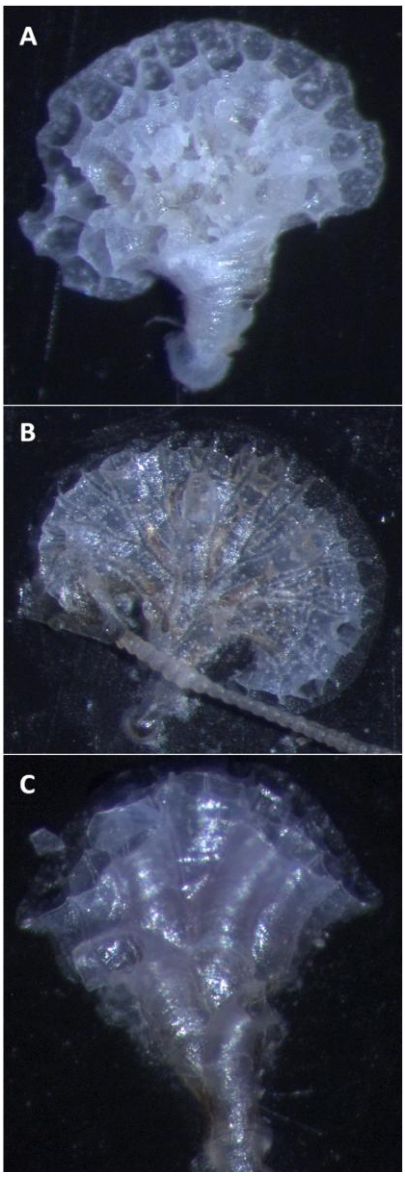


Figure 4.23: Microscopic images of Tubulipora colonies taken from panels using a Leica light microscope. A = Tubulipora 1 dominant species across all panels, B= Tubulipora 2 from St Abbs winter 2012/2013, C = Tubulipora 3 from Millport winter 2012/2013.

4.7 Summary and Conclusions

Using mineralogical analysis, environmental measurements and community analysis, patterns of variability were investigated in the $MgCO_3$ content in skeletal calcite of marine bryozoans deposited over a three year period on settlement panels at sites around Scotland. No statistically significant differences in environmental measurements, community structure or skeletal $MgCO_3$ in calcite were attributable to differences in depth. Significant differences were detected between sites and with seasonal changes in seawater temperature.

The following patterns were detected between sites:

- The North Atlantic current and the influence of Lusitanian/ Boreal species result in similar ecology and bryozoan communities in Millport and Orkney.
- North Sea water, the influence of Arctic waters and the dominance of Boreal species characterizes St Abbs and also links it to Orkney sites.
- Millport and St Abbs were the least similar sites environmentally and had the most different bryozoan communities.
- Overall wt% $MgCO_3$ in skeletal calcite in samples from different sites was found to be statistically different to each other. Only St Abbs and Orkney featured similar mineralogy and this is attributed to the similar temperature regime between Orkney and St Abbs.

The following seasonal trends were detected:

- Regular seasonal variation in seawater temperature was detected at all sites with both Millport and Orkney exhibiting inter-annual variation in winter temperatures caused by the North Atlantic Oscillation.
- At all sites species richness was higher in the summer than the winter.
- A statistically significant (or nearing significance) positive relationship was detected between wt% $MgCO_3$ in calcite and seawater temperature for all species.

Data from this experiment suggest that seawater temperature at the time of deposition is the primary causative factor in determining Mg content in bryozoan calcite for the widely distributed study species. This concurs with previous studies that have shown increasing $MgCO_3$ deposition in calcite at warmer temperatures. It is not possible to determine from this experiment alone, however, whether the mineralogical variability is thermodynamically

driven or is due to temperature driven biological factors such as increased feeding, metabolism, growth rates or reproduction. Although the underlying mechanisms of $MgCO_3$ in calcite variability with temperature as yet remain unclear, this study emphasizes the importance of multi-year studies, sample replication and accurate measurement of environmental conditions as well as consideration of site and species specific biological factors.

Chapter 5: Investigating the effects of the intra-species "vital effect" and environmental conditions on skeletal mineralogy of Bryozoa from Antarctica and Scotland

5.1 Abstract

Bryozoans exhibit a highly variable chemistry within their calcium carbonate skeletons. Previous publications have predominantly attributed this to environmental factors such as seawater temperature, however, previous to this study, the patterns and scale of this variability had not been examined in detail.

The locations chosen for this study were the contrasting stenothermal and eurythermal regions of Ryder Bay, on the West Antarctic Peninsula and Scapa Flow in Scotland. In each of these regions three/four study species were chosen and a minimum of five sites were selected with similar depth and physical properties. During expeditions to Antarctica and Scotland in 2012 specimens of each species were collected from each site by SCUBA diving and simultaneously environmental variables were recorded. In order to eliminate all sources of non-environmental skeletal variation specimens were collected in the equivalent, Austral or Boreal summer, month in each region and animals were of the same size, age and breeding status. X-ray diffraction was used to quantify the wt% $MgCO_3$ in calcite and wt% aragonite present in the skeletons of over 1000 bryozoan specimens. Skeletal variability was examined alongside environmental measures in order to elucidate patterns and major contributors to mineralogical differences.

The results of this investigation show that, despite the different seasonal stability of the regions, Polar and UK bryozoans are remarkably similar in their skeleton variability. This is attributed to the comparable temperature variability experienced by bryozoans in each region during the deposition period for the extracted skeleton fraction. The chemical composition of skeletons from sites in both Antarctica and the UK were statistically different from each other for all species. Antarctic bryozoans in particular are shown to be more mineralogically distinct between sites than expected and this is ascribed to the high degree of habitat fragmentation caused by ice disturbance and currents driving directional adaptation of isolated populations to local environmental conditions. No species from either region are found to have the expected positive correlation between wt% $MgCO_3$ in calcite and temperature; however, three species display a negative response of decreasing wt% $MgCO_3$ in calcite with increasing temperature. This unexpected mineralogical response to temperature by species from both regions introduces doubt about the validity of bryozoan wt% $MgCO_3$ in calcite as a palaeo-environmental proxy for seawater temperature and raises questions about the potential success of some species under future climate change and ocean acidification scenarios.

5.2 General Introduction

Past investigations into links between mineralogy and environmental conditions in the Bryozoa have been primarily concerned with temperature. The earliest studies observed increasing aragonite in regions with warmer seawater (Lowenstam 1954; Carver & Rucker 1969). Some have investigated the use of stable oxygen isotopes as a proxy for seawater temperature (Smith & Key 2004; Lombardi et al. 2008; Knowles et al. 2010). Lombardi et al. (2008) compared oxygen isotope and MART derived temperatures against wt% $MgCO_3$ in calcite to attempt to elucidate the accuracy of mineralogy as a temperature proxy (Lombardi et al. 2008) . Further studies have investigated the relationship between latitude and mineralogy (Smith et al. 2006; Kuklinski & Taylor 2009; Taylor et al. 2009; Smith & Girvan 2010; Loxton et al. 2012), with latitude usually considered a proxy for seawater temperature. There have been no studies to date comparing environmental measurements collected directly from the sampling site to bryozoan mineralogy.

Latitudinal studies have all either reinforced the expectation of a positive correlation between sea-water temperature and mineralogy in bryozoans (Smith et al. 1998; 2006; Taylor et al. 2008; Kuklinski & Taylor 2009; Taylor et al. 2009; Loxton et al. 2012) or been inconclusive (Steger & Smith 2005; Smith & Girvan 2010).

5.2.1 The "muddy water"

Publications assessing links between bryozoan mineralogy and environmental conditions have highlighted the possible existence of a number of potentially confounding patterns, many of which are difficult to quantify. In the case of large scale latitudinal patterns, factors such as salinity, food availability, growth rates and disturbance vary with latitude alongside temperature (see Chapter 3 for details) and the scale of their individual or synergistic impact on mineralogy is not yet understood.

Additional "muddy water" which can affect bryozoan mineralogy includes biological influences over different temporal scales. Short timescale variations in mineralogy, occurring during a colonies lifetime, can be influenced by the physiology of the animal (Weiner et al. 2001). Variations in mineralogy between isolated populations can be driven by directional evolution in abbreviated timescales (<1000 generations) as a reaction to

environmental conditions (Cheetham et al. 1994) while evolution of a species-wide change in morphology occurs over millennia (Lynch 1990).

Following the first report of links between mineralogy and seawater temperature by Urey et al. (1951), a follow-up paper was quickly released by Epstein et al. (1951), expanding this into a usable scale, but in which he also observed that not all mineral deposition was occurring in isotopic equilibrium, and that "the presence of a physiological effect in the case of certain groups of animals such as the echinoderms and corals, and plants such as a coralline algae, has seemed probable ….". This physiological effect on mineralogy subsequently came to be known in biogeochemistry as the "vital effect" and can be known to override or mask mineralogical responses to environmental factors such as temperature (Weiner et al. 2001).

Mineralogical variation within and between colonies has been observed in a number of bryozoan studies (Crowley & Taylor 2000; Lombardi et al. 2008; Kuklinski & Taylor 2009; Loxton et al. 2012). Scheiner et al. (1991) and subsequently Cheetham et al. (1995) attributed this phenotypic plasticity to environmental cues at the micro (within colony) and macro (between colony) scale. Biomineralization studies in other phyla have since expanded this view to attribute many mineralogical responses to physiological processes, termed the "vital effect", triggered by these micro and macro-environmental cues (Weiner et al. 2001). The physiological processes driving mineralogy can include differences in feeding (e.g. Lombardi et al. 2008), breeding (e.g. Barker et al. 2005) and metabolic rate (e.g. Portner et al. 2000).

The scale of "vital effect" varies between phyla, and the usual response to this from the biogeochemistry community has been to identify "reliable" species to use for palaeoenvironmental studies and ignore the rest. However, as Weiner et al. (2001) highlight, "in the absence of a deep understanding of the vital effects, it is well nigh impossible to know when the recording is really faithful".

Although a few bryozoan studies have alluded to the existence of a vital effect, and discussed environmental versus biological control (Schafer & Bader 2008; Kuklinski & Taylor 2009; Taylor et al. 2009; Smith & Girvan 2010), the scale of the vital effect on bryozoan mineralogy has not yet been quantified and, as yet, it is not known whether bryozoan skeletons can be considered "faithful recorders" of environmental conditions.

Over the multi-generational timescale, specialisation to an ecological niche can occur in isolated populations. Lynch (1990) identifies this as directional selection, also known as directional evolution. This is where ecological conditions help to drive speciation by selecting for the most favourable genetic mutations and drift (Cheetham et al. 1994). Lynch (1990) considers this as a secondary source of phenotypic variation in the majority of cases, although Cheetham et al. (1994) identifies that directional selection, if working in concert with "founder effects" (Futuyma & Moreno 1988), can induce phenotypic differentiation in significantly less than 1000 generations (Cheetham et al. 1994). The "founder effect" is where a small population shows increased sensitivity to genetic drift due to an increase in inbreeding and low genetic variation (Futuyma & Moreno 1988). The strength of the founder effect is dependent on the relative genetic isolation of a population and the ability of a species to inbreed and self-fertilise. Studies by Hunter and Hughes (1993), on the temperate bryozoan, *Celleporella hyalina*, identify that self-fertilization can occur in completely reproductively-isolated colonies. This view reinforces earlier studies on *Epistomia bursaria* (Linnaeus, 1758) by Dyrynda & King (1982) and self-fertilisation has also since been observed in the genus, *Membranipora.* Ostrovsky (2010) observes that generally cross-fertilization is the rule among Bryozoa, but "selfing" is reserved for 'emergency' situations.

The relative isolation of populations is caused by a combination of larval and gamete dispersive abilities and physical constraints to gene-flow. Broadcast spawners are likely to achieve greater genetic out-crossing than brooders, which have a lower dispersal capacity, and therefore have a lower chance of gene pool mixing (Wilson et al. 2009). Marshall et al. (2006) observe that larger, lecithotrophic larvae, delay settlement as they have a greater yolk reserve and can therefore achieve greater dispersal. Goldson et al. (2001) summarize larval patterns in the observations that gene flow is constrained by the pelagic larval phase, which in turn is limited by size in lecithotrophic larvae; they note that restricted larval dispersal allows genetic differentiation to arise over distances of just meters.

Physical habitat fragmentation can also contribute to the isolation of populations as flow rates affect the dispersal of gametes and larvae and influence the likelihood of out-breeding. Studies by Goldson et al. (2001) on *C. hyalina* in the high flow areas of the Menai Strait found that strong flow can act as a barrier between populations, directing gametes and

larvae and reducing gene-flow outwith the direction of the current. In Antarctica habitat fragmentation can also be caused by substrate disturbance; Pöhlmann (2011) found that grounding of sea-ice during winter and elevated scouring in Spring and Summer have strong effects on the genetic population structure of the limpet, *Nacella concinna* by reducing effective population sizes and giving rise to larger impacts of genetic drift and higher occurrence of inbreeding.

Evolutionary-driven phenotypic differentiation is described by Cheetham (1994) as a combined effect of random genetic drift and mutation counterbalanced by the effects of stabilizing selection; the ability of a species to "correct" random genetic excursions from the phenotypic optimum. Stabilizing selection is based on the observation that morphological diversification within lineages occurs most rapidly in the early stages of genetic isolation and becomes progressively slower (Lynch 1990), as such the effect of stabilizing selection is stronger in species which have undergone a longer period (usually measured in generations) since isolation and have achieved greater morphological stasis.

5.2.2 Known knowledge gaps

Within the phyla Bryozoa there are still significant knowledge gaps about biomineralization. Indications of environmentally driven patterns in mineralogy have been detected, but as yet it has not been possible to quantify the effect of biological control on mineralogy or conclusively prove the existence of the vital effect. As such there is still uncertainty as to whether bryozoans can be used as "faithful recorders" of palaeontological conditions.

Knowledge gaps are primarily attributable to the limited understanding of the biological process of mineralization in bryozoans. A process for mineralization at the cellular level has been proposed (see Chapter 1), but as yet there have been no publications indicating genes, proteins or biochemical pathways which may control mineralogy in the phyla.

Mineralogical studies on bryozoans to date have also been heavily constrained by the mixed origin and limited availability of samples and associated metadata on environmental conditions. It is hoped that this dedicated large-scale study will help to clarify environmental patterns, prove the existence of the vital effect and offer a gauge of the usefulness of bryozoans as palaeontological recorders.

5.2.3 A tale of two regions

Comparisons between the bryozoan skeletal mineralogy of one or more geographical regions have been conducted by authors in the past (e.g. Smith et al. 2006; Kuklinski & Taylor 2009) in order to investigate patterns over latitudinal gradients. None of these studies, however, have included replicated collection regimes between the regions or large numbers of samples.

The regions in this study, Antarctica and the UK (Orkney), were chosen for their different environmental characteristics. Antarctica has a highly stenothermal environment, with an annual temperature range of up to three degrees (ºC) a year (Portner et al. 2000). By contrast the UK is traditionally eurythermal with seasonal temperature fluctuations of over 15ºC common in the North Sea (Portner et al. 2000). A dedicated, high-replicate study of a temperate region can offer interesting data on the range of variability seen within individual species, differences between sites, and relationships between mineralogy and environmental conditions. The addition of a contrasting Polar region to the study allows comparisons between eurythermal and stenothermal mineralogical adaptations and responses.

5.2.4 Introducing Antarctica

Following the breakup of Gondwana, the Antarctic continent was formed from the coming together of two separate fragments; East and West Antarctica. The sea remained temperate for much of the Tertiary but has become progressively colder and more isolated since then. The Antarctic Circumpolar Current (ACC) was created after the separation of the Tasman Rise and the opening of Drake's Passage (33 and 28Ma respectively) completed the deep water circulation around the continent (Barnes & Conlan 2007). This isolated the continent from species transfer. Over the past 2Ma Antarctica has been covered by a dynamic ice cover and has been subject to cycles of glacial expansion and retreat (Nicol et al. 2000). During periods of glacial maxima the ice extended to the continental shelf, obliterating any marine life in the shallow waters, and allowing total recolonization as the ice retreated. Evidence is emerging now of a few ice free refugia which allowed directional evolution, pockets of specialized Antarctic species (Peck et al. 2006) and were the source for recolonization of the shallow subtidal zone during interglacial periods (Convey et al. 2009a).

Antarctica is often referred to as relatively constant environment compared to the rest of the world and the temperature has certainly remained low and constant since the creation of the ACC, varying over just a few degrees (ºC) a year. Although Antarctica is currently in a long (10,000yr) period of glacial minima, the shallow sub-tidal zone remains a dynamic environment, subject to high levels of disturbance. Wave action and disturbance, in the winter from the ice foot and anchor ice, and in the summer from intense iceberg scour, combine to make Antarctica a highly disturbed system compared to other latitudes (Barnes & Conlan 2007).

Antarctica is also an area that is currently undergoing a high level of anthropogenic driven change. The West Antarctic Peninsula is one of the fastest warming regions on Earth (Meredith 2005) and the Southern Ocean is also at higher risk of ocean acidification than other oceans due to its naturally low saturation levels of carbonate (Convey et al. 2009b). It is predicted that the Southern Ocean will become under-saturated with regards to aragonite, during winter, as early as 2030 (Fabry et al. 2009; Orr et al. 2005; McNeil & Matear 2008). Moy et al. (2009) have already shown that shell weights of modern Foraminifera have 30-35% lower shell weights than Holocene specimens from the same Antarctic region. The climate has changed before around Antarctica, but not at the rates currently being experienced, and this is giving organisms limited time to adapt (Doney et al. 2009).

MINERALOGY OF ANTARCTIC BRYOZOA

The first study on the mineralogy of Antarctic bryozoans was conducted by Borisenko and Gontar (1991) and published in Russian. Additional studies by Taylor et al. (2009) and Loxton et al. (2012) added further mineralogical analyses of Antarctic bryozoans.

In total, the mineralogy of 45 species has been analysed from Antarctica (number of specimens = 99).These previous analyses represent an estimated 15% of Antarctic bryozoans (Kuklinski 2013) with the majority remaining unstudied. The majority of mineralogical data on Antarctic bryozoans is derived from single analyses with just three species featuring five or more replicates (maximum 16 replicates). Little is known about the mineralogical range within species.

There is still a lot that is not known about the mineralogy of bryozoans in Antarctica. At present over 85% of the known species in Antarctica are unanalysed and repeat analyses have been conducted on just six species. As a result, little is known about the range of

mineralogical variability between sites and seasons that Antarctic bryozoans exhibit. To date there have been no direct investigations into a possible link between the mineralogy of Antarctic bryozoans and environmental conditions.

Until recently it was believed that Antarctic species featured exclusively low Mg-calcite (LMC) and no aragonite (Borisenko & Gontar 1991). Recent publications, however, report a mean mineralogy of IMC for the region and traces of aragonite (Taylor et al. 2009; Loxton et al. 2012). These findings suggest that Antarctic mineralogical assumptions need to be revisited and reconsidered.

5.2.5 Introducing Orkney

Orkney is an archipelago of seventy islands, situated to the North East of the Scottish mainland; Islands vary in size and many are small and uninhabited. The waters of Orkney are typically shallow and the coastline is predominantly a high energy rocky habitat (Rouse et al. 2013). The intertidal and subtidal substrate is primarily rocky, with boulders, gravel and occasional subtidal mud deposits (Bennett et al. 1998). Tide-swept communities are common in the deeper waters while kelp forests dominate the sheltered sub-tidal zone (Bennett et al. 1998). Orkney is classified as temperate with a sea temperature range of 5 to 13ºC and annual rainfall of 90-100mm; it is influenced by the North-East Atlantic Drift which brings warm waters northwards along the West coast of Britain. Orkney is reported as being relatively stable compared to the rest of the UK due to its lack of extreme temperature fluctuations and the high year round humidity on the islands (Jones 1975).

PAST STUDIES OF BRYOZOA IN ORKNEY

In the 19th century nineteen specimens of bryozoans were collected from Orkney with the earliest specimens recorded from 1847 (Rouse 2010). There followed a lull of nearly a century with just 2 specimens being collected in the 1970s and 1980s before a resurgence of interest in the late twentieth century. The siting of an oil terminal at Flotta in Scapa Flow provided the impetus for many of the more recent studies of marine ecology, monitoring projects and impact studies in the region although these earlier impact assessments neglected the Bryozoa, probably due to difficulties in identification.

An environmental impact assessment in 1993 by De Kliujver (1993)included a survey of 18 sublittoral, hard substratum sites around Orkney, adding 379 specimens to the bryozoan

record. This was followed by annual governmental commissioned surveys of the region by SNH, JNCC and SEPA (Bennett et al. 1998), many of which contributed records and specimens to the bryozoan collections at the National Museum of Scotland and the Natural History Museum and continue to the present day. In the last few years, the influence of "citizen science" groups has grown, with groups such as Seasearch (Marine Conservation Society 2012), providing bryozoan distribution records from Orkney.

Prior to the commencement of this study, 91 species of bryozoan had been recorded from Orkney (n=2296) (Rouse 2010). Orkney is situated at the boundary between Temperate and Boreal regions and this is reflected in the range of species to be found in the archipelago. Species distribution may be influenced by the North-East Atlantic Circulation from the West coast of the UK (Bennett et al. 1998), and, to a lesser extent, exchange with the North Sea to the East and with cold Northern waters via Norway and Iceland (Scottish Government 2011). Rouse et al. (2013) report that the region has a higher than average taxonomic distinctness when compared to other regions of Scotland, and this is primarily attributed to the habitat heterogeneity of the region.

MINERALOGY OF SCOTTISH BRYOZOA

As discussed in Chapter 3, there have been relatively few published mineralogical studies featuring bryozoan species which are present in Scotland (Clarke & Wheeler 1922; Schopf & Allan 1970; Poluzzi & Sartori 1974; Borisenko & Gontar 1991; Smith et al. 2006; Taylor et al. 2009). The publications summarise the analysis of 41 species (n=148), of which specimens collected from Scottish waters were used in the analysis of 5 species (n=8). See Appendix A for full details.

Chapter 3 has added significantly to our understanding of the mineralogical categorisation of Scottish species. In addition, Chapter 4 has added replicate studies linking seasonal variations in environmental conditions to skeletal mineralogy. As yet, however, there has been little work looking at mineralogical variability between sites at the less than 10km scale, or the relative influence of environmental conditions on this variability. In addition a study with high (>50) replication will allow a far greater understanding of the intra-species variability of mineralogy and may add to the discussion of relative biological and environmental control of mineralogy.

5.3 Aims and Objectives

5.3.1 Summary of purpose of study

The purpose of this study is to conduct a high-replicate (>100 per species), multi-site study on the skeletal mineralogy of bryozoans from Antarctica (a stenothermal region) and Orkney (a eurythermal region). This will allow comparison between the mean mineralogy and range of skeletal variability for the species from the different regions and the investigation of comparative mineralogical responses to environmental and biological influences.

5.3.2 Objectives of study

1. Determine the mean mineralogy and intra-species variability of three UK and four Antarctic species (>100 replicates per species) and compare this between regions.
2. Investigate how skeletal mineralogy varies between sites and compare this between the two study regions (5+ sites from each region with >12 replicates from each site).
3. Discuss mineralogical variability with relation to environmental and biological influences.

5.4 Materials and Methods

5.4.1 Sample collection

This study is based on the mineralogical examination of 1014 specimens of seven bryozoan species collected from Adelaide Island, Antarctica (four species, n=584) and Orkney, Scotland (three species, n=430).

ANTARCTIC STUDY REGION

The study region was within an approximate 6km radius of Rothera Scientific base, Ryder Bay, in the West Antarctic Peninsula (68ºS, 68ºW) (Fig. 5.1). Ryder Bay is characterised by

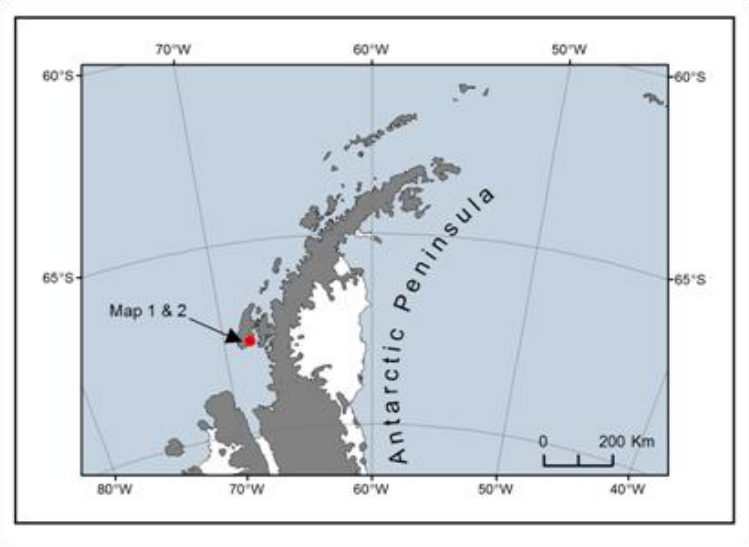


Figure 5.1: Map indicating location of Ryder Bay on the West Antarctic Peninsula.

seasonal coverage of fast-ice (frozen sea surface) and the shallow benthos is subject to extensive ice scour (Barnes 1995) from floating icebergs during the austral summer months. The benthic sea temperature at the time of material collection, in Jan-Feb 2012, was measured at 0.09°C (+/- 0.2ºC), but varies annually from approximately –1.8 to 1°C (from BAS RaTS dataset, unpublished data). The salinity regime of near bottom waters at the time of material collection was measured at 31psu (+/- 0.35psu), and varies annually from approximately 31 to 34 psu (BAS logger data). See Section 5.5.1 for a full environmental profile for the area.

Six local (<10km scale) collection sites (1, 2a, 3, 4, 5, 6) were selected within Ryder Bay, with similar depth, 8.5m (+/- 2.5m) and substrate (boulders/cobbles) (Fig. 5.2). Sites were

an average of 3.85km apart (ranging from 425m to 8km distant from each other) (Tab. 5.1). All sample collecting took place by SCUBA within a three week period (Jan-Feb 2012).

Close proximity (<10m scale) collection sites (2a, 2b and 2c) were located in Lagoon Back Bay (Fig. 5.2) and were of similar depth, 7m (+/- 1m) and substrate (boulders/cobbles on silt). Close proximity sites were an average of 5m apart (ranging from 1.6m to 7.3m distant from one another) (Fig. 5.2 inset map 2). Close proximity sample collecting, at sites 2a, 2b and 2c, took place by SCUBA on a single dive in January 2012.

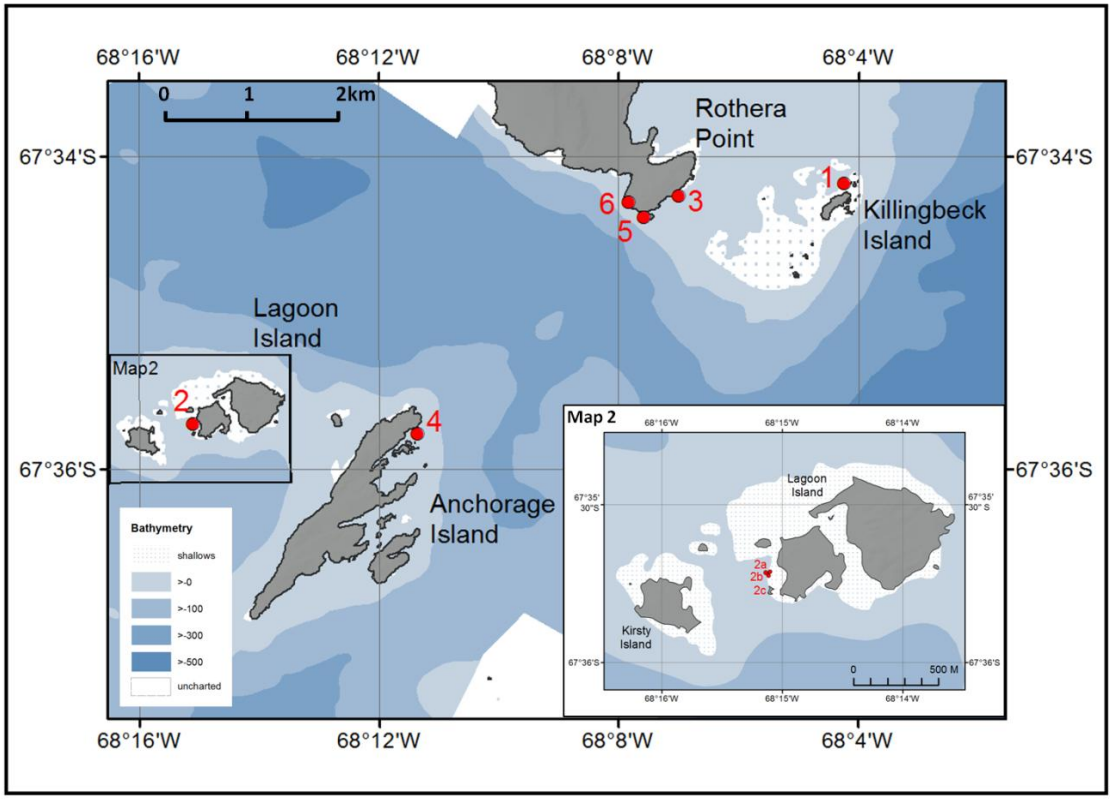


Figure 5.2: Map indicating local collection sites (1-6) in Ryder Bay. Inset (map2) shows location of close proximity sites in Lagoon Back Bay.

ORKNEY STUDY REGION

Orkney is an archipelago of islands situated 16 km North-East of mainland Scotland (58-59ºN, 2-3W), at the point of confluence of North Atlantic and North Sea waters (Fig. 5.3). At the centre of this island group lies Scapa Flow, a semi-enclosed marine basin covering an area of approximately 130 km^2 (Joyce 2004) where water depths range between 10-70m. Tides and currents circulate through Hoy Sound, in the northwest and Hoxa Sound in the South which open to the Atlantic Ocean and Pentland Firth respectively.

Table 5.1: Matrix showing Antarctic site details and direct distance (m) between sites.

site name depth (m)	Latitude Longitude	SITE ⇨ ⇩	1 ⇩	2a ⇩	2b ⇩	2c ⇩	3 ⇩	4 ⇩	5 ⇩	6 ⇩
Killingbeck	-67°34'10.60"	1 ⇨		8008	8008	8008	1980	5920	2350	2550
depth: 10.5m	-68°4'14.79"									
Lagoon Back Bay	-67°35'43.02"	2a ⇨			7.3	6.1	6220	2500	5790	5650
depth: 6m	-68°15'7.14"									
Lagoon Back Bay	-67°35'43.14"	2b ⇨				1.6	6220	2500	5790	5650
depth: 7m	-68°15'7.68"									
Lagoon Back Bay	-67°35'43.14"	2c ⇨					6220	2500	5790	5650
depth: 7.5m	-68°15'7.56"									
East Beach	-67°34'15.36"	3 ⇨						4274	425	600
depth: 10m	-68°07'0.48"									
Anchorage	-67°35'48.45"	4 ⇨							3840	3790
depth: 11m	-68°11'30.67"									
Cheshire	-67°34'22.92"	5 ⇨								292
depth: 10m	-68°7'31.05"									
Honeybucket	-67°34'18.44"	6 ⇨								
depth: 8m	-68°7'50.82"									

The benthic sea temperature at the time of material collection, in May 2012, was measured at 8.2ºC (+/- 0.02ºC), but is highly variable through the year with a range from 5 - 14ºC (Marine Scotland 2012). The salinity regime of near bottom waters at the time of material collection was measured at 33psu (+/- 0.42psu), and varies annually from 30-35psu (Marine Scotland 2012). See Section 5.5.1 for full environmental profile for the area.

Figure 5.3: Map showing location of Orkney islands in the UK

Five local (~10km scale) collection sites (1-5) were selected within Scapa Flow, with an average depth of 9.5 (+/- 3.5m) and substrate (boulders/cobbles) (Fig. 5.4). Sites were an average of 6 km apart (ranging from 1.7 km to 11.8 km distant from one another). All sample collecting took place by SCUBA in a single week in May 2012.

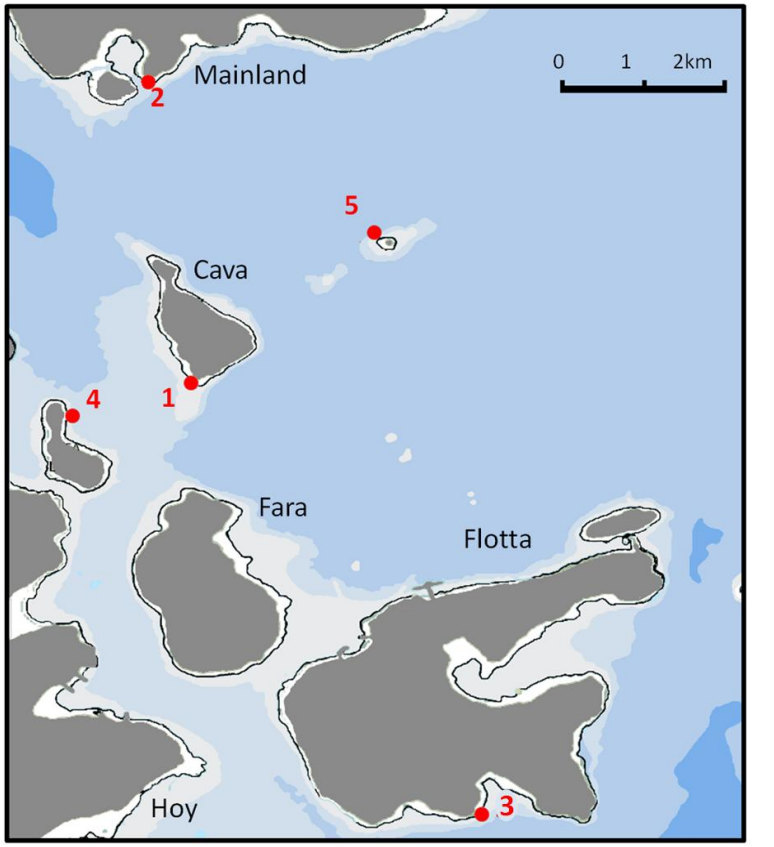


Figure 5.4: Map indicating local collection sites (1-5) in Scapa Flow, Orkney

METHOD OF SPECIMEN COLLECTION

All species selected are encrusting bryozoans which reside on the underside of rocks. Suitable rocks with the desired species were selected underwater and carefully placed in collecting bags. Care was taken during the lifting process to minimise crushing of colonies.

ENVIRONMENTAL MEASUREMENTS

In both Antarctica and Orkney benthic sea temperature and conductivity were measured at each site every minute during sample collection using a HOBO U24-002 salt water conductivity logger; instrument accuracy is reported to be 0.1ºC for temperature (measurable range -2 - 36ºC) and 3% of conductivity reading (HOBO 2013b). Conductivity

was converted to Practical Salinity Units, as defined on the Practical Salinity Scale, (McDougall et al. 2009) using the Gibbs function of sea-water.

In Orkney, between October 2010 and March 2013, hourly seawater temperature measurements were recorded using an HOBO UA-002-64 temperature logger at 6m and 12m benthic sites in Scapa Flow (6m site: 58º 55' 300N, 003º 06' 579W , 12m site: 58º 55' 273N, 003º 06' 548W). Logger accuracy is reported to be 0.5ºC for temperature in a measurable range between -20 to 70ºC (HOBO 2013a). In Antarctica, British Antarctic Survey (BAS) long-term logger data were provided from the RaTS dataset (RaTS, H.Venebles, unpublished data), collected from Cheshire Island using the protocol described by Clarke et al. (2008).

In Orkney, *in situ* water samples were collected by SCUBA from each site in 500ml borosilicate glass bottles. In order to prevent any further biological activity in the stored sample, each sample was poisoned within one hour of collection with a saturated solution of mercuric chloride (7 g/100 ml de-ionized water) in a 0.02% volume ratio. Carbonate analysis was undertaken at the National Oceanography Centre, Southampton following the protocols of Dickson et al. (2007). Dissolved Inorganic Carbon (DIC) was determined using coulometric titration; Total Alkalinity (TA) was determined using closed-cell titration. The precision of DIC and TA measurements obtained using this method is reported as +/- 0.05% or better (Dumousseaud et al. 2011). The measurement of DIC, TA, temperature and salinity allows the calculation of the other variables of the carbonate system through the use of thermodynamic constants. The CO2SYS program (Lewis & Wallace 1998) was used for the recalculation of the carbonate system variables using the thermodynamic constants of Mehrbach et al. (1973) refitted by Dickson & Millero (1987). Carbonate analysis was conducted according to the methods of Dumousseaud et al. (2011).

5.4.2 Specimen processing

The species selected for analysis from Antarctica were the cheilostomatous bryozoans, *Antarctothoa antarctica* Moyano & Gordon, 1980, *Fenestrulina rugula* Hayward & Ryland, 1990, *Hippadenella inerma* (Calvet, 1909), and *Inversiula nutrix* Jullien, 1888. The study species were selected on the basis of being well-known, common and abundant Antarctic species with distinctive features for identification (Fig. 5.5). Specimens were identified to species level under a dissection (stereo) microscope using the monograph of

Hayward (1995). Scanning electron microscope (SEM) images of each species were taken on a small selection of specimens in order to confirm identification (Fig. 5.5).

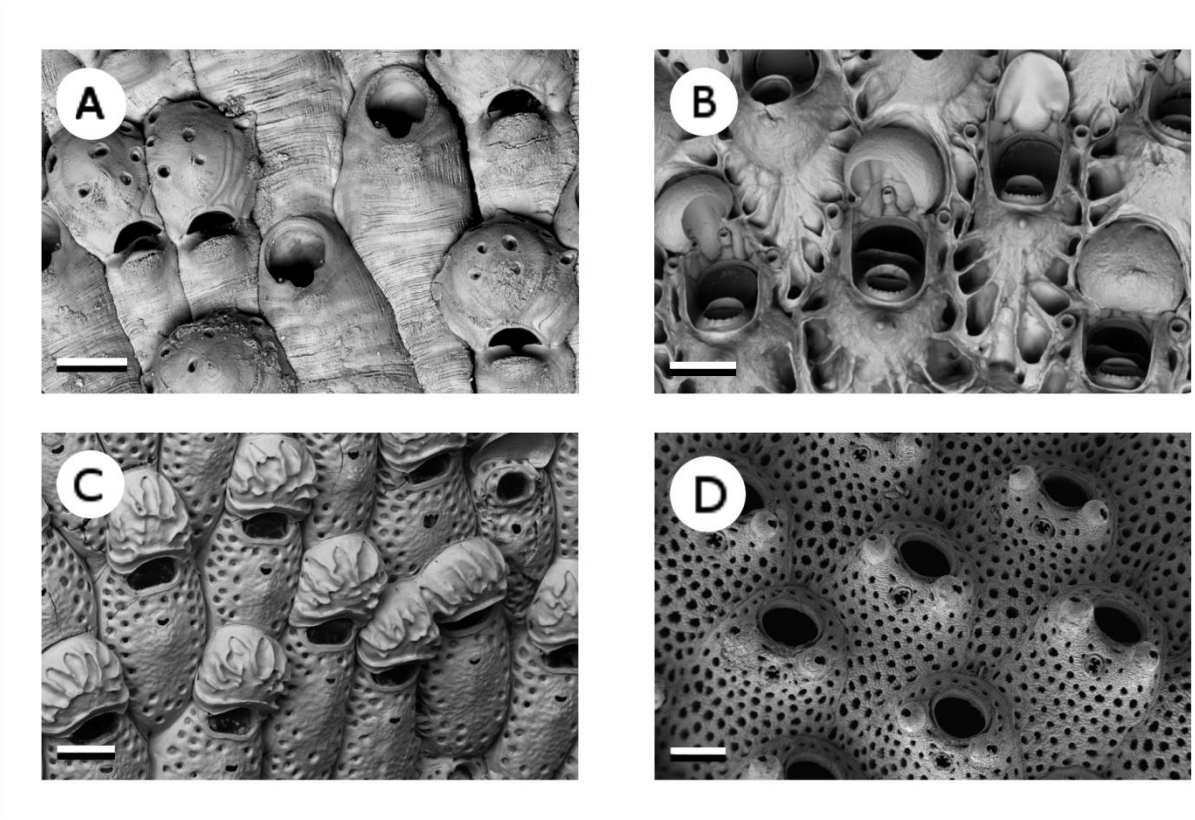


Figure 5.5: Scanning Electron Microscope images of the study species. A = Antarctothoa antarctica, scale = 200µm, B = Hippadenella inerma, scale = 100µm. C= Fenestrulina rugula, scale = 100µm. D= Inversiula nutrix, scale = 200µm

Detailed growth-rate data are available for *A. antarctica* and *F. rugula* as measured by Bowden (2005) from 2001-2003 at Rothera Research Station in Ryder Bay. These indicate that during the peak growing months of the austral summer the species are growing at 0.45mm/month and 0.66mm/month respectively. At this growth-rate the 2mm extracted sample would have been deposited in the 3-5 months preceding collection. No growth-rate data are available for *H. inerma* or *I. nutrix* and so this rate of deposition plus a 50% margin for error is taken as the approximation of the time period of deposition for Antarctic species (i.e. six months). Temperature for this predicted deposition period was extracted from the BAS long-term logger dataset collected from Cheshire (Site 5).

The species selected for analysis from Orkney were the cheilostomatous bryozoans, *Microporella ciliata, sensu strictu* (Pallas, 1766), *Membraniporella nitida* (Johnston, 1838), and *Escharella immersa* (Fleming, 1828). The study species were selected on the

basis of being well-known, common species with distinctive features for identification (Fig. 5.6). Specimens were identified to species level under a dissection (stereo) microscope using the monographs of Hayward & Ryland (1998;1999) and the re-description of *Microporella ciliata* by Kuklinski & Taylor (2008). Scanning electron microscope (SEM) images of each species were taken on a small selection of specimens in order to confirm identification (Fig. 5.6).

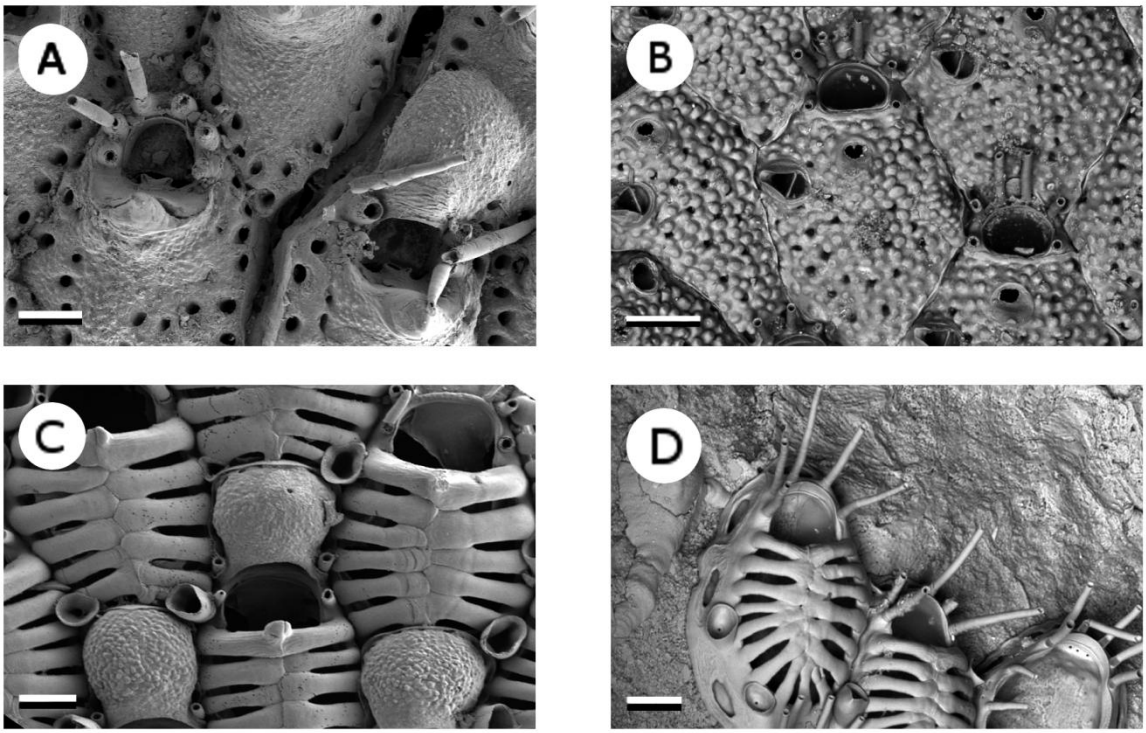


Figure 5.6: Scanning Electron Microscope images of lightly bleached specimens of the study species. A = Escharella immersa, scale = 100µm, B = Microporella ciliata, scale = 100µm. C and D= Membraniporella nitida, scale = 100µm. D shows early ontogeny

All specimens were alive when collected and were subsequently rinsed in fresh water and air-dried for a minimum of one month before samples were extracted for analysis. Individual colonies of similar diameter were selected for sampling and samples were extracted from the growing edge of the colonies, containing the most recently deposited skeletal material (in the last 6 months); a minimum of 5 zooids was extracted for each sample (approximately the outermost 2mm from the growing edge of the colony). As far as possible care was taken to ensure that no substrate (e.g. coralline algae) or epibionts were included within the sample as they could potentially contaminate results through their added mineralogies.

Voucher specimens and extracted skeletal material have been lodged in the Bryozoa collections of the Natural History Museum, London (NHM) under the following registration numbers: Antarctic material - NHMUK 2013. 10.30.1-928; Orcadian material - NHMUK 2014.1.10.261-331, 2014.1.10.332-422, 2014.1.10.575-634, 2014.1.10.683-725 and 2014.1.10.727-813.

5.4.3 Analysis techniques

Mineralogical analyses were conducted at the NHM, London following the protocol described in Section 2.4.3.

Where required to confirm identification SEM was undertaken with a low-vacuum instrument (LEO 1455- VP) capable of imaging large, uncoated specimens using back-scattered electrons. Specimens were bleached (10% domestic bleach) in an ultra-sonic bath, rinsed, dried and carbon mounted on aluminium stubs prior to imaging.

5.4.4 Data sources, processing and presentation

Distribution data of Scottish species were downloaded from Ocean Biogeographic Information System (OBIS) (2010) with 897 records extracted for the three species (*Membraniporella nitida*, n=25, *Microporella ciliata*, n=584, *E. immersa*, n=288). Distribution data for the four Antarctic species were downloaded from SCAR-MarBIN Portal, a regional OBIS node for the Antarctic (De Broyer & Danis 2013) with 134 records extracted for the four species (*H. inerma*, n=18, *A. antarctica*, n=35, *F. rugula*, n=20, *I. nutrix*, n=61). Only data points identified to species level from scientifically reliable sources have been included. A species distribution map for the UK was prepared using ArcGIS geographical information system (ESRI 2011) .

STATISTICS AND DATA ANALYSIS

All mineralogical measurements underwent transformation and were tested for normality and equal variance and subsequently non-parametric statistical analysis was conducted. Statistical methods are described in Section 3.4.4.

5.5 Results

In this study the wt% $MgCO_3$ in calcite of 584 Antarctic and 480 Orcadian specimens was quantified. This study represents the largest multi-replicated dataset generated for any Bryozoa to date.

5.5.1 Antarctic bryozoans

Two of the four species, *F. rugula* (n = 190) and *H. inerma* (n = 145), were found to have intermediate Mg-calcite (IMC, 4 - 8 wt% $MgCO_3$ in calcite) with mean wt% $MgCO_3$ in calcite (+/- standard deviation, SD) of 4.92 (+/- 0.772) and 5.02(+/- 0.679) respectively. The remaining species, *A. antarctica* (n = 159) and *I. nutrix* (n = 90), were found to have a low Mg-calcite (LMC, 2 - 4 wt% $MgCO_3$ in calcite) skeleton with mean wt% (+/- SD) of 0.60 (+/- 0.531) and 2.64 (+/- 0.257) respectively (Fig. 5.7).

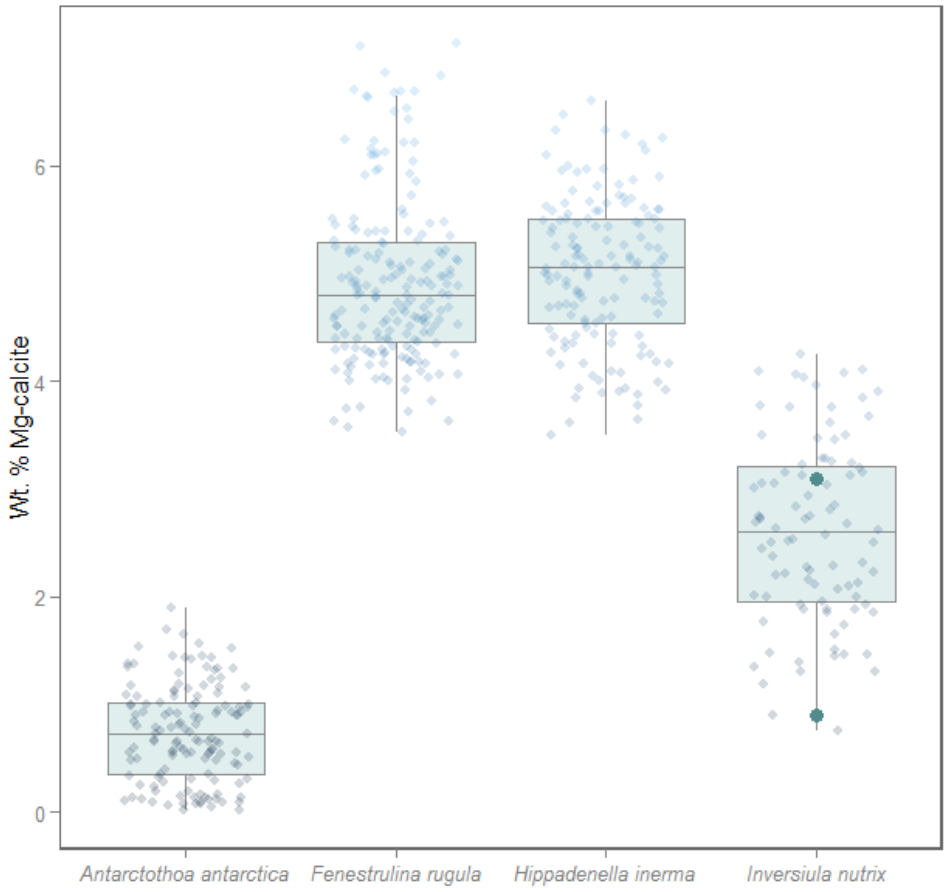


Figure 5.7 Box plots showing wt% $MgCO_3$ in calcite for Antarctic species. Box shows standard deviation around mean. Tail indicates range. Scatterplot indicates spread of samples. Previous analyses by Taylor et al. (2009) are indicated with dark circles. Wt. % Mg-calcite refers to wt% $MgCO_3$ in calcite.

Two of the species were found to be calcite dominated bimineralisers, with low levels of aragonite present. *F. rugula* was found to contain low levels of aragonite ranging between 0-14.5% (mean = 2.53%, stdev = 3.39%) of total calcium carbonate present. *I. nutrix* was likewise found to contain trace levels of aragonite ranging between 0-3.27% (mean = 0.69%, stdev = 1.07%) of total calcium carbonate present. The remaining species, *H. inerma* and *A. antarctica* were found to be entirely calcitic. ANOVA analysis shows there to be a statistically significant difference in wt% $MgCO_3$ in calcite between species (Kruskall Wallis: $\chi^2 = 418.694$, $p < 0.001$). Tukey *posthoc* testing reveals this to be the case for all species ($p<0.001$) with the exception of *F. rugula* and *H. inerma* which show no statistically significant difference to each other ($p=0.553$). The similarity between *F. rugula* and *H. inerma* can be seen in Figure 5.8.

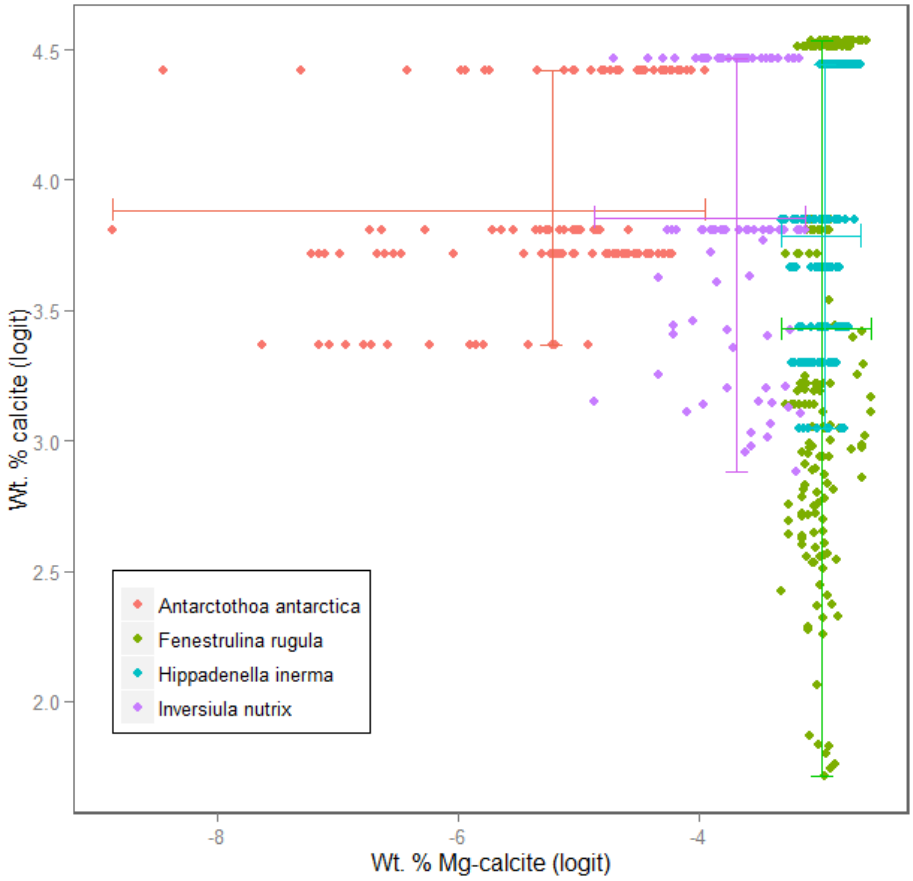


Figure 5.8: Scatterplot showing the range and spread of specimens around the mean wt% $MgCO_3$ in calcite (logit) and wt% calcite (logit). Wt. % Mg-calcite refers to wt% $MgCO_3$ in calcite.

MINERALOGICAL VARIABILITY

Local sites (<10km apart): Data were collected for three species, *F. rugula*, *A. antarctica* and *H. inerma*, from six sites less than 10km apart (1, 2a, 3, 4, 5, 6). Results of a Kruskall Wallis one-way ANOVA analysis showed that the mean wt% $MgCO_3$ in calcite was different between sites for all three species (Tab. 5.2).

Table 5.2: Kruskall Wallis one-way ANOVA results testing the null hypothesis that there is no difference in wt% $MgCO_3$ in calcite for samples of a single species collected from different sites. All data were logit-transformed prior to analysis.

H_0 There is no difference in Mg-calcite (logit) for samples of a single species collected from different sites					
Species	DF	χ2	p-value	significance	H_0 rejected?
Fenestrulina rugula	6	111.38	<0.0001	****	☑
Antarctothoa antarctica	6	50.4299	<0.0001	****	☑
Hippadenella inerma	6	56.9467	<0.0001	****	☑
Inversiula nutrix	2	4.6675	0.097		☒

Posthoc Mann-Whitney *U* tests show that wt% $MgCO_3$ in calcite was statistically significantly different among sites in 17 out of 30 comparisons (Tab. 5.3).

Close proximity sites (<10m apart): Data were collected for four species, *F. rugula, A. antarctica, H. inerma* and *I. nutrix* at three sites less than 10m apart (2a, 2b, 2c). Results of the ANOVA analysis show that the mean wt% $MgCO_3$ in calcite was different among close proximity sites for the species *F. rugula* (Kruskall Wallis: $\chi^2 = 26.5767$, $p<0.001$) and *A. antarctica* (Kruskall Wallis: $\chi^2 = 20.2055$, $p<0.001$). Posthoc Mann-Whitney U tests indicated that mean wt% $MgCO_3$ in calcite from each site was statistically different only for *F. rugula* (sites 2a&2c and 2b&2c) and *A. antarctica* (2a&2b and 2a&2c,) as represented in Table 5.3. There was no statistically significant difference in mean wt% $MgCO_3$ in calcite among close proximity sites for *H. inerma* or *I. nutrix.*

Wt% $MgCO_3$ in calcite variability among species and all sites is illustrated in Figure 5.9. Considering variability across all scales, Site 1, Killingbeck Island, was the most mineralogically distinct, with wt% $MgCO_3$ in calcite differing statistically in 17 of the 18 species/site combinations (Tab. 5.3); the only exception being *A. antarctica* and site 5, Cheshire Island. Site 5, Cheshire Island was the next most mineralogically distinct site with wt% $MgCO_3$ in calcite differing significantly from its neighbours in 10/12 species/site combinations. The least mineralogically distinct site was site 4, Anchorage Island, with

wt% $MgCO_3$ in calcite which differed significantly in only 4 out of 15 species/site combinations.

Table 5.3: Results of posthoc Mann-Whitney U tests comparing different sites. Presence of symbol indicates significance. Dash indicates no measurement.

Site	1	2a	2b	2c	3	4	5	6
1		● ○ ■ -	● ○ ■ -	● ○ ■ -	● ○ ■ -	● ○ ■ -	● - -	- - ■ -
2a			○	● ○	-	-	● ○ - -	- - -
2b	*Fenestrulina rugula* = ●			●	○ -	-	● ○ - -	- - -
2c	*Antarctothoa antarctica* = ○				● ○ -	● -	○ - -	- - -
3	*Hippadenella inerma* = ■					-	● ○ - -	- - -
4	*Inversiula nutrix* = □						● ○ - -	- - -

The most mineralogically distinct species among sites were *F. rugula* and *A. antarctica* which both differed statistically in 14 out of 21 site combinations. *H. inerma*, in contrast, showed a statistically distinct mineralogy in just 6 of the 21 site combinations.

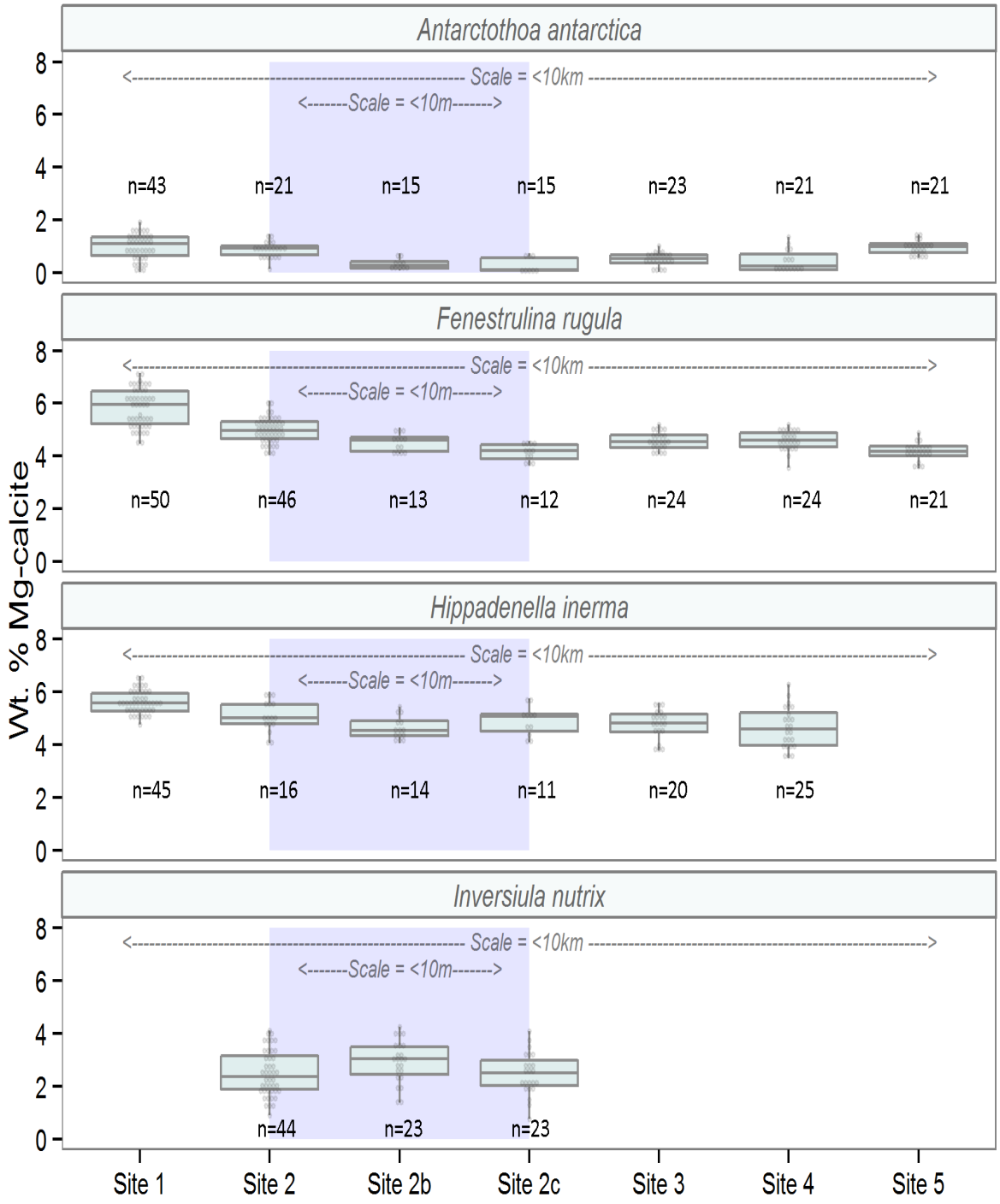


Figure 5.9: Box plots showing wt% $MgCO_3$ in calcite for Antarctic species at different sites. Box shows standard deviation around mean, tail indicates range. Shaded area indicates close proximity sites (< 10m apart). Wt. % Mg-calcite refers to wt% $MgCO_3$ in calcite.

SPECIES DISTRIBUTION

Species distributional data are limited for Antarctica with between 18 and 61 records each for the species studied here and collection being biased towards the permanent Antarctic research bases and well-studied regions. The data that are available indicate that *H. inerma* is the most wide ranging species with a latitudinal distribution of 56.7 latº (21.2S – 77.8S), this is followed by *A. antarctica* (23.5 latº), *I. nutrix* (17.1 latº) and finally *F. rugula* with a range of just 12.8 latº (Fig. 5.10).

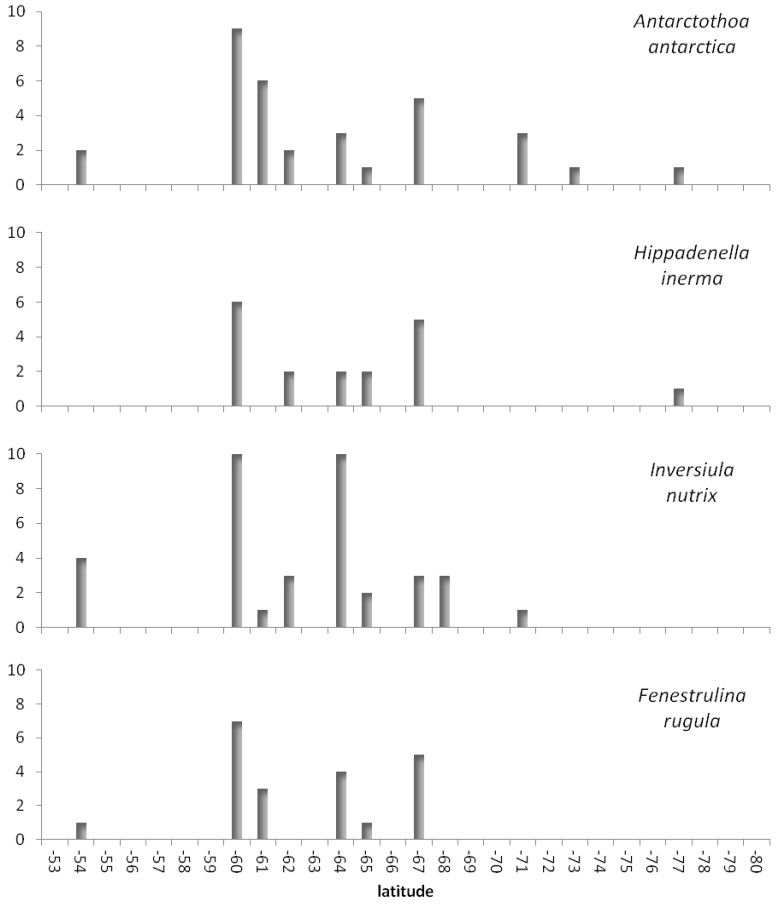


Figure 5.10: Species latitudinal distribution data for Antarctica study species.

ENVIRONMENTAL ANALYSIS

Temperature data measured during sample collection at the sites were compared to data from the same date and time (+/- 2hours) extracted from the BAS long-term logger dataset at Cheshire Island. Overall the long-term data were found to be statistically different from collected data (GLM ANOVA: $F = 14.93$, $p<0.001$). *Posthoc* analysis, however, shows no statistical difference between the BAS long-term logger data and collected data for site 5,

Cheshire Island (Tukey's test: difference of means (DOM) = 0.038, p=0.999) and site 1, Killingbeck (Tukey's test: DOM = - 0.038, p=0.999). The comparison between temperature measured during sample collection (Tab. 5.4) and the BAS long-term logger data at site 5, Cheshire Island, enabled "ground truthing" of the data collected. As the study loggers datasets and the BAS long term logger at Cheshire Island were not statistically different, a high degree of accuracy in the HOBO loggers is indicated.

Temperature measured during sample collection was found to be statistically different among sites (GLM ANOVA: F = 36.48, p<0.001). *Posthoc* tests illustrate that sites are statistically similar in only 2/10 cases (Tukey's test: sites 2a to 3, p=0.469; sites 1 and 5, p=0.892). No statistically significant relationship was detected between wt% $MgCO_3$ in calcite and salinity for any of the study species.

Table 5.4: Physical and environmental characteristics for study sites at the time of collection.

	Site 1	***Site 2a***	***Site 2b***	***Site 2c***	***Site 3***	***Site 4***	***Site 5***	***Site 6***
	Killingbeck		*Lagoon Back bay*		*East Beach*	*Anchorage*	*Cheshire*	*Honeybucket*
date	31/01/2012		24/01/2012		30/01/2012	08/02/2012	28/01/2012	18/01/2012
time (HH:MM)	13:00		18:00		20:00	13:00	15:00	
temperature (°C)	-0.0675	0.295	0.207	0.155	0.299	0.0925	-0.146	0.431
salinity (psu)	31		31.4		30.93	30.52	31.33	-
depth (m)	10.5	6	7	7.5	10	11	10	8

The only statistically significant correlations between environmental conditions and wt% $MgCO_3$ in calcite for the Antarctic bryozoan species tested are negative correlations for *H. inerma* (Kendall's correlation: p<0.001, Kendall's tau = -0.322) and *A. antarctica* (Kendall's correlation: p<0.001, Kendall's tau = -0.235). For these two species the wt% $MgCO_3$ in calcite decreases as the sea-water temperature increases.

During the estimated period of skeleton deposition the BAS long-term logger data shows the sea-water temperature at Cheshire (site 5) ranged though 2.47ºC (minimum = -1.80ºC, maximum = 0.67ºC), with the midday mean fluctuating day-to-day by up to 0.95ºC (Fig. 5.11). Between August 2011 and January 2012 the mean day-to-day sea-water temperature range was 0.09ºC.

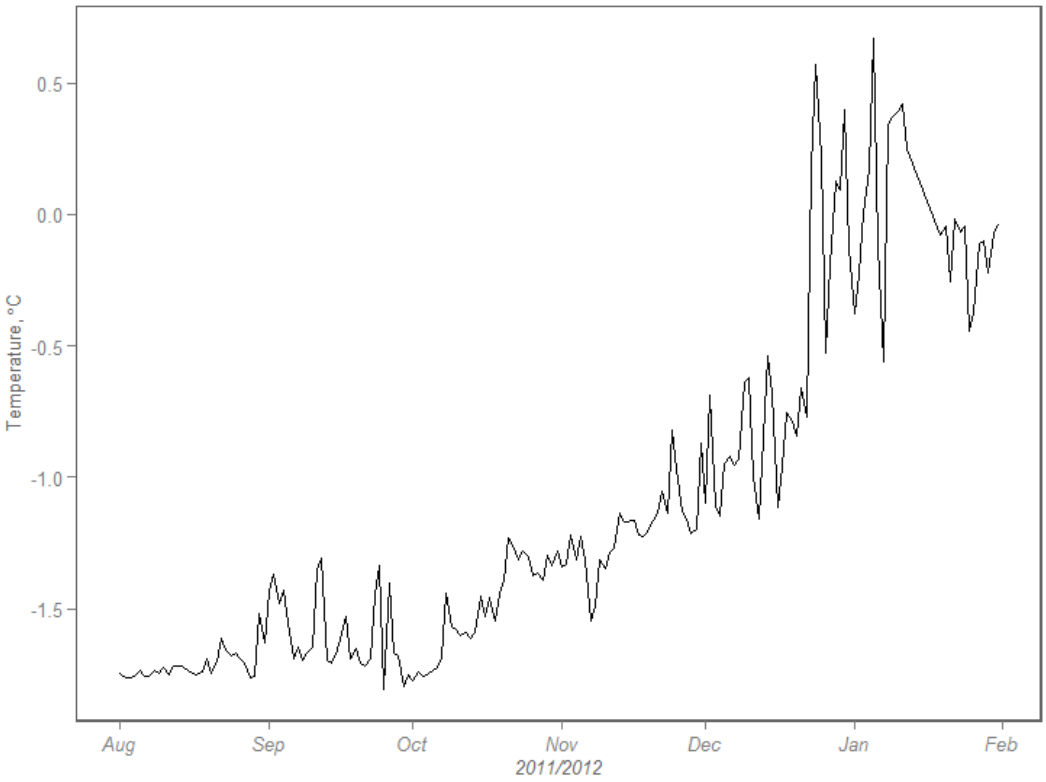


Figure 5.11: Graph showing mean midday temperature at Site 5, Cheshire during the predicted skeletal deposition period preceding collection. Long-term logger data collected by the British Antarctic Survey as part of the RaTS dataset (Clarke et al. 2008)

5.5.2 Orcadian bryozoans

In this study the wt% $MgCO_3$ in calcite and wt% aragonite of 480 specimens of three bryozoan species was quantified. All of the species, *Membraniporella nitida* (n=139), *Microporella ciliata* (n=145), and *E. immersa* (n=146), were found have intermediate Mg-calcite (IMC, 4-8 wt% $MgCO_3$ in calcite) with mean wt% $MgCO_3$ in calcite (+/- standard deviation, SD) of 6.25 (+/- 0.648), 6.88 (+/- 0.638) and 5.66 (+/- 0.612) respectively (Fig. 5.12).

One of the species, *Membraniporella nitida*, was found to be entirely calcitic. *Microporella ciliata* and *E. immersa* were both found to be calcite dominated bimineralisers with varying volumes of aragonite present. *Microporella ciliata* was found to contain levels of aragonite ranging between 1 and 66wt% (mean = 22.7wt% aragonite, SD +/- 18.96%) of total calcium carbonate present. *E. immersa* was, likewise, found to contain levels of aragonite

ranging between 1 and 75wt% (mean = 31wt% aragonite, SD +/- 18.69 wt%) of total calcium carbonate present.

ANOVA analysis shows a statistically significant difference among species in wt% $MgCO_3$ in calcite (wt% $MgCO_3$ in calcite: $p<0.001$, $F=134.21$) and proportion of aragonite (wt% aragonite: $p<0.001$, $F=143.3$). Tukey *posthoc* testing reveals this to be the case for both wt% $MgCO_3$ in calcite and wt% aragonite for all species ($p<0.001$ in all cases).

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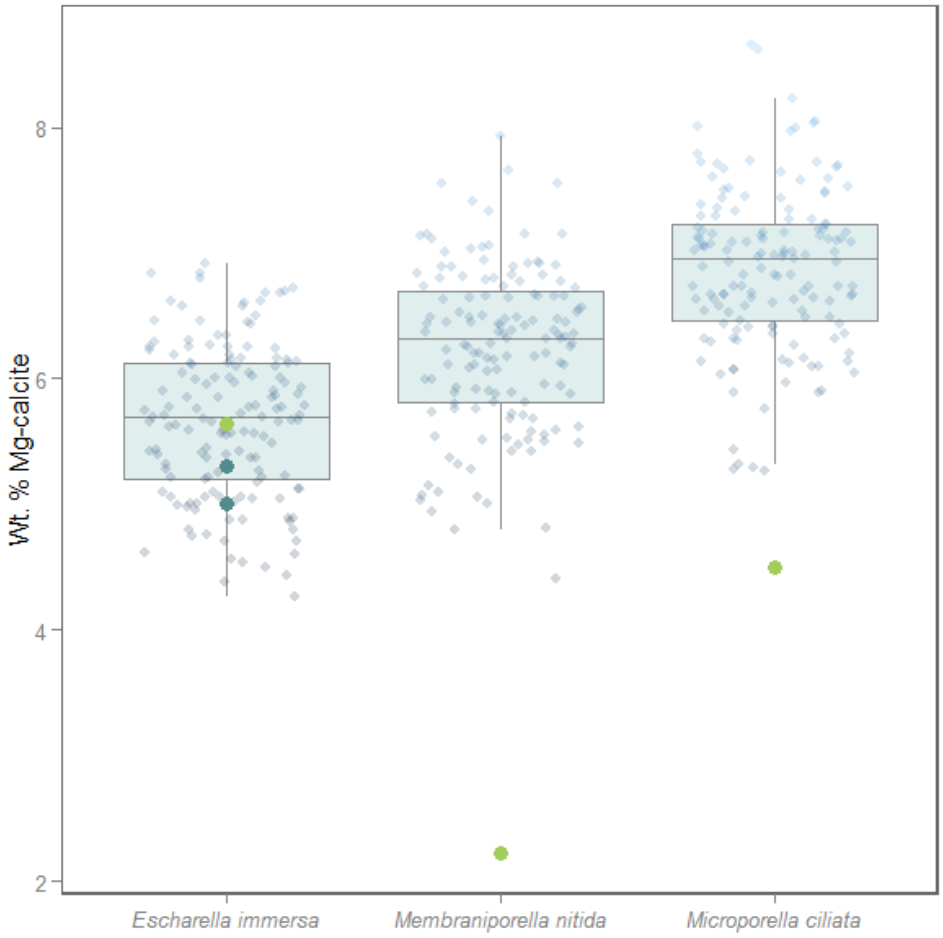

Figure 5.12: Box plots showing wt% $MgCO_3$ in calcite for Orcadian species. Box shows standard deviation around mean. Tail indicates range. Previous analyses by Taylor et al. (2009) are indicated with dark circle, previous analyses from earlier chapters of this thesis are indicated with green circles. Wt. % Mg-calcite refers to wt% $MgCO_3$ in calcite.

The combined wt% $MgCO_3$ in calcite and wt% calcite, or the "mineralogical space" a species occupies, gives each species a "mineralogical signature" as can be seen in Fig. 5.13.

Discriminant Analysis shows that an unknown individual from Orkney could be assigned to "species" from analysis of wt% $MgCO_3$ in calcite and wt% calcite alone with an accuracy of over 76% (*E. immersa* 72.6% correctly assigned, *Microporella ciliata*, 64.1% correctly assigned, *Membraniporella nitida*, 92.1% correctly assigned).

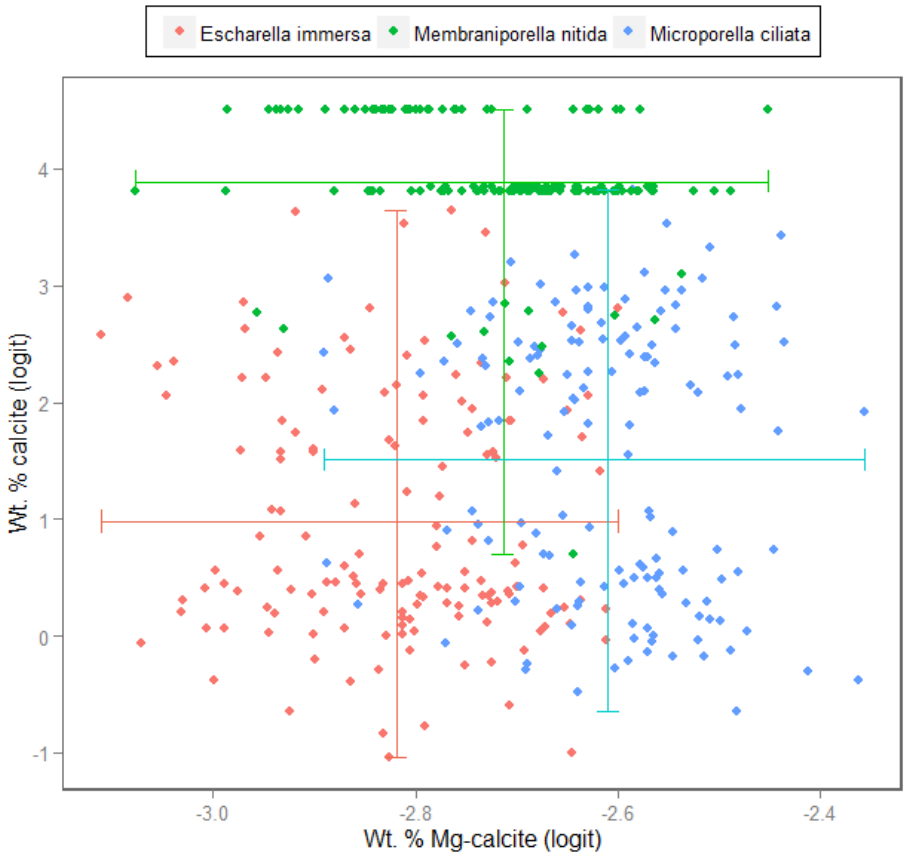

Figure 5.13: Scatter plot showing the "mineralogical space" occupied by each species. Error bars indicate range. Wt. % Mg-calcite refers to wt% $MgCO_3$ in calcite.

MINERALOGICAL VARIABILITY

Data were collected for three species, *Membraniporella nitida*, *Microporella ciliata* and *E. immersa*, at five sites less than 12km apart. Results of a Kruskall Wallis one-way ANOVA analysis showed that the mean skeletal wt% $MgCO_3$ in calcite was different among sites for all three species (Tab. 5.5).

Posthoc Mann-Whitney *U* tests show that wt% $MgCO_3$ in calcite was statistically significantly different among sites in 18 out of 30 cases (Tab. 5.6).

Table 5.5: Kruskall Wallis one-way ANOVA results testing the null hypothesis that there is no difference in wt% $MgCO_3$ in calcite for samples of a single species collected from different sites. All data were logit-transformed prior to analysis.

Species	DF	χ2	p-value
Membraniporella nitida	4	31.685	<0.0001
Microporella ciliata	4	21.2206	0.0003
Escharella immersa	4	43.6428	<0.0001

Table 5.6: Results of Mann-Whitney posthoc analysis comparing wt% $MgCO_3$ in calcite among different sites. Significant differences among sites are indicated by the presence of the following symbols: Membraniporella nitida = filled circles, Microporella ciliata = empty circles, Escharella immersa = filled squares.

Site	1	2	3	4	5
1		○ ■	● ○ ■	● ○	● ○
2			● ○ ■	● ○	● ■
3				■	
4					■
5					

Kruskall Wallis one-way ANOVA analysis also shows that the mean wt% aragonite in skeletons is different among sites for all species (Tab. 5.7).

Table 5.7: Kruskall Wallis one-way ANOVA results testing difference in wt% aragonite for samples of a single species collected from difference sites. All data were logit-transformed prior to analysis.

Species	DF	χ2	p-value
Membraniporella nitida	4	75.4892	<0.0001
Microporella ciliata	4	13.1659	0.0105
Escharella immersa	4	23.8135	<0.0001

Posthoc Mann-Whitney *U* tests shows that the mean skeletal wt% aragonite was statistically significantly different among sites in 18 out of 30 cases, exactly the same as the variability seen for wt% $MgCO_3$ in calcite (Tab. 5.8).

Table 5.8: Results of Mann-Whitney posthoc analysis comparing Wt% aragonite among different sites. Significant differences among sites are indicated by the presence of the following symbols: Membraniporella nitida = filled circles, Microporella ciliata = empty circles, Escharella immersa = filled squares.

Site	1	2	3	4	5
1		● ○	● ○ ■	● ■	● ■
2			● ○ ■	● ■	○ ■
3				○	
4					○
5					

Examination of the Mann-Whitney *posthoc* analysis of both skeletal wt% $MgCO_3$ in calcite and wt% aragonite reveals that sites 1 and 2, South Cava Island and Holm of Houghton, are the most mineralogically distinct of all the study sites, differing statistically from 9 of the 12 species/site combinations. The least mineralogically distinct for both measures of skeletal mineralogy is site 5, Barrel of Butter, which differed significantly in only 5 out of 12 species/site combinations.

Variability in skeletal wt% $MgCO_3$ in calcite and wt% aragonite among species and sites can be seen in Figure 5.14. All species show the same skeletal mineralogical distinctness among sites, differing statistically in 50% (6/12) of sites. Although overall mineralogical distinctness is the same for wt% aragonite and wt% $MgCO_3$ in calcite, the species which are distinct among sites are different for the two mineralogical measurements.

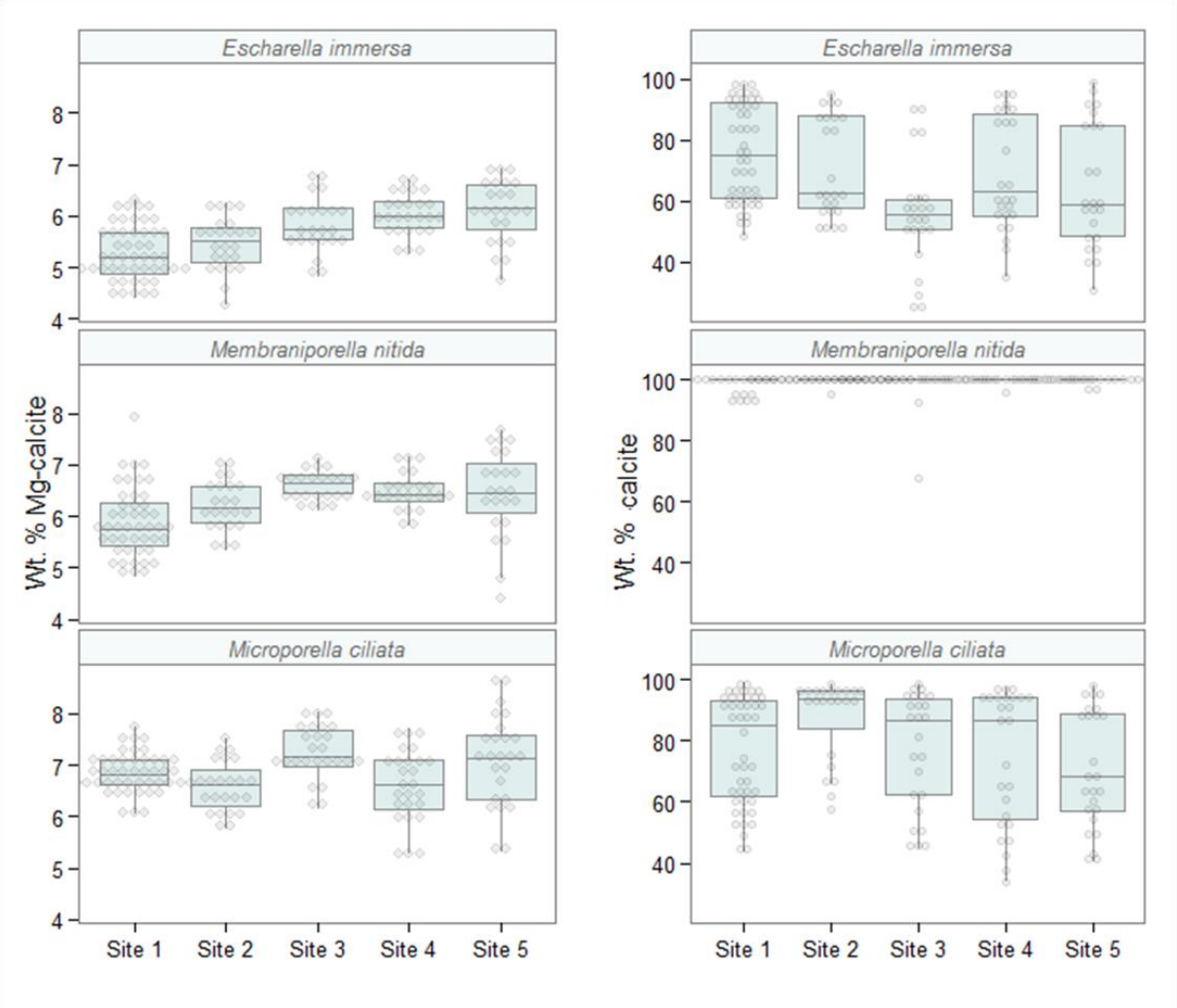

Figure 5.14: Box-plots showing the distribution of wt% $MgCO_3$ in calcite (left) and calcite (right). Boxplots show mean (line, standard deviation around mean (box) and range (tails). Dot-plot shows distribution of data points. Wt. % Mg-calcite refers to wt% $MgCO_3$ in calcite.

SPECIES DISTRIBUTION

Species distributional data are of varying volumes for the Orcadian species with between 25 and 584 records each for the species studied here (*E. immersa*, n=288, *Membraniporella nitida*, n=25, *Microporella ciliata*, n=584). The data indicate that *Microporella ciliata* is the most wide ranging species with a latitudinal distribution of 33.2 latº (39.9N – 76.1N), this is followed by *E. immersa* (12.2 latº) and *Membraniporella nitida* with a range of just 10.3 latº.

ENVIRONMENTAL ANALYSIS

The long term data series show no statistical difference (ANOVA) between 6m and 12m depths (Fig. 5.15).

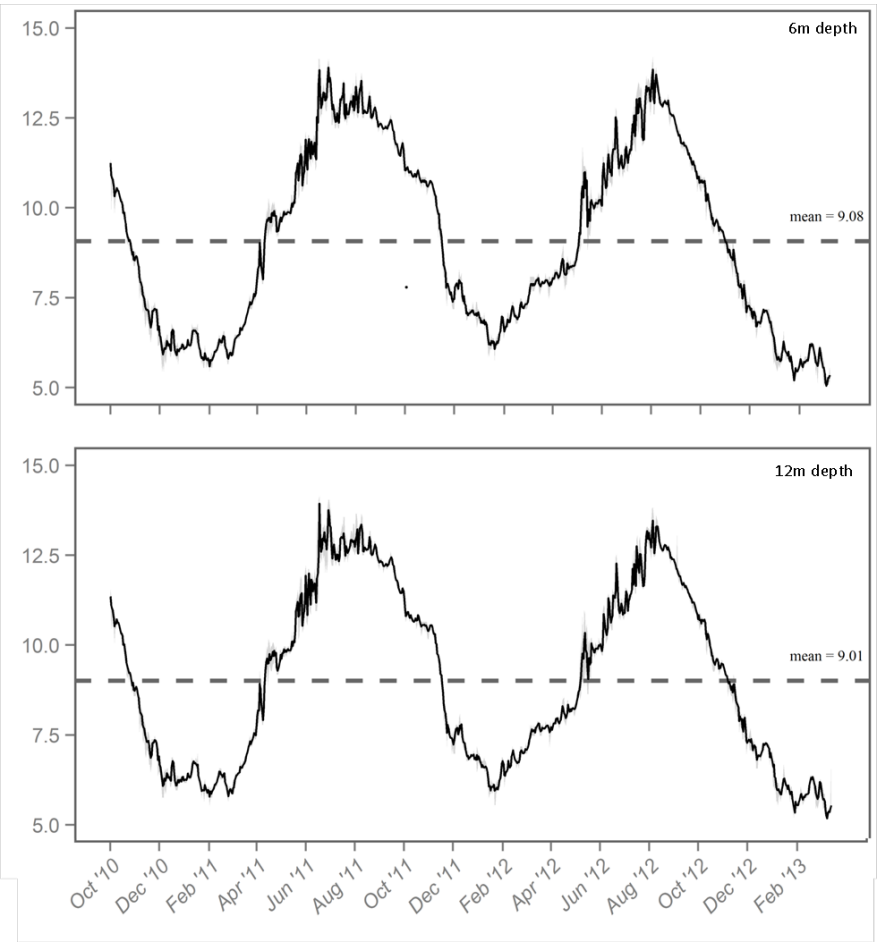

Figure 5.15: Benthic seawater temperature in Scapa Flow at 6m (top) and 12m (bottom) depths. Data was collected between October 2010 and March 2013. Black line indicates mean midday temperature; grey shading shows daily maximum and minimum temperatures. Multi-year mean is indicated with a dashed line. Data taken from Chapter 4.

Temperature data measured during sample collection at the sites were compared to data from the same date and time (+/- 2hours) extracted from the long-term data series at 12m depth. The site data were found to deviate from the long-term data by a mean of just 0.12 ºC (maximum deviation = 0.21ºC, minimum deviation = 0.05ºC) indicating a high degree of temperature homogeneity within Scapa Flow

Environmental measurements taken from the individual sites also show little seawater temperature variability among sites (mean = 8.23ºC, SD +/- 0.07ºC) with only slightly higher variability in salinity (mean = 33.01psu, SD +/- 0.49psu) (Tab. 5.9).

Table 5.9: Physical and environmental characteristics for study sites at the time of collection.

	Site 1 *South Cava*	***Site 2*** *Houton*	***Site 3*** *Flotta*	***Site 4*** *Rysa Little*	***Site 5*** *Barrel of Butter*
date	03/05/2012	09/05/2012	10/05/2012	06/05/2012	03/05/2012
temperature (°C)	8.222	8.274	8.334	8.19	8.1475
salinity (psu)	33.49	32.41	32.59	33.17	33.40
depth (m)	10.5	6	10	11	10
Total alkalinity (TA)	2288.2	2293.3	2086.9	2290.4	3685.3
Dissolved inorganic carbon (DIC)	2129.2	2096.3	2086.9	2085.5	3713.5
pH	8.036	8.137	8.186	8.146	7.518
CO_3	119.5	143.5	159.8	147.6	64.2
W ca	2.87	3.46	3.85	3.55	1.54
W ar	1.81	2.19	2.43	2.24	0.97

The skeletal composition of *E. immersa* shows a statistically significant negative correlation between site temperature and wt% $MgCO_3$ in calcite and positive correlations between site salinity and total alkalinity (TA) with wt% $MgCO_3$ in calcite (Tab. 5.10). The skeletal composition of *Membraniporella nitida* shows a negative correlation between site salinity and wt% $MgCO_3$ in calcite and positive correlations between pH, CO_3^{2-} and $\Omega_{Calcite}$ with wt% $MgCO_3$ in calcite (Tab. 5.10).

Table 5.10: Results of Kendall's correlation analysis comparing wt% $MgCO_3$ in calcite and wt% aragonite values for samples of single species to site environmental variables (Tab. 5.9). All mineralogy data were logit-transformed prior to analysis. X indicates no statistically significant correlation detected.

		Wt. % Mg-calcite			Wt. % aragonite		
		E.immersa	*M.nitida*	*M.ciliata*	*E.immersa*	*M.nitida*	*M.ciliata*
temperature	*p* :	0.002	x	x	x		0.018
	T :	-0.191					-0.146
depth	*p* :	x	x	x	x		0.034
	T :						0.131
salinity	*p* :	0.025	0.001	x	0.005		x
	T :	0.138	-2.095		-0.173		
total alkalinity (TA)	*p* :	0.007	x	x	x		x
	T :	0.166					
pH	*p* :	x	0.002	x	0.028		x
	T :		0.200		0.135		
CO_3^{2-}	*p* :	x	0.002	x	0.028		x
	T :		0.200		0.135		
Ω calcite	*p* :	x	0.002	x	0.028		x
	T :		0.200		0.135		

p = p-value; T = Kendall's tau

The skeletal composition of *E. immersa* shows a negative correlation between wt% aragonite and site salinity and positive correlations between wt% aragonite and pH, CO_3^{2-} and $\Omega_{Calcite}$ (Tab. 5.10). As salinity decreases, and pH, CO_3^{2-} and $\Omega_{Calcite}$ increase, there is an increase in wt% skeletal aragonite (and a corresponding decrease in wt% calcite). The skeletal composition of *Microporella ciliata* shows weak statistically significant correlations between wt% aragonite and site temperature and depth. The wt% aragonite value decreases (and calcite increases) with temperature; wt% aragonite increases (and calcite decreases) with increasing depth (Tab. 5.10).

5.5.3 Antarctica vs. Scotland

COMPARATIVE MINERALOGY

Wt% $MgCO_3$ in calcite for Antarctic species (across all sites) is statistically different to the wt% $MgCO_3$ in calcite for UK species (Kruskall-Wallis, χ^2=531.3048, $p<0.001$). All species are statistically different to each other (χ^2=777.5061, $p<0.001$, Mann-Whitney U-test $p<0.001$ for all cases). There is no statistical difference between the standard deviation (χ^2=0.7008, $p=0.403$) of wt% $MgCO_3$ in calcite of Antarctic and British species (Fig. 5.16).

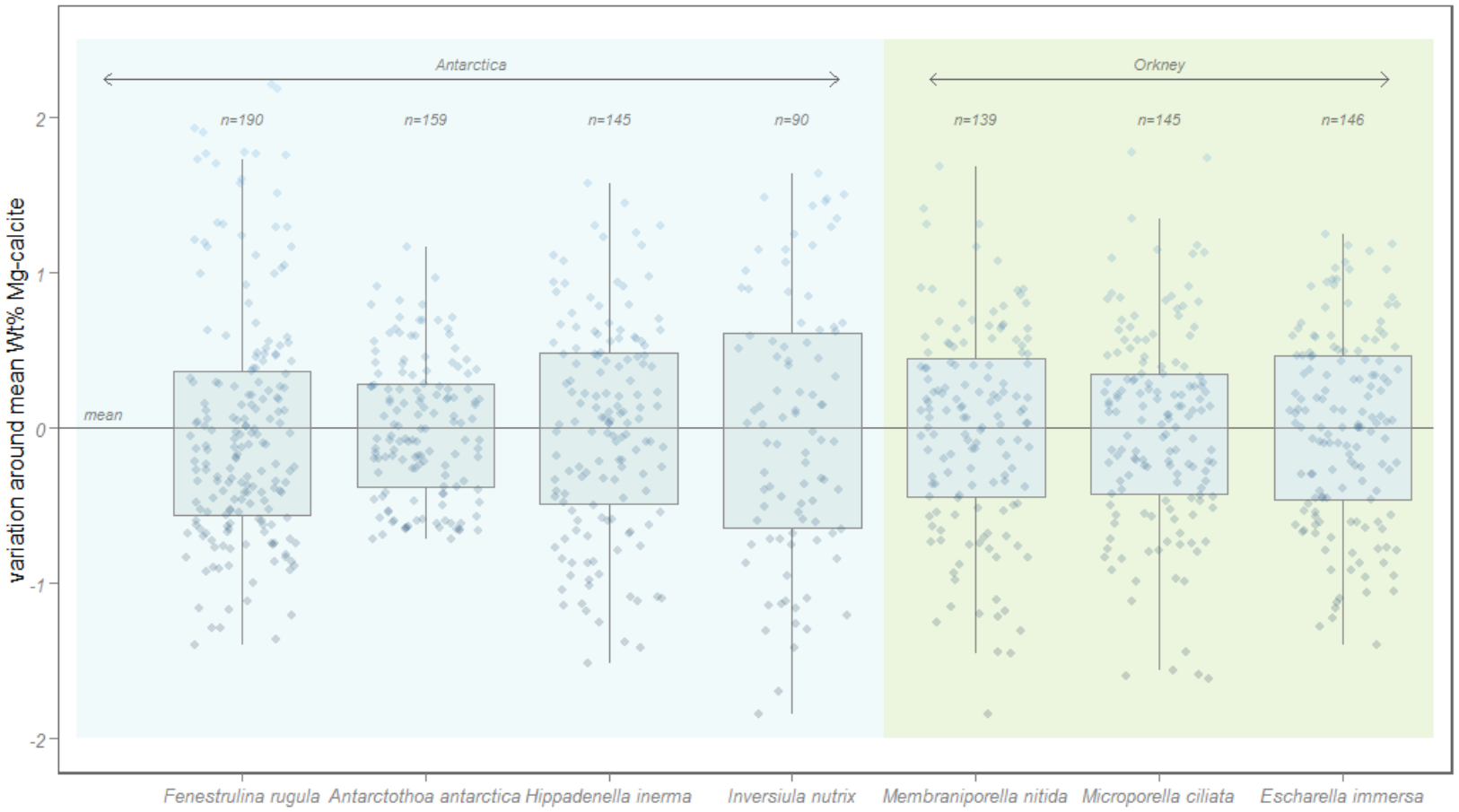

Figure 5.16: Box-plots showing the variation around the mean wt% $MgCO_3$ in calcite for Antarctic and Orcadian species. Boxplots show mean (line), standard deviation around mean (box) and range (tails). Dot-plot shows distribution of data points. Blue box indicates Antarctic species, green box indicates Orcadian species. Wt. % Mg-calcite refers to wt% $MgCO_3$ in calcite.

5.6 Discussion

The aim of this study was to conduct a high-replicate (>100 per species), multi-site study on the skeletal mineralogy of bryozoans from Antarctica (a stenothermal region) and Orkney (a eurythermal region). This aim was achieved through the analysis of 1014 specimens of seven species.

5.6.1 Species mineralogy

Two of the species studied here have been previously analysed for mineralogical content. Taylor et al (2009) analysed specimens of *I. nutrix* from the Antarctic and *E. immersa* from the UK. They reported that the two specimens of *I. nutrix* which they analysed from King George Island had LMC (0.9 and 3.1 wt% $MgCO_3$ in calcite) which concurs with the low Mg-calcite categorisation of the results presented here. Previous analyses of *E. immersa* found the two specimens analysed from the Isle of Man and Dyfed in Wales to be IMC (5 and 5.3 wt% $MgCO_3$ in calcite respectively) and bimineralic (15 and 9 Wt% aragonite respectively). Analysis in a previous Chapter (3) of this thesis, found *E. immersa* from St Abbs to be IMC and bimineralic (5.64 wt% $MgCO_3$ in calcite and 13.65% wt% calcite). Again this concurs with the categorisation of the specimens examined in this study and all fall within the range of specimens analysed. These results have been gathered by different researchers at different times with similar findings indicating that the method used in this study is reasonably robust.

The remaining Orcadian species, *Microporella ciliata* and *Membraniporella nitida*, have both been previously analysed (Chapter 3). The results presented in this study are higher than the previously analysed specimens (Fig. 5.12). A specimen of *Microporella ciliata* (collected from 12m Eday Sound, Orkney 5/9/2011, 14°C) showed a wt% $MgCO_3$ in calcite of 4.5, compared to this study's mean of 6.88. A specimen of *Membraniporella nitida* (collected from 12 m, St Abbs, 4/8/2011, 13°C) had wt% $MgCO_3$ in calcite of 2.22, compared to this study's means of 5.66. This indicates there may not be a strong relationship between temperature and wt% $MgCO_3$ in calcite in these species as, if data followed the proxy that wt% $MgCO_3$ in calcite increases with temperature, it would have been expected that the wt% $MgCO_3$ in calcite would be higher in the more southerly St Abbs specimen. It is more probable that the differences in measured wt% $MgCO_3$ in calcite

are attributable to morphological differences caused by age and associated damage to the specimens. Both species also have their highest settlement rates in Sept/Oct (Maughan 2000) and *Microporella ciliata* has a slow growth rate of 1mm per month (Maughan 2000). Specimens from Chapter 3 were therefore at least a year old when sampled. Both *Microporella ciliata* and *Membraniporella nitida* are morphologically complex, featuring, spines, ovicells and avicularia. It is possible that in older samples, some of these complex features had been lost or damaged through either wear and tear or grazing. This may in turn have impacted the resultant mean wt% $MgCO_3$ in calcite for the specimens analysed from Chapter 3. In contrast the specimens from this Chapter possibly settled in Sept/Oct 2011 and were relatively young "pristine" colonies with all their morphologically complex features intact. Localisation of $MgCO_3$ in the calcite of complex skeletal features will be discussed in detail in Chapter 6.

TAXONOMY/PHYLOGENY/FAD

Phylogenetic data are available for the Orcadian species, *Microporella ciliata* and *E. immersa* (Waeschenbach et al. 2012) and the Antarctic species, *A. antarctica* (Wright et al. 2008). If taxonomic stabilityis assumed to the Section level, then the additional phylogenetic groupings of the *Hippothoomorpha*, *Lepraliomorpha* and *Umbonulomorpha* sections can be used to review patterns of mineralogy. The species that have been phylogenetically characterized from these taxonomic Sections are well clustered in the published phylogeny (Waeschenbach et al. 2012) and it can be tested whether other species from these Sections also adhere to the mineralogy patterns discussed in Chapter 3 (Tab. 5.11). All *Hippothoomorpha* species from Chapter 3, for which both phylogeny and mineralogy are available, exhibit LMC and are calcite dominated. This pattern is continued here with the very low wt% $MgCO_3$ in calcite and 100% calcite shown by *A. antarctica*.

Previously analysed species (Chapter 3) from the Section *Lepraliomorpha* exclusively feature IMC and the majority feature bimineralic or aragonite dominated skeletons. This pattern for wt% $MgCO_3$ in calcite is continued for the *lepraliomorphs* analysed here; *H. inerma*, *F. rugula* and *Microporella ciliata* all feature IMC and both *F. rugula* and *Microporella ciliata* show some aragonite. Only three species of *Umbonulomorpha* have been analysed previously (Chapter 3) but all are characterized by a bimineralic skeleton and LMC/IMC.

Table 5.11: Table showing taxonomic relationships among study species and associated first appearance date (FAD) of the family, distributional range and mineralogy.

Section	Superfamily	Family	Genus	Species	family FAD	genera in family	species in genus	distributional range (lat °)			mean Mg-calcite	mean Wt% calcite
								family	genus	species		
Acanthostegomorpha	*Cribrilinoidea*	*Cribrilinidae* Hincks, 1879	*Membraniporella* Smitt, 1873	*nitida* (Johnston, 1838)	93.5 Ma	27	12	158.1	117	10.33	6.25	0
Hippothoomorpha	*Hippothooidea*	*Hippothoidae* Busk, 1859	*Antarctothoa* Moyano, 1987	*antarctica** Moyano & Gordon, 1980	85.8 Ma	7	15	159.1	32.6	23.5	0.6	0
Lepraliomorpha	*Schizoporelloidea*	*Buffonellodidae* Gordon & d'Hondt, 1997	*Hippadenella* Canu & Bassler, 1917	*inerma** (Calvet, 1909)	47.8 Ma	5	6	54.6	54.6	56.65	5.02	0
		Microporellidae Hincks, 1879	*Fenestrulina* Jullien, 1888	*rugula** Hayward & Ryland, 1990	20.5 Ma	10	60	159.1	138.4	12.8	4.92	2.53
			Microporella Hincks, 1877	*ciliata* (Pallas, 1766)	20.5 Ma	10	100	159.1	159.1	36.16	6.88	22.7
Umbonulomorpha	*Adeonoidea*	*Inversiulidae* Vigneaux, 1949	*Inversiula* Jullien, 1888	*nutrix** Jullien, 1888	28.1 Ma	1	4	53	53	17.05	2.64	0.69
	Lepralielloidea	*Romancheinidae* Jullien, 1888	*Escharella* Gray, 1848	*immersa* (Fleming, 1828)	71.3 Ma	19	42	159.7	159.7	12.21	5.66	31

* antarctic species

This trend is continued with *I. nutrix* and *E. immersa* which both feature some aragonite and IMC or LMC. No phylogenetic analysis of *Membraniporella nitida* or the *Acanthostegomorpha* section has been conducted to date.

Species mineralogy can also be compared with published patterns associated with bryozoan families. Four of the families examined here, *Cribrilinidae*, *Hippothoidae*, *Microporellidae* and *Romancheinidae*, have been previously analysed by Smith et al. (2006) or Taylor et al. (2009). *Cribrilinidae* and *Hippothoidae* are both reported to be calcitic which concurs with the data presented here.

The published family means for wt% $MgCO_3$ in calcite also closely correlate to the analysed means from this study. *Membraniporella nitida*, family *Cribrilinidae*, has a mean of 6.25 wt% $MgCO_3$ in calcite (published family mean = 6.9 wt% $MgCO_3$ in calcite (Smith et al. 2006), range = 4.5) while *A. antarctica*, family *Hippothoidae*, has an analysed mean of 0.6 wt% $MgCO_3$ in calcite (published family mean = 3 wt% $MgCO_3$ in calcite (Taylor et al. 2009), range = 7).

Microporellidae and *Romancheinidae* are reported as bimineralic or calcite dominated mix families (Taylor et al. 2009) which again concurs with the data showing all three species from these families have some aragonite. The two species from family *Microporellidae, F. rugula* and *Microporella ciliata* have wt% $MgCO_3$ in calcite of 4.92 and 6.88 respectively. This is close to the published family mean of 5.2 wt% $MgCO_3$ in calcite (range = 5.2) (Taylor et al. 2009). *E. immersa*, family *Romancheinidae*, features 5.66 wt% $MgCO_3$ in calcite, close to the published family mean of 7.5 wt% $MgCO_3$ in calcite and well within the family range (7). There is no detected relationship between family First Appearance Date (FAD) and mineralogy.

DISTRIBUTIONAL RANGE/ ENDEMISM / SPECIALIZATION.

The families, genera and species have widely varying distributional range as can be seen in Table 5.11. There is a statistically significant relationship between the midpoint of distributional range of the genera (in degrees from equator) and the mean wt% $MgCO_3$ in calcite of species (linear regression, p=0.018, F=12.14, R^2=0.65) which indicates that the region in which a genus has evolved may have some influence on wt% $MgCO_3$ in calcite. This pattern is reinforced at the species level if we look at some examples from the Antarctic.

A. antarctica, *F. rugula* and *I. nutrix* have the lowest wt% MgCO3 in calcite of the analysed species at 0.6, 4.9 and 2.6wt% respectively. They are also all endemic, i.e. only found within the Antarctic polar front (Barnes & Conlan 2007), and all have lower wt% $MgCO_3$ in calcite then their family means. Publications show that the family *Hippothoidae* has a broad distributional range of 159 lat^o with a midpoint at 1.6^{O}N, and it has a mean wt% $MgCO_3$ in calcite of 3 (range=7) (Wright et al. 2008). The species *A. antarctica*, however, has a comparatively tiny endemic distributional range of 23.5 lat^o. This is reflected in the low mean wt% $MgCO_3$ in calcite of 0.6, which is 2.4wt% below the average for the family

(Taylor et al. 2009). These patterns provide evidence that, since the first appearance of the families, these endemic Antarctic species have undergone adaptive evolution where the mineralogy of the species has become specialised for the environment within which it has evolved. *H. inerma* may also be endemic, as stated by Hayward et al. (1991; 1995) however OBIS distribution data extend its range into Chile. This may, however, be an artefact due to incorrect identification of the vicariant species *H. margaritifera* (Moyano 1999).

LIFECYCLE

A possible contributing factor to the differences among species mineralogy is the lifecycle stage of the different species at the time of collection. Mineralogy of bryozoan skeletons has been linked to astogeny, the process of aging (Bone & James 1993; Kuklinski & Taylor 2009; Smith & Girvan 2010), and to growth rates (Barnes et al. 2007; Smith 2007 Kuklinski & Taylor 2008). Both astogeny and growth rates vary according to a bryozoans lifecyle. The growth rate during the period of skeleton deposition has been shown to affect wt% $MgCO_3$ in calcite (Taylor et al. 2009) and has and the same authors have shown that at periods of faster growth more wt% $MgCO_3$ in calcite is incorporated within calcite. Age of the colony and the level of astogeny/secondary calcification can directly impact the proportion of aragonite in the skeleton. In order to assess lifecycle stage of specimens settlement data have been collated from the literature for all species except *H. inerma*, for which no data are available, and are summarized in Figures 5.17 and 5.18 which show the relative settlement times of the species in the year preceding collection plotted against the monthly mean midday seawater temperature.

Literature indicates that *Microporella ciliata* and *Membraniporella nitida* have the most settlement during the Spring/Summer (Denitto et al. 2007; Maughan 2000). This concurs with data presented by Coma et al. (2000) who observe that in cold temperate waters benthic suspension feeders exhibit increased activity and breeding in the Spring and Summer. Coma et al. (2000) document that food availability is low in the winter, due to inhibition of phytoplankton growth caused by the increased depth of the mixed layer and short daylight hours. Food availability data are limited, however, and so usually temperature is used as a proxy, as food and temperature tend to positively correlated (Coma et al. 2000). Although chlorophyll data are not available for Scapa Flow for the spring and

summer months, a correlating decrease in nutrients can be seen during spring and summer months, which could potentially be explained by increased phytoplankton.

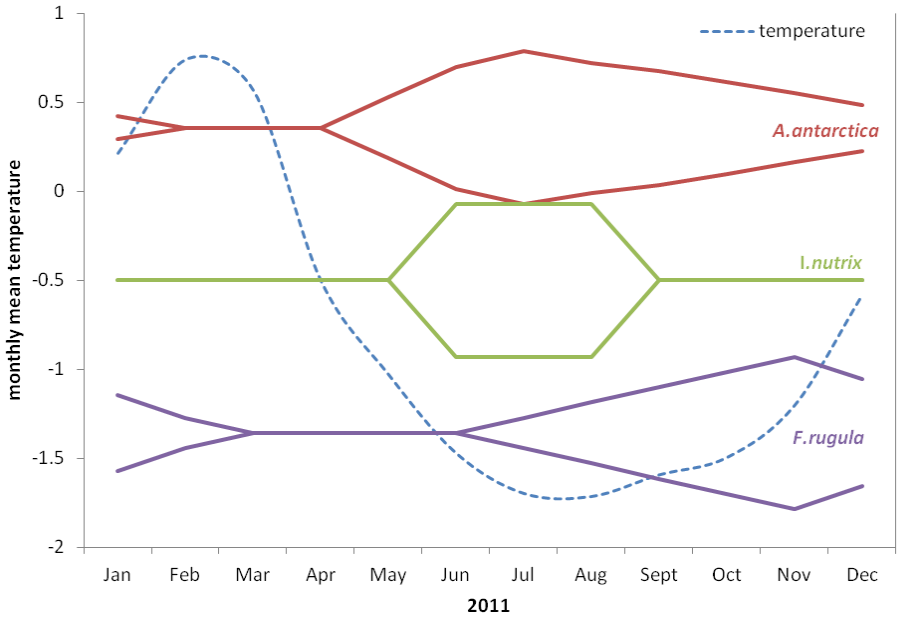

Figure 5.17: relative settlement times of the Antarctic species in the year preceding collection plotted against the monthly mean midday seawater temperature. Settlement data collated from Bowden et al. (2006).

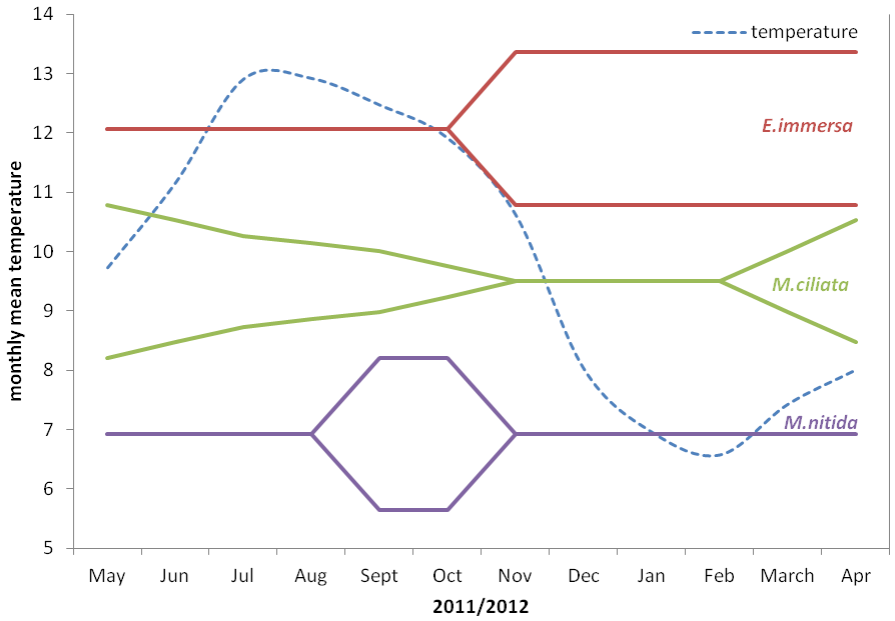

Figure 5.18: Relative settlement times of the Orcadian species in the year preceding collection plotted against the monthly mean midday seawater temperature. Settlement data collated from Chapter 4, Denitto et al. (2007) and Maughan (2000).

Data presented in Chapter 4 however, show that *E. immersa* has a solely winter breeding cycle, settling between November and April only. This is similar to the settlement of

Antarctic species which is greatest during the winter months for *A. antarctica* and *I. nutrix* (Bowden et al. 2006). *F. rugula* also features some settlement in the winter months although peak settlement occurs at the beginning of the summer (Bowden et al. 2006). For *I. nutrix* there is no settlement outside the winter months (Bowden et al. 2006). Publications by Barnes et al. (1995), Coma et al. (2000) and Bowden (2005) indicate that the activity, feeding and reproduction of some Antarctic benthic species are less constrained by seasonality of phytoplankton than in temperate waters. This is explained by Coma et al. (2000) as being due to the availability of both nano-plankton and micro-plankton in Antarctic waters. Species which can feed on nano-plankton, which unlike micro-plankton is present in the water column for much of the year, are less dependent on food availability as a settlement cue (Coma et al. 2000). The prevalence of winter bryozoan settlement in Antarctica is attributed by Bowden (2005) to be an adaptive response to an environment where ice-scouring, disturbance and grazing peaks during the summer. Many Antarctic bryozoan predators, such as urchins, limpets and asteroids, feature a lifecycle which is closely linked to the seasonal micro-phytoplankton summer blooms and therefore feature reduced activity during the remainder of the year (Brockington & Clarke 2001). Bowden (2005) observes that bryozoa would encounter less predator grazing if they settle in the winter rather than the summer.

From the size of the colonies (~1cm diameter) and the published settlement times it can be estimated that the Orcadian species were at least 6 months post-settlement at the time of collection. It is possible that specimens of *E. immersa* were from the end of the winter settlement from 2010/2011, in which case the greater age in comparison to the other Orcadian species may be a contributing factor to the higher level of secondary calcification (31% wt.% aragonite). In contrast the Antarctic species are estimated to be at least 10 months old (*A. antarctica* ~ 11months, *I. nutrix* ~ 16months, *F. rugula* ~ 10months). During sample extraction for all species approximately 2mm of skeletal material was extracted from the growing edge. Using published growth data, the time period during which this was deposited can be predicted, and, therefore, the environmental conditions the animal was exposed to during this period.

Growth rate data for *Microporella ciliata* were published by Ball et al. (1995), and estimates gave a deposition rate of 1mm per month. This indicates that, in the current study,

the extracted skeleton sample (2mm) was deposited in the two months prior to collection. No growth rate data are available for other Orcadian species and so this rate of deposition +50% (margin for error) will be adopted as an approximation of the time period for deposition for all Orcadian species (i.e. three months). More detailed growth rate data are available for *A. antarctica* and *F. rugula* as measured by Bowden (2005) from 2001-2003 at Rothera Research Station. This indicates that during the peak growing months of the austral summer the species are growing at 0.45mm/month and 0.66mm/month respectively. At this growth rate the 2mm sample would have been deposited in the 3-5months preceding collection. No growth rate data are available for *H. inerma* or *I. nutrix* and so this rate of deposition + 50% (margin for error) will be taken as the approximation of the time period of deposition for Antarctic species (i.e. six months).

The temperature measurements during the sample deposition time are plotted in Figures 5.19 and 5.20. The deposition of the skeletal sample in Orcadian species occurred during the coldest part of the year in Orkney (Fig. 5.20) and this is indicated by the mean

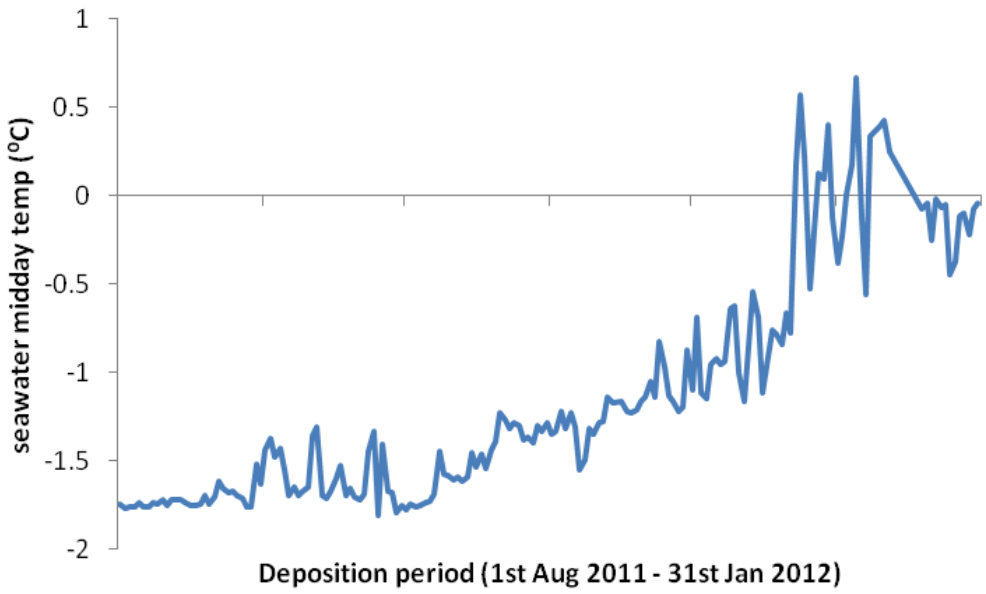

Figure 5.19: Mean midday seawater temperature in Ryder Bay, Antarctica during the estimated period of sample deposition

temperature during the time of deposition of 7.37°C, less than the annual mean temperature for Orkney of 9.89°C. Similarly the deposition period for the Antarctic samples was

during the transition period between the austral winter and summer (Fig. 5.19). This is evidenced by the mean temperature during the deposition period of -1.14^{o}C, less than the annual mean of -0.815^{o}C. This may indicate that the wt% $MgCO_3$ in calcite measurements recorded for the species from both regions are lower than if samples had been collected during warmer times of the year and future measurements will likely raise the means for the species.

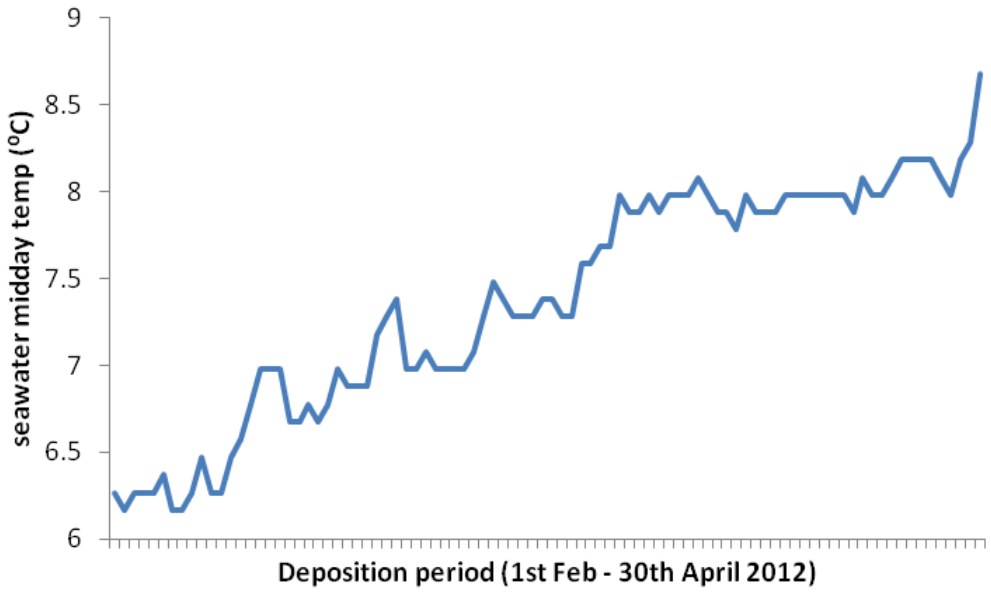

Figure 5.20: Mean midday seawater temperature in Scapa Flow, Orkney during the estimated period of sample deposition

5.6.2 Variability of mineralogy among sites

Environmental explanations

Relationships between bryozoan wt% $MgCO_3$ in calcite and environmental parameters were investigated and it was found that: i) the relationship between wt% $MgCO_3$ in calcite and temperature was inconsistent among species; ii) the predicted positive correlation between sea-water temperature and wt% $MgCO_3$ in calcite was not exhibited in any of the species examined; iii) no relationship between wt% $MgCO_3$ in calcite and salinity was detected in Antarctic bryozoans; and iv) the relationship between wt% aragonite and temperature was found to be inconsistent between the two bimineralic Orcadian species.

For species showing no relationship between temperature and wt% $MgCO_3$ in calcite it is possible that the temperature variability among sites is too low to cause a significant change in Mg incorporation. Controlled culture experiments, undertaken to "calibrate" the relationship between wt% $MgCO_3$ in calcite and temperature in Foraminifera, have shown that Mg incorporation within calcite can be expected to increase by approximately 8-10% per 1º C (Lea et al. 1999). The temperature differences among sites in both Antarctica and Orkney in this study are fractions of a degree and so any increase in wt% $MgCO_3$ in calcite caused by temperature may be below the levels of detection. This theory concurs with Antarctic data published by Rathburn & Decker (1997) on benthic Foraminifera, which also failed to detect any significant temperature related pattern of Mg/Ca in Antarctic specimens from -2 – 0.2ºC, in contrast to Foraminifera from temperate and tropical regions.

When using wt% $MgCO_3$ in calcite as a proxy for sea-water temperature, it is assumed that that biomineralization is influenced by environmental conditions alone (Urey et al. 1951). If species mineralogy is deposited asynchronously with environmental parameters then the biomineralization process is considered to be under the influence of the "vital effect" (Weiner & Dove 2001). If sea-water temperature is assumed to be the sole controlling factor of mineralogy then, given the consistently low sea-water temperatures in Antarctica, we would predict that the skeletons of endemic bryozoan species would be composed of LMC. In this study it was found that the mean wt% $MgCO_3$ in calcite differed significantly among species and that two species, *H. inerma* and *F. rugula* were composed of IMC. A study by Taylor et al. (2009) also found that the dominant mineralogical type of Antarctic bryozoans was IMC with 19/22 species featuring this form. It is suggested that the results of the two studies provide evidence for a 'vital effect' in the skeletal composition of some Antarctic bryozoans, and further speculated that phylogenetic affinity may be influencing mineralogical composition.

Analysis of Orcadian species showed that mean skeletal wt% aragonite was different among sites for all species. Aragonite is more energetically expensive to build than calcite (Anderson & Crerer 1993) and this observation predicts that in more energetically favourable, warmer conditions aragonite will increase. In this study, however, the relationship between environmental conditions and aragonite deposition was inconsistent between the two bimineralic species examined and no species showed the predicted

positive relationship between temperature and aragonite deposition. *E. immersa* showed no correlation between temperature and aragonite deposition and although wt% aragonite decreased with depth for this species, long-term environmental measurements showed no correlation between temperature and depth between 6 and 12m. This result is, therefore, unlikely to be caused by any variation in temperature related to depth, and instead may be indicative of site associated biological drivers. *Microporella ciliata* featured an inverse relationship of decreasing aragonite with increasing seawater temperature, contrary to the expected trend.

The relationship between wt% $MgCO_3$ in calcite and environmental temperature was found to be inconsistent among the species investigated. The negative trend of decreasing wt% $MgCO_3$ in calcite with site temperature, seen in the species *A. antarctica*, *H. inerma* and *E. immersa*, cannot be explained by thermodynamics and provides evidence that biological processes are likely influencing the biomineralization process. This reverse wt% $MgCO_3$ in calcite: temperature relationship has not before been discussed in bryozoans although there is some published evidence that this has been observed before. Crowley & Taylor (2000) published a study on the mineralogy of New Zealand bryozoans. In this study isotopic proxies were discussed, however, data was also published for mol% $MgCO_3$ in calcite and temperature. If these data are plotted, then we can see that the majority of species do not appear to follow the expected trend of increasing mol% $MgCO_3$ in calcite with temperature (Fig. 5.21). The species *Hornera squamosa* shows a statistically significant negative correlation between mol% $MgCO_3$ in calcite and temperature (p=0.033, -0.748). Crowley & Taylor (2000) also observe that *Heteropora cf neozelandica* features a lower equilibrium oxygen precipitation temperature that indicates that the species favours calcification at below mean annual temperature values. The simultaneous detection of unexpected aragonite in this species, however, made the authors doubt the measurement of chemical (mole% MgCO3) and isotopic variables and no further conclusions were drawn (Crowley & Taylor 2000).

Urey et al. (1951) suspected that some animals deposited mineralogy out of synch with environmental conditions and this was subsequently proved in phyla such as the echinoderms (Lowenstam 1954). From these observations arose the idea of good and bad phyla for use as palaeoenvironmental recorders (Weiner et al. 2001). In recent years,

however, the situation has been becoming more complicated, even for phyla traditionally considered faithful recorders, such as planktonic Foraminifera (Weiner et al. 2001). It is perhaps no surprise then, that, in the phylum Bryozoa, many of which are complex mineralizers, there are also some species which are "bad recorders" of palaeoenvironmental temperature and instead feature a strong vital effect.

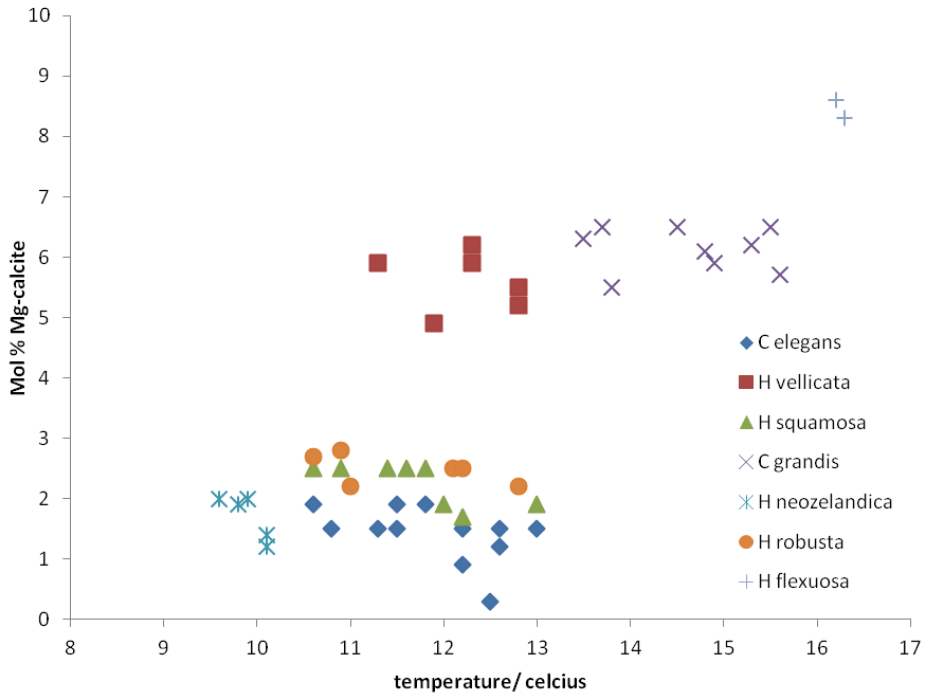

Figure 5.21: Scatterplot showing mol% $MgCO_3$ in calcite plotted against temperature for seven New Zealand species of bryozoan. Data taken from Crowley & Taylor (2000).

Biological explanations for mineralogical plasticity among sites

Variability in skeletal mineralogy can be considered a phenotypic differentiation within a species. Lynch (1990) attributed phenotypic differentiation among populations to evolutionary and/or environmentally induced change. Evolutionary driven phenotypic differentiation is described by Cheetham et al (1994) as a combined effect of random genetic drift and mutation counterbalanced by the effects of stabilizing selection; the ability of a species to "correct" random genetic excursions from the phenotypic optimum.

Morphological diversification within lineages occurs most rapidly in the early stages of genetic isolation and becomes progressively slower (Lynch 1990), as such the effect of

stabilizing selection is stronger in species that have undergone a longer period (usually measured in generations) since isolation and have achieved greater morphological stasis.

A population study on the temperate bryozoan, *C. hyalina*, by Goldson et al. (2001) shows that gene flow in bryozoans can be limited over distances as small as 10 m, due to a combination of the short pelagic phase of lecithotrophic larvae, and local physical hydrography. All of the species in the Antarctic component of this study brood lecithotrophic larvae, and the close proximity sites (2a, 2b, 2c), which are hydrographically heterogeneous, are most likely within swimming distance of each other for larvae and gametes (Wendt 2000). The resulting population connectivity might explain the similarity in wt% $MgCO_3$ in calcite among the Antarctic sites at the <10m scale. At the local scale (<10km) in Antarctica physical hydrography is likely to play a much stronger role in population connectivity. The dominant flow in Ryder Bay is from North-East to South-West although in winter a shallower current has been documented flowing in the opposite direction, northwards (Wallace et al. 2008; Beardsley et al. 2004) (Fig. 5.22). This pattern of flow could be providing a larval and gamete dispersal route between sites 2, 3, and 4. Evidence for population connectivity between Anchorage Island (site 4) and East Beach (Site 3) was found by Hoffman et al. (2012) during fine scale genetic population studies on the broadcast spawning limpet, *Nacella concinna*. The current study shows that wt% $MgCO_3$ in calcite in bryozoan skeletons from sites 2, 3 and 4 is similar. It is suggested that this $MgCO_3$ similarity may be related to the connectivity of the populations from sites 2, 3 and 4. Physical hydrography may also explain why Killingbeck Island (site 1) has the most distinct wt% $MgCO_3$ in calcite for all three species. Killingbeck is isolated from the other sites by the North - South direction of water flow and, with its shallow surrounding waters, it may also be subject to greater habitat fragmentation than other sites caused by iceberg scour (Fig. 5.23) and ice anchor disturbance (Hoffman et al. 2011; Hoffman et al. 2012). Habitat fragmentation at Killingbeck could be enhancing the founder effect (Futuyma & Moreno 1988) and over successive generations the limited gene pool would accentuate the impact of directional evolution on phenotypic characteristics such as skeleton chemistry (Cheetham et al. 1995), distinguishing its population from other nearby sites.

In Antarctica, *F. rugula* is reproductively most active at the beginning of the austral summer (Bowden et al. 2006). In contrast *A. antarctica* releases most larvae in July, during

the austral winter (Bowden et al. 2006). No reproduction information is available for *H. inerma.* Reproducing during the winter, when the sea surface is covered in fast ice and the water in the shallows is less impacted by wind-driven currents, may limit larval dispersal in *A. antarctica* and result in less population connectivity than *F. rugula*. It is suggested that differential breeding periods may help explain the inconsistency of the environmental response among species.

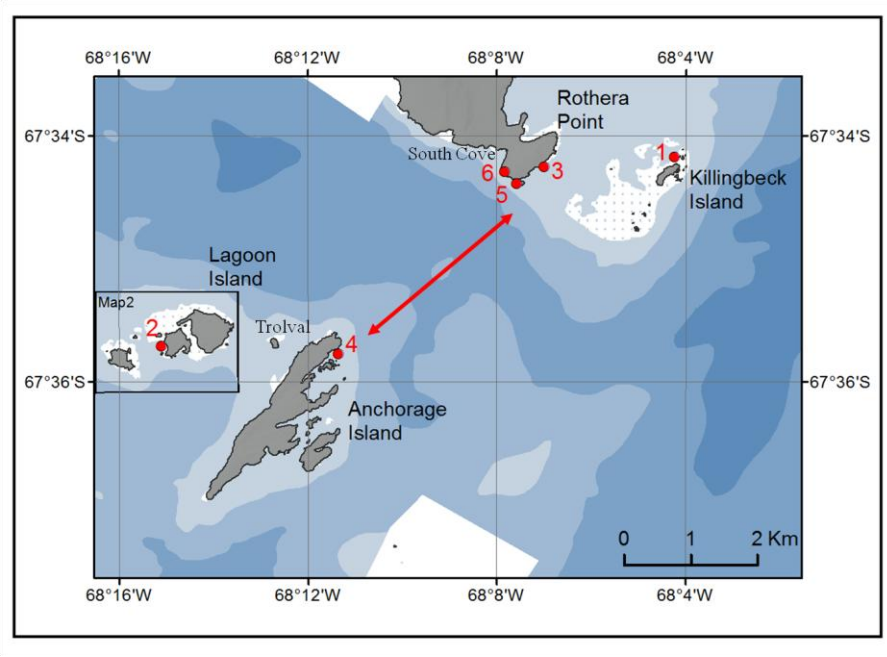

Figure 5.22: Map of sample sites in Ryder Bay. Red arrow indicates current flow. (Wallace et al. 2008; Beardsley et al. 2004)

Figure 5.23: Photograph taken from towards Killingbeck Island (site 1). The mountains of Pourqois Pas can be seen in the distance. Note icebergs in the channel between Rothera point and Killingbeck

Orkney features a highly tidal hydrological regime (Fig. 5.24). The main tidal flows come in and out of Scapa Flow from the North-West and the South, both at an average flow rate of ~0.25m/s (Marine Scotland 2012). The island cluster in the West of Scapa Flow cause a complex funneling of the tidal current between these sites. Fara, in particular creates an almost cyclical flow around the island at all points of the tide and the South of Flotta is prone to eddies as the Southern tidal in-flow meets the ebbing flow from Fara (Orkney Islands Council 2013). The least mineralogical distinct site from the Orcadian component of the study is the Barrel of Butter, site 5, which is in the centre of Scapa Flow and is, therefore, subject to incoming flow, larvae and gametes from both the South and the North-West. It is particularly indistinct from Flotta, site 3, with no statistically significant difference in any species for either wt% $MgCO_3$ in calcite or wt% aragonite, despite its geographical distance from Flotta (>7.5km). Lecithotrophic larvae from the temperate bryozoan, *C. hyalina*, which are of a comparable size to the species used in this study, were found to be able to swim for up to 4 hours, with a preference to settle within an hour of release (Goldson et al. 2001). If similar planktonic behaviour is assumed for the comparably sized larvae from the current study, then during periods of peak tide the dispersal potential would be up to 14.4km, with distances of over 3.6km achieved in one hour. This would allow transport of larvae from Flotta to the Barrel of Butter in just over two hours, within the feasible time limit for successful metamorphosis upon settlement (Wendt 1998). It is therefore possible that the mineralogical indistinctness between Barrel of Butter and Flotta is caused by population interconnectivity. The most mineralogically distinct sites are South Cava (site 1) and Houton Bay (site 2). The cyclical tidal flow around Fara appears strong at all points of the tide (Orkney Islands Council 2013) and may be acting to isolate South Cava and Houton Bay from the south of Scapa Flow, helping to explain why all species from these sites are mineralogically distinct from Flotta populations.

It is suggested that in both Orkney and Antarctica, relative population connectivity strongly contributes to the observed wt% $MgCO_3$ in calcite and wt% aragonite variability among study sites. To confirm this hypothesis, population genetics would need to be conducted on bryozoans from the study sites to quantify geneflow and genetic distance among individuals from the different locations. These population connectivity data could then be compared to the observed differences in wt% $MgCO_3$ in calcite and wt% aragonite.

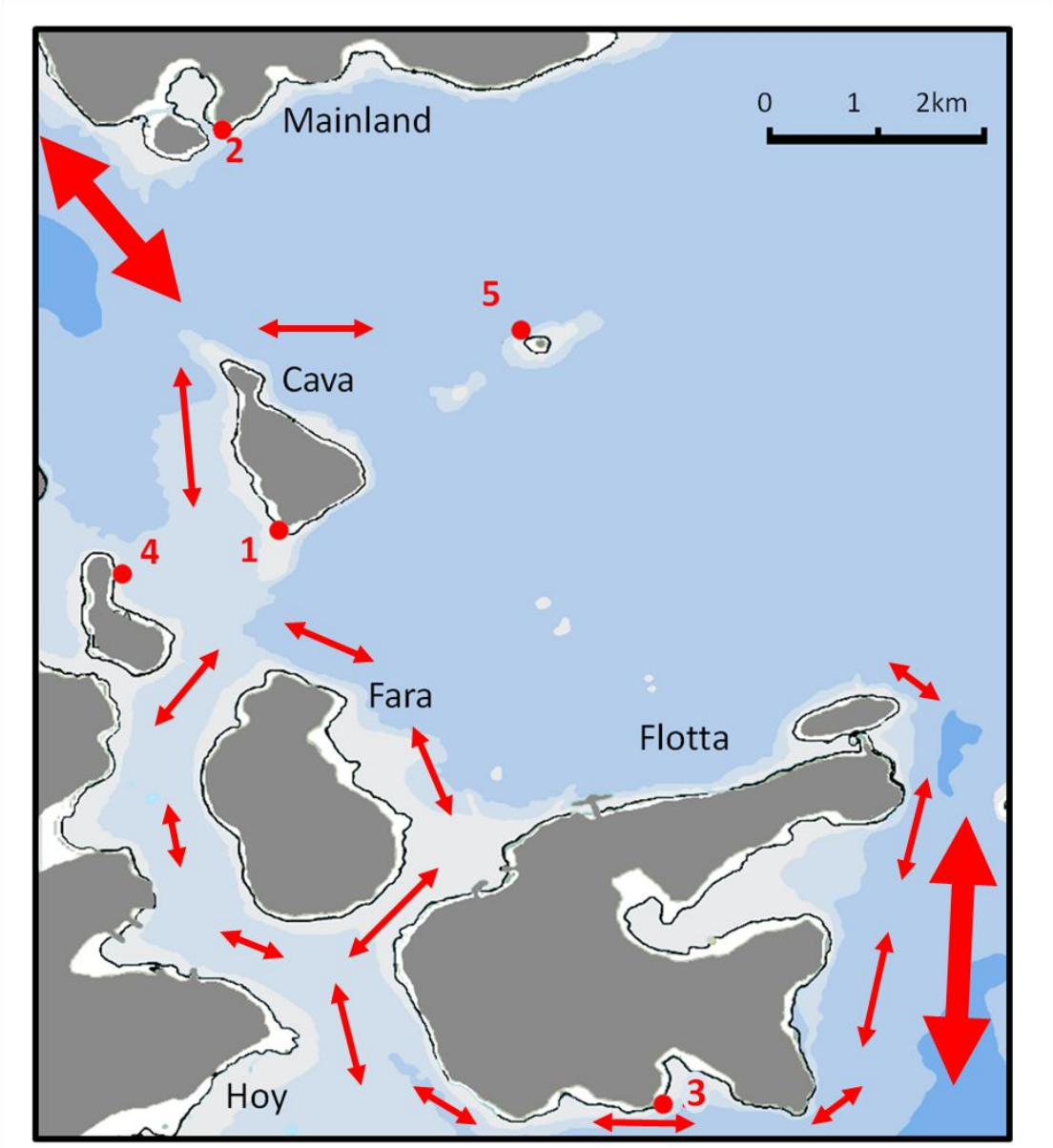

Figure 5.24: Map showing tidal flow in Scapa Flow as indicated by the admiralty tidal stream atlas. (UK Hydrographic Office 1986)

5.6.3 Comparison between regions: Antarctica vs UK

SPECIES MINERALOGY

Previous mineralogical studies (Borisenko & Gontar 1991; Taylor et al. 2009) have produced a mean mineralogy of 1.6 wt% $MgCO_3$ in calcite in Antarctic species. The four

Antarctic species in this study vary widely around this mean and are higher than this in three of the four species examined. Two species, *H. inerma* and *F. rugula* produce IMC rather than the expected LMC. In Orkney, by comparison the three species examined all produce similar wt% $MgCO_3$ in calcite which is close to the mean for Scotland, 5.86 wt% $MgCO_3$ in calcite (see Chapter 3). The difference from published means for the regions is most likely attributable to the breadth of the analysis conducted. Previous Antarctic analyses have only covered 21 out of an estimated 300 + Antarctic species, less than 7% of the species (Kuklinski 2013), while the mineralogical analysis in Chapter 3 of this thesis covered 79% of Scottish species. It might be expected that, as more Antarctic species are analysed, more variability in mineralogy may be found.

MINERALOGICAL PLASTICITY

There was no statistical difference between the range or plasticity of wt% $MgCO_3$ in calcite for Antarctic or Orcadian species. All of these species are ascophorans and can be compared to the wt% $MgCO_3$ in calcite range of other ascophorans from the literature (Smith et al. 2006; Taylor et al. 2009; Smith & Girvan 2010) and Chapter 3. Thirteen ascophoran species, each of which have been analysed more than ten times, have a mean range of 2.92 wt% $MgCO_3$ in calcite (min = 1.3, max = 5.7), this is closely comparable to the mean range of wt% $MgCO_3$ in calcite for the Antarctic (3.165 wt% $MgCO_3$ in calcite) and Orcadian (3.19 wt% $MgCO_3$ in calcite) species from this Chapter. The mineralogical plasticity of ascophorans seems highly conserved regardless of mean wt% $MgCO_3$ in calcite or distributional range of the species. This may be an indication of evolutionary / phylogenetic roots shared by all ascophorans. Many qualitative genetics models regard phenotypic plasticity as a trait in its own right, which is "either controlled by genes which are separate from those that determine the trait mean and/or has differential expression of the plasticity gene under different environmental conditions" (Cheetham et al. 1995) . If this were to be proven, the "plasticity gene" may be highly conserved between genetically related taxa, such as ascophorans. It should be noted however that no genetic work has been carried out on mineralogy genes in bryozoans and so the existence of and/or relative expression of a plasticity gene is conjecture at this stage. Many years of genetic research would be required before mineralogical plasticity could be reliably attributed to a "plasticity gene".

The proportion of the species plasticity that is attributed to within and among site variations can be compared for Antarctica and Orkney. Antarctica has an average ratio of 57:43, compared to Orkneys 66:34 (within: among site variation in wt% $MgCO_3$ in calcite).

There are three possible reasons why more of the mineralogical plasticity is attributed to among site variations in Antarctica when compared to Orkney.

1) Sampling methodology: Sampling of sites in Antarctica was over a longer period (3 weeks compared to Orkneys single week), possibly allowing for more seasonal influence on mineralogy among sites. In addition sampling at individual sites was only conducted by one diver in Antarctica compared to two divers in Orkney. As a result Orkney sites may have come from a slightly wider collecting zone and subsequently encompassed more microhabitats.

2) The relative influence of stabilizing selection: In Antarctica species are generally thought to be slower growing, take longer to reach maturity and have longer generation times than in temperate waters (Barnes 2000). This might mean that Antarctic species which diverged at the same time as temperate species, would have cycled through less generation time since divergence and therefore be under weaker influence of stabilizing selection (Lynch 1990). Stabilising selection acts to correct mutation and prevent genetic drift into phenotypic diversification (Cheetham 1986). If the effect of stabilizing selection is weaker in Antarctica then morphological and mineralogical specialization may be able to occur at a faster rate.

3) Habitat fragmentation: In Antarctica sites are more isolated from gene flow by strong currents/flow and habitat fragmentation caused by iceberg scour, ice anchors (Hoffman et al. 2011; Hoffman et al. 2012) and relative time since habitat creation following glacial retreat (Golledge et al. 2010). Habitat fragmentation would enhance the founder effect accentuating the effect of directional evolution on mineralogy. In concert with inbreeding and self-fertilisation, habitat fragmentation could, therefore, give rise to more specialized mineralogy for the local environmental conditions.

It is has been reported in Antarctica that other invertebrate species exhibit a high rate of phenotypic variation and genetically distinct populations over short spatial distances (Wilson et al. 2009; Peck & Clark 2012; Hoffman et al. 2012), and this has been primarily

attributed to habitat fragmentation and gene flow limitations. It is therefore probable that the increased amount of among-site variations in Antarctica, compared to temperate waters, is a legitimate observation, rather than an artifact caused by sampling bias.

TEMPORAL SCALE OF ENVIRONMENTAL VARIABILITY.

Antarctica is an environment with low seawater temperatures between -1.8ºC and +2ºC (Grange et al. 2011; Clarke et al. 2008) which are considered to be constant; experiencing temperature fluctuations of just 3-4 degrees per year (Portner et al. 2000). Antarctic marine invertebrates are therefore regarded as being highly stenothermal (Peck & Barnes 2009), only able to function efficiently in a very narrow range of temperature (Peck & Barnes 2004). By comparison the North Sea has been shown to experience an annual fluctuation of up to 15 ºC (Portner et al. 2000) and temperate species generally considered to be eurythermal, with a correspondingly wider thermal tolerance window.

This wide-held view of the stability of Antarctica versus temperate regions is based on annual and seasonal temperature fluctuations of temperature. Daily and within-season temperature fluctuations at shallow near-shore sites tell a different story, however. BAS Logger data from Cheshire (site 5) record that midday temperatures in Antarctica vary day-to-day by an average of 0.156 ºC during austral summer (May-Oct 2011) and 0.09 ºC during winter (Nov 2011 – April 2012). In comparison logger data from 12m in Scapa Flow (Chapter 4) record that midday temperatures vary day-to-day by an average of 0.09ºC year round with no seasonal specific peaks in variability.

As discussed in Section 5.6.1 the differences in published growth rates between Antarctic and temperate species translates as a different estimated deposition period for the extracted samples (Fig. 5.18 and 5.19). The six month growth period preceding collection in Antarctica is when temperatures and nanoplankton concentrations are at their highest and growth rates are correspondingly high. By contrast the three months prior to collection in Orkney were during winter, when the lowest annual temperatures and low phytoplankton concentration result in the slowest annual growth period. Both regions experienced a

comparable range of variation of 3°C during the sample deposition periods although the day-to-day variability is significantly higher in Antarctica (Kruskall-Wallis test, p=0.004) at 0.14°C compared to Orkneys 0.09 °C. These data offer a view that is contrary to the standard reporting of Antarctica as an extremely stable environment in comparison to temperate regions. The comparable variability in temperature over this shorter temporal scale offers a potential explanation as to why there is a remarkably similar degree of mineralogical plasticity between Ryder Bay and Scapa Flow samples in this study.

WHAT DOES THIS MEAN IN THE CONTEXT OF WIDER SCIENCE?

BIOLOGICAL VS ENVIRONMENTAL CONTROL OF MINERALOGY

Biological involvement in the control of mineralogy is evidenced by the data at both the phylogenetic and physiological level. At the deeper phylogenetic level the data show conserved mineralogical preferences within families. An example of this is *F. rugula* which features IMC, in keeping with the family mineralogy, despite the expected low wt% $MgCO_3$ in calcite for the region. The existence of a vital effect is proven to be particularly strong in the three species, *A. antarctica*, *H. inerma* and *E. immersa*, which all exhibit a trend of decreasing wt% $MgCO_3$ in calcite with increasing sea-water temperature. This trend is contrary to what we would expect given the seawater chemistry and is evidence for strong physiological involvement in Mg incorporation in calcite for these species.

There is also significant evidence for the influence of environmental conditions on mineralogy. wt% $MgCO_3$ in calcite and wt% aragonite deposition is significantly lower in Antarctica than for Orkney caused by directional evolution over palaeontological timescales driving species mineralogy away from the ancestral norm and towards that which is most environmentally favourable for each region.

The data from this Chapter suggest that environmental and biological controls of mineralogy are not mutually exclusive but work in concert with each other. Significant correlations between temperature and mineralogy were detected, with environmental conditions causing the trigger for differentiation and the biology of the species determining the direction and scale of the mineralogical response.

MINERALOGY AS AN ENVIRONMENTAL PROXY

The data presented in this Chapter provide no conclusive evidence for a correlation between skeletal mineralogy and salinity, depth or carbonate chemistry. Half of the species showed no significant correlation between temperature and wt% $MgCO_3$ in calcite, while the remaining species, *A. antarctica*, *H. inerma* and *E. immersa*, showed a negative correlation between temperature and wt% $MgCO_3$ in calcite. This is contrary to the positive relationship between wt% $MgCO_3$ in calcite and temperature that is reported to be expected for bryozoans (Lowenstam 1954). This published relationship is based primarily on regional comparisons (Smith et al. 2006; Kuklinski & Taylor 2009), or studies which use latitude (Lowenstam 1954; Smith et al. 2006; Taylor et al. 2009) or isotope measurements (Lombardi et al. 2008) as a temperature proxy. This Chapter provides data to show that the relationship between seawater temperature and mineralogy in bryozoans is more complex than has been previously believed and that species are often exerting strong control over their own calcification. The relationship between wt% $MgCO_3$ in calcite and temperature in the Bryozoa should therefore not be assumed but should be proven for each species before it can be reliably used as a palaeoclimatic proxy.

Mineralogy in a warming and acidifying ocean

There is no statistical difference in mineralogical plasticity for temperate and Antarctic species over the experimental range of temperature variation. This suggests that species in both regions may respond to rising sea temperatures to a similar degree.

Gap analysis and next steps

The data presented in this Chapter are the first fine-scale comparison of bryozoan mineralogy between regions. Limitations in environmental data and site inter-relatedness, however, mean that the potential causes for these patterns cannot yet be considered conclusive.

The explanations investigated in this Chapter would be further strengthened by improved distribution data for *H. inerma* and *Microporella ciliata*. At present the distribution data for both of these are unreliable due to taxonomic confusion and accurate distribution data would help in the exploration of patterns relating to endemism and cold specialization. In addition genetic analysis to investigate the inter-relatedness of bryozoan populations from the different study sites would be useful in proving habitat fragmentation in Antarctica.

5.7 Summary and Conclusions

Each species studied has a skeletal mineralogy which is determined by a combination of biological and environmental factors. Species show conserved mineralogical traits with their phylogenetic relations. Antarctic species have also become somewhat specialized for the cold environment with lower wt% $MgCO_3$ in calcite and lower wt% aragonite than temperate regions.

There is no significant difference between the mineralogical plasticity of species from Antarctica and Orkney. This is attributed in part to the shared phylogenetic and mineralogical ancestry of ascophorans but is also likely a result of the similar environmental variability in the two regions during the time of sampling. When viewed from an annual perspective Antarctica is an extremely stable environment in comparison to temperate regions. During the growth period of the samples, however, there was a comparable temperature range between the regions and greater day-to-day variability in Antarctica.

All species show statistically significant differences between sites with Antarctica showing greater mineralogical distinctness between study sites than Orkney. Differing mineralogy between sites can be primarily attributed to population connectivity. The most mineralogically distinct sites in Antarctica show evidence of habitat fragmentation caused by ice scour and strong, constant currents preventing out-breeding with other sites.

Isolated populations show a higher degree of adaptation for local environmental conditions, with temperature being the main factor influencing mineralogy. No species show any evidence of the expected relationship of increasing wt% $MgCO_3$ in calcite with temperature but three species show statistically significant negative correlations between wt% $MgCO_3$ in calcite and temperature. The three species are a mixture of Antarctic endemic and Arctic/Boreal species.

Understanding the relationship between wt% $MgCO_3$ in calcite and temperature for different species could potentially allow future modeling of which species might be ‘winners and losers’ under predicted climate change and ocean acidification conditions.

Chapter 6: Investigating relationships between bryozoan mineralogy and skeletal morphology at the ultra, micro and macro scale.

6.1 Abstract

The morphology of bryozoan skeletons and calcification methods have been studied since the 19th century. To date, however, investigations to assess links between $MgCO_3$ and morphology have been limited to a few colony (macro) scale studies. Evidence has been found in marine invertebrates for the differential deposition of aragonite and $MgCO_3$ for mechanical advantage but this is yet to be proven in bryozoans.

In this study the relationship between bryozoan skeletal mineralogy and morphology was investigated at the ultra, micro and macro scale. Techniques included ultrastructure imaging of skeletal fabrics, staining of skeletons using $MgCO_3$ and aragonite-specific stains and the application of MCMCglmm models to assess the effects of phylogeny and morphological complexity on mineralogy.

Findings show that mineralogy and morphology are closely interlinked at the ultra, micro and macro scale with additional relationships proven with phylogeny and ecological preference of species. The crystal size of ultrastructure fabric was found to increase with decreasing $MgCO_3$ content. All species analysed also show differential deposition of $MgCO_3$ in skeletal features with the highest $MgCO_3$ content found in more genetically evolved, morphologically complex species. Study species are shown to utilize the mechanical properties of aragonite and $MgCO_3$ for ecological advantage allowing them to colonise abrasive and fast-flowing habitats.

The observed relationship between mineralogy and morphology raises concerns about the future of bryozoan species with complex skeletons under ocean acidification conditions. Localisation of dissolution-susceptible fabrics in specific skeletal features may lead to selection against morphologically complex species and those which utilise aragonite and $MgCO_3$ to occupy challenging ecological niches. Further study is recommended to elucidate the mechanical properties associated with the differential mineralogy of bryozoan skeletons and the potential effects of ocean acidification on vulnerable species.

6.2 Introduction

6.2.1 Ultrastructure in bryozoans

Early studies on bryozoan skeletal microstructure were conducted using thin section techniques by authors including Calvet (1900), Ostroumoff (1903) and Levinson (1909). One of the earliest observations of skeletal microstructure in bryozoans was recorded by Levinson (1909), who documented "a very fine striation parallel to the distal lines" in *Flustra foliacea*. A more detailed study by Bronstein & Prenant (1936) on calcitic cheilostomes identified three microstructural arrangements of calcitic crystals, including "chevron" and "fan" patterns, which were reported as common across some species and often conserved within taxonomic groups; these would later become known as "fabrics". A collection of microstructure studies followed, with investigations of the soft parts responsible for calcification (Lutaud 1957; 1961; Bobin & Prenant 1968; Banta 1968a; 1968b; 1969), although these were limited by the extent of magnification available (Sandberg 1971). More information on the ultrastructure of the skeleton became available following the commercial release of the first scanning electron microscopes (SEM) in the late 1960's (Taylor & Weedon 2000). The first study on Cyclostome ultrastructure was conducted by Söderqvist (1968), who identified two distinct ultrastructure fabrics in *Crisia eburnea* and a common laminar "stacked" fabric in a further four Cylostome species. Boardman & Cheetham (1969) produced a review comparing Cheilostome and Cyclostome microstructure and in the same year Cheetham et al's (1969) study on the cheilostome, *Metrabdotos,* used a combination of ultrastructure imaging, staining and X-ray diffraction to investigate both ultrastructure and spatial distribution of aragonite and calcite. Sandberg (1971) consolidated previous cheilostomatous ultrastructure studies as well as adding observations on additional Recent samples. Sandberg (1971) investigated the relationship between ultrastructure and mineralogy and looked for commonalities between species, recognising six fabrics; transverse fibrous, parallel fibrous, lamellar, cell-mosaic, crystal stacks and massive layer. Sandberg (1977a; 1983) continued to build on this work in subsequent book Chapters. Tavener-Smith & Williams (1972) conducted the first broad survey on the ultrastructure of both extant and fossil cheilostomes and cyclostomes, examining more than twenty extant species. Tavener-Smith & Williams (1972) observed that the skeletal ultrastructure of cyclostomes was less variable than cheilostomes with cyclostomes featuring no more than two types of fabric. Brood (1972) conducted further

ultrastructure studies on cyclostomes, identifying three wall fabric suites, structures of sequential layers of distinct fabrics which he noted were conserved between taxonomic groups. Brood (1976) published a further study describing the secretion of these cyclostomatous fabric suites. Ross (1975; 1976; 1977) proposed an alternative mechanism for Cyclostome wall secretion, however this received little support and some structures have since been re-identified (Weedon & Taylor 1996). Ristedt (1977) specifically investigated the primitive cheilostomatous genus *Membranipora*, examining the ultrastructure of five species and finding substantial skeletal differences between them, an observation later proved correct by the reorganisation of four of the species into alternative genera (Weedon & Taylor 2000). Interestingly, the study recorded *M. membranacea* as calcitic, although it is now widely recognised that true *M. membranacea* is aragonitic (Bone & Wass 1990; Taylor & Monks 1997). Bigey (1977; 1982) compared recent skeletal ultrastructures of two Recent cyclostomes to Palaeozoic bryozoans, and offered further evidence for conserved fabric suites between species (Taylor & Weedon 2000). Williams (1990) composed a biomineralization review of lophophorates, allowing direct comparison between ultrastructure fabrics and mechanisms in the Brachiopoda and Bryozoa. Schäfer (1991) offered further evidence for laminar ultrastructures in cyclostomes, specifically with relation to brood chambers. A suite of detailed publications followed which investigated the ultrastructure of cyclostomes skeletons, both Recent and fossil (Boardman et al. 1992; Taylor & Jones 1993; Weedon & Taylor 1995b; Taylor et al. 1995; Weedon & Taylor 1996; 1997; 1998; Weedon 1997; 1998). In these cyclostome studies they identify six conserved fabrics and five distinct wall fabric suites. A suite is considered a succession of fabrics which appear in a conserved order. Similarities were noted between species and other marine groups and ultimately the data was used to construct a phylogeny of cyclostome bryozoans based on ultrastructure features of 29 genera (Taylor & Weedon 2000). Further studies were published on cheilostome ultrastructure including a detailed description for the new genus, *Jellyella* (Taylor & Monks 1997) and an examination of seventeen species of primitive cheilostomes which revealed seven distinct fabric types and eight fabric suites (Weedon & Taylor 2000). Weedon & Taylor (2000) discuss the similarities and differences between fabric types in cheilostomes and cyclostomes, taking the existence of two cheilostome specific fabrics, rod-like and wall-perpendicular prismatic, as evidence of independent evolution of calcification in the cheilostomes. In 2003 a study on the fabrics of cheilostome ooecial walls (Ostrovsky et al. 2003) found four fabrics

previously described by Weedon & Taylor (2000). Gontar (2009) claimed a new cyclostome "rough chevron" fabric, however, the images are not a high enough magnification to confirm this as distinct from fabrics reported by Taylor & Weedon (2000).

ULTRASTRUCTURE FABRICS

Granular fabric (Fig. 6.1A): This is also referred to as wedge shaped crystallites and is described by Taylor & Weedon (2000) as the outermost fabric in many cyclostomes. Weedon & Taylor (2000) describe a similar fabric of the same size and fibrillar substructure in the surface of cryptocysts and exterior walls in primitive cheilostomes, described as Wedge Shaped Granular. Crystallites are 1-2µm x 0.2-1µm in size (Taylor & Weedon 2000; Weedon & Taylor 2000).

Planar spherulitic fabric (Fig. 6.1B): Taylor & Weedon (2000) describe this fabric as strips of acicular crystallites flat against a wall surface in cyclostomes. Weedon & Taylor (2000) also found elongate parallel-aligned strip-like units in the spines and gymnocyst of cheilostomes, with crystals measuring 0.5-3µm in width and 5-20µm in length; Weedon & Taylor (2000) concluded they are a common fabric between the two Orders.

Transverse fibrous fabric (Fig. 6.1C): In cyclostomes this is described as elongated imbricated crystallites growing in the plane of the interior and exterior wall surface with long axes transverse to the distal direction of wall growth (Taylor & Weedon 2000). Crystals measure1-5 µm in width and greater than 30 µm long, although full crystal length is rarely visible as they are embedded in the wall. This fabric is not seen by Weedon & Taylor (2000) in primitive cheilostomes, although it is observed that it has the same orientation as rhombic semi-nacre and in cyclostomes "matures" into rhombic semi-nacre. Cheetham et al. (1969) observed this fabric composed of aragonite crystallites in the cheilostome *Metrabdotos*.

Foliated fabric (Fig. 6.1D): In cyclostomes this presents as flattened, imbricated crystallites growing parallel to the proximo-distal direction of wall growth (Taylor & Weedon 2000). Like transverse fibrous, this fabric is also not seen by Weedon & Taylor (2000) in primitive cheilostomes. Crystallographically this fabric is very similar to

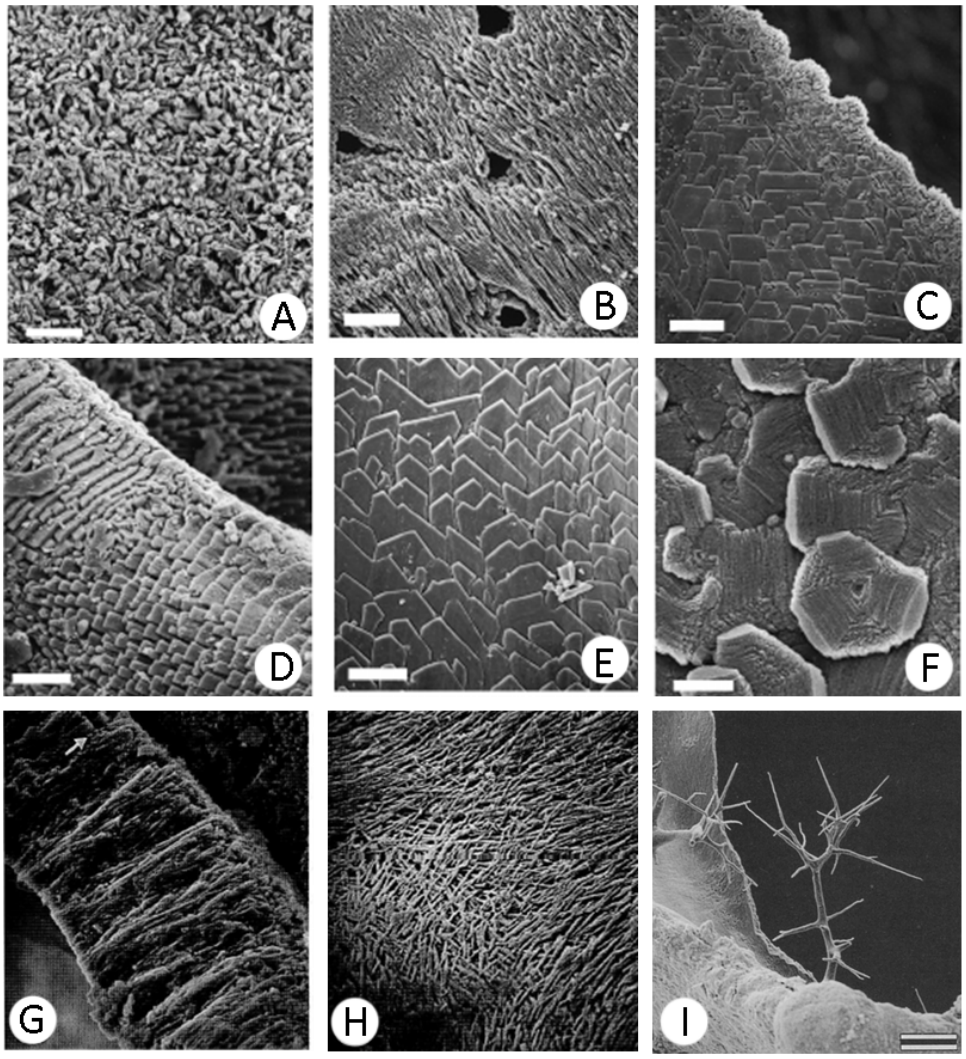

Figure 6.1:Commonly recognized fabrics. A: granular fabric; B: Planar spherulitic fabric; C: Transverse fibrousfabric; D: foliated fabric; E: Rhombic semi-nacre; F: Hexagonal semi-nacreous; G: wall-perpendicular prismatic; H: rod-like fabric; I: moulded fabric. Images reproduced from: A-F - Taylor & Weedon (2000); G & H – Weedon & Taylor (2000); I – Taylor & Monks (1997)

transverse fibrous fabric, with the angle of orientation distinguishing the two (see Figure 6.2).

Rhombic semi-nacre (Fig. 6.1E): Platey fabric of wall parallel sheets consisting of rhombic tablets with frequent screw dislocations and scattered small, newly-formed crystallites (Taylor & Weedon 2000; Weedon & Taylor 2000). Fusion between crystals is common. This fabric is found in both cheilostomes and as a mature wall surfaces in cyclostomes (Taylor & Weedon 2000). In cyclostomes crystals can have a hexagonal outline, but the interfacial angles are never consistently 120º, as they are in cyclostomes

Hexagonal semi-nacre, distinguishing it from the hexagonal semi-nacreous fabric (Weedon & Taylor 2000)

Hexagonal semi-nacreous (and pseudofoliated) fabric (Fig. 6.1F): This fabric differs in crystallographic orientation and shape to other laminar fabrics. It is based on hexagonal tablets of calcite, with the c-axis perpendicular to the wall surface (Taylor & Weedon 2000). It has only been found in cyclostomes so far.

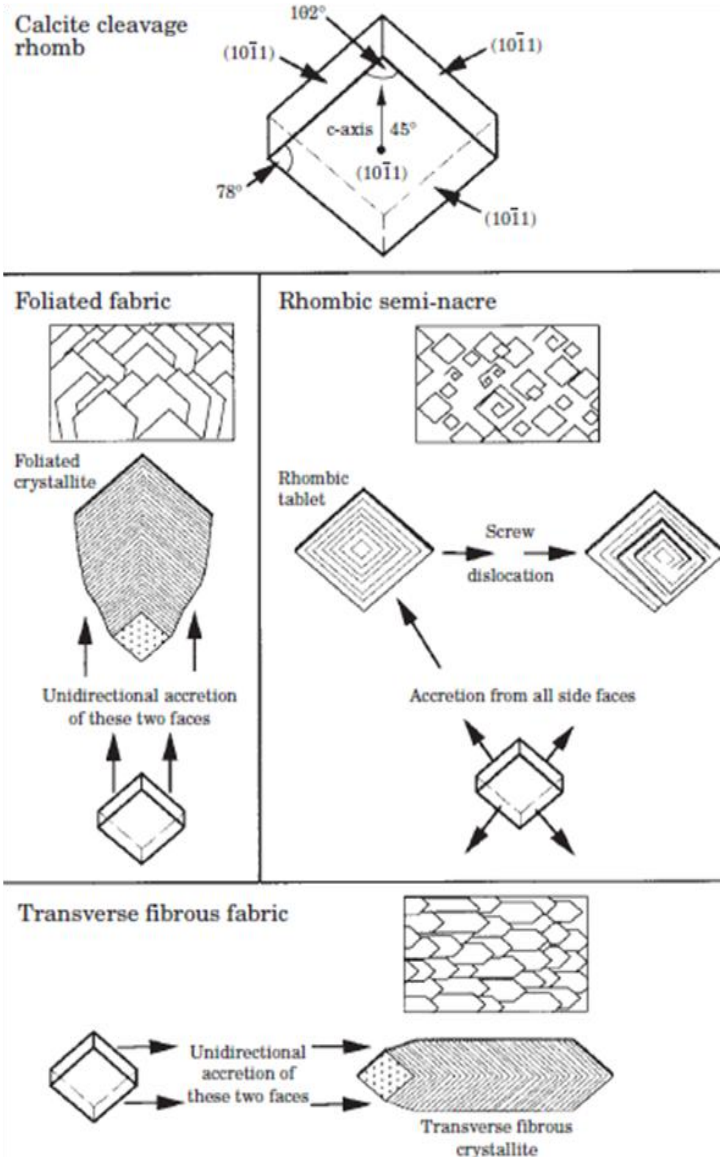

Figure 6.2: Diagrammatic representation of the growth of laminar fabrics from the calcite cleavage rhomb aligned with one of the (1011) faces parallel to the wall surface, and with the c-axis at 45° to the wall surface. The fabric type is determined by the direction of accretion. Reproduced from Taylor & Weedon (2000)

Wall perpendicular prismatic fabric (Fig. 6.1G): This is the most common fabric found in primitive anascan species studied by Weedon & Taylor (2000), occurring predominantly as calcite but also as aragonite in *M. membranacea*. Densely packed bundles of acicular (needle-shaped) crystallites (each 50-100nm diameter) are aligned perpendicular to the wall surface. Bundles truncate abruptly against each other or are separated by thin spaces (100-300nm) (Weedon & Taylor 2000). This fabric has not been observed in cyclostomes.

Rod-like fabric (Fig. 6.1H): Composed of parallel-aligned, densely-packed elongated crystallites which have a consistent rod or spindle shape. Individual crystallites are 100-500nm in diameter and up to 2µm in length (Weedon & Taylor 2000). This fabric is reportedly common in cheilostomes (Weedon & Taylor 2000) but has not been observed in cyclostomes.

Weedon & Taylor (2000) further describe the laminar fabrics "irregular platey", seen in *Aetea*; and "fibrous platey", observed in *Jellyella*. Each of the fabrics has been found in two species of a single genus. The images and descriptions make it difficult to distinguish between these and the laminar fabrics identified in cyclostomes by Taylor & Weedon (2000). Taylor & Weedon (2000) further describe a "moulded fabric" of non-angular single crystals which was observed to form structures such as the mural spines and spikes often seen in cyclostomes. This moulded fabric also appears to describe the newly formed spicules seen in *Jellyella eburnea* by Taylor & Monks (1997) and could therefore be a common fabric in both cyclostomes and cheilostomes (Fig. 6.1I).

Several studies have been published which discuss potential methods for skeletal deposition (e.g. Lutaud 1957; Lutaud 1961; Banta 1968a; Banta 1968b; Banta 1969; Tavener-Smith & Williams 1972) and these are discussed in detail in Chapter 1. Although conducted over forty years ago, the discussion by Tavener-Smith & Williams (1972) remains the most exhaustive of potential differentiation in methods of deposition related to different ultrastructures. Common across all calcifying bryozoans studied to date, calcium carbonate deposition is reported to primarily occur on the epithelium or the inner surface of the periostracum, which act as seeding sheets for crystal development (Tavener-Smith & Williams 1972). Tavener-Smith & Williams (1972) suggest that different fabrics in cheilostomes are formed dependent on whether crystals are seeded within epithelium folds, on the outer part of epithelium folds, bound between the periostracum and epithelial sheet or on "organic sheets" which can be an extension of the periostracum. Sandberg (1971;

1977) corroborates this theory and observes that "planar spherulitic" fabric is deposited as "one-sided" growth on interior epithelial walls. The laminar fabrics in particular are described as being formed from a simultaneous secretion of carbonate and protein by the epithelial cells which forms interconnected protein sheets which "mould" the laminae (Tavener-Smith & Williams 1972). In both cyclostomes and cheilostomes Tavener-Smith & Williams (1972) observe organic partitions, lined with proteinaceous membranes, within which distinct fabrics are deposited.

The fabrics described earlier in this Section are based primarily on the description of calcitic crystals, however, aragonite crystals are shown to form the same fabrics as calcite in some cases. Weedon & Taylor (2000) observe aragonite to form "wall-perpendicular prismatic" in primitive cheilostomes, and Sandberg (1971; 1977; 1983) further observed aragonitic "planar spherulitic" and "transverse fibrous" fabrics. Tavener-Smith & Williams (1972) and Sandberg (1977; 1983) report no differential mechanism between deposition of aragonite and calcite in bryozoans. No known studies have been conducted comparing the $MgCO_3$ content of calcite in different bryozoan ultrastructures.

6.2.2 Mineral localisation in bryozoans

Some of the earliest bryozoan mineralogical analyses (Clarke & Wheeler 1917; 1922) were performed using Meigen's stain (Meigen 1901) allowing an estimation of the presence and spatial location of aragonite and calcite. This work continued with studies looking at the composition and spatial distribution of the polymorphs in bimineralic species (e.g. Cheetham et al. 1969; Carver & Rucker 1969; Sandberg 1971; Carson 1978; Cheetham & Thomsen 1981; Smith & Girvan 2010) . In these studies it was observed that calcite was usually the skeletal framework in bryozoans, later overlaid with a secondary layer of aragonite. The localisation of aragonite as a secondary layer was confirmed by Taylor et al. (2007) using Raman spectroscopy, and it was also observed that many fine-scale skeletal structures, such as ovicells, avicularia and orifice rims were predominantly calcitic. Taylor et al. (2009) also identified that aragonite was localised on the base of free-living bimineralic bryozoans. Schopf & Allan (1970) were the first to use a microprobe on Bryozoa and this was utilised more recently by Schäfer & Bader (2008). A microprobe is able to provide quantitative information on the spatial distribution of elements, such as Mg, although it is unable to detect the different calcium carbonate polymorphs. In the past decade there has also been a resurgence of staining techniques, including the first use of

Titan Yellow on bryozoans, which offers information on the spatial distribution of $MgCO_3$ in calcite (Bone & James 1993; Smith & Girvan 2010; Smith & Lawton 2010). Both microprobe and Titan Yellow techniques offer information on the spatial distribution of Mg in bryozoan skeletons and, although they cannot be considered quantitative, studies have revealed some recurring patterns. Smith & Lawton (2010) and Smith & Girvan (2010) found HMC to be concentrated in the bases and older parts of erect colonies.

No known studies have looked at the spatial distribution of Mg-calcite at the zooidal scale. The mechanical advantage of mineral localisation has been considered in some studies (Smith et al. 2006; Smith & Girvan 2010; Smith & Lawton 2010), although this has been restricted to the discussion of secondary calcification (both aragonitic and HMC), the "hardness" this may provide in an abrasive environment or as a defence against predators, and the "reinforcement" this provides when located at a colony base.

6.3 Aims and Objectives

6.3.1 Summary of purpose of study

The interaction between bryozoan skeletal mineralogy and morphology has been little studied and, as a result, is currently poorly understood. The aim of this study is to increase the data available on the crystal ultrastructure, spatial distribution of minerals and morphological complexity of bryozoan skeletons and interrogate these for patterns relating to bryozoan mineralogy, phylogeny and colony form. Results will be discussed in relation to the mechanical properties associated with different mineralogies/fabrics and potential evolutionary adaptation of species for competitive and ecological advantage.

6.3.2 Objectives of study

1. Investigate and document the ultrastructure fabric in the skeletons of selected bryozoans. Ultrastructure fabrics will be measured and compared to images from previous publications resulting in a combined dataset which will be investigated for phylogenetic and mineralogical patterns.
2. Utilise mineral specific staining techniques to investigate patterns of spatial localisation of Mg-calcite and aragonite in the skeletal features of bryozoans.
3. Investigate potential relationships between bryozoan skeletal morphological complexity, mineralogy and phylogeny.
4. Discuss the mechanical properties associated with skeletal mineralogy and ultra-morphology and the potential ecological advantages they may confer to bryozoans.

6.4 Materials and Methods

6.4.1 Sample collection

This study is based on the morphological examination of specimens of bryozoan species collected from Antarctica (four species) and Scotland (fourteen species). All specimens were collected either intertidally or by SCUBA diving with the Heriot-Watt Scientific Dive team. Details of specimen processing methods can be found in Section 3.4.1

6.4.2 Analysis techniques

IMAGING

Where required to confirm identification SEM was undertaken according to methodology detailed in Section 3.4.1.

Ultra-SEM was used to examine the crystal size and structures of species and was conducted on specimens which had been bleached for 72 hours (10% domestic bleach diluted with tap water) in an ultra-sonic bath, rinsed in fresh tap water, dried, mounted on aluminium stubs and sputter coated with gold. Imaging was undertaken using a Carl Zeiss Ultra Plus Field Emission SEM at the EMMA unit of the Natural History Museum, London.

STAINING

Specimens selected for aragonite staining were etched in 5% acetic acid for 5 seconds, rinsed and dried and then boiled in 10% $CO(NO_3)$ (Meigen's solution; prepared as per Suzuki et al. (1993)) for 10 minutes before rinsing and drying. Specimens undergoing staining for magnesium were etched in 5% acetic acid for 30 seconds, dried and then immersed in Titan Yellow dye (prepared as described by Choquette and Trusell (1978)) for 20 minutes. Staining was fixed using 20% sodium hydroxide. Stained specimens were imaged using a Zeiss light microscope.

6.4.3 Data sources, processing and presentation

Ultrastructure images in Section 6.5.1 were examined to determine the ultrastructure fabrics present and the dominant fabric. The cross-sectional area of the dominant fabric was calculated using the maximum width and height of crystals which were considered to be "representative" of the fabric in that species. Author's images were added to from

published literature as detailed in Table 6.1. Only publications on British species with high quality images and distinct crystals were used. Calculated cross-sectional areas were *log* transformed prior to further analysis.

Plates were prepared using GNU Image Manipulation Program (GIMP) (Kimball & Mattis 2012).

6.4.4 Statistics and data analysis

Wt% $MgCO_3$ in calcite data were obtained from Chapters 3 and 5 and tested for normality using Anderson-Darling normality tests and homogeneity of variance using Levine's test of equal variance. Where the criteria for parametric testing were satisfied data were analysed using a generalised linear model (GLM) ANOVA. Where the criteria for parametric testing were not satisfied non-parametric Mann-Whitney U-tests were used. p-values for all analyses were calculated; this is a measure of the strength of theevidence and is based on the traditional probability of error of 0.05. Only when the p-value is less than 0.05 is the evidence considered statistically significant. Statistics and data analysis were conducted using MINITAB release 14 software (2004) and R programming environment (R Core Team 2013).

PHYLOGENETIC COMPARATIVE METHODS

Phylogenetic data and branch lengths were taken from the recent publication of Waeschenbach et al. (2012a) as described in Chapter 3. The phylogeny was input into the R statistical language (R Core Team 2013) using Newick coding. Kembel et al.'s (2010) methodology for Comparative Phylogenetic Methods was followed. Blomberg's K (Blomberg et al. 2003) was calculated as a measure of underlying phylogenetic signal for the trait of morphology using the "multiPhylosignal" function in the Picante package for R (Kembel et al. 2010; 2013).

MARKOV CHAIN MONTE CARLO GENERALIZED LINEAR MIXED-MODEL (MCMCGLMM)

For model compatibility the phylogenetic tree from Waeschenbach et al. (2012a) was ultra-metrically scaled for evolutionary time using the "chronos" function in the APE package for R (Paradis et al. 2013). All mineralogical measurements, taken from Chapters 3, 4, 5 and 6, underwent weighted average transformation in the context of beta regression (Eq. 1) following the methodology of Smithson & Verkuilen (2006).

Equation 1: Weighted average transformation in the context of beta regression. n=total data points

$$\frac{y(n-1)+0.5}{n}$$

This initial transformation ensured that no data points equalled 0 or 1 and prepared the data for subsequent transformation using the logit function. The logit function (Eq. 2) takes account for the fact that the mineralogical measurements used are proportions (p) (Warton & Hui 2011).

Equation 2:logit function

$$logit\ (p) = \ln\left(\frac{p}{[1-p]}\right)$$

The MCMCglmm library (Hadfield 2009), for the R statistical language (R Core Team 2013), was used to model the comparative effects of phylogeny and morphology on skeletal mineralogy. For the mineralogical measurements of Wt% Mg-calcite and Wt% calcite seven generalised linear mixed-models were fitted; a full model looking at the combined effects of phylogeny, cross-sectional area of dominant crystal form and morphological complexity; three further models looking at the mixed effects of phylogeny & morphological complexity, phylogeny & cross-sectional area of dominant crystal form and morphological complexity & cross-sectional area of dominant crystal form. Three further models look at phylogeny, cross-sectional area of dominant crystal form and morphological complexity alone as predictors.

MCMCglmm generates model parameters using a Markov chain Monte Carlo (MCMC) algorithm (Sorensen & Gianola 2002) which are then applied to the generalized linear mixed-model. For each model 65,000 iterations were run and mixing and convergence were assessed using the methods implemented in the CODA package (Cowles 2013). All models showed consistent parameter estimates and low values for autocorrelation, indicating that the models were well mixed and had reached convergence. The fit of the models to the mineralogical data was compared using the deviance information criterion (DIC); models with low DIC are preferred (Hadfield 2012; Smith et al. 2013).

6.5 Results

6.5.1 Skeletal ultrastructure

The ultrastructure of fourteen bryozoan species, comprising ten UK and four Antarctic species, are presented in Figures 6.3 – 6.5.

Table 6.1: Summary table of species Mg-calcite, ultrastructure fabric present and (log) cross-sectional area of dominant fabric. Wt% Mg-calcite refers to wt% $MgCO_3$ in calcite.

Species	*Wt% Mg-calcite*	Ultrastructure fabric								dominant fabric x-section area
		1	***2***	***3***	***4***	***5***	***6***	***7***	***8***	*log(nm^2)*
Aetea anguina[5]	7.86		x	x						4.70
Annectocyma tubulosa[3]	6.4				x	x	x	x		5.18
*Antarctothoa antarctica**	0.6			x			x			6.18
Cellaria fistulosa (IMC phase)	7.6	x		x	x		x			4.85
Cellaria fistulosa (LMC phase)	1.9									7.19
Celleporella hyalina[2]	0.2			x			x			7.45
Cellepora pumicosa[1,2]	6.9					x	-			5.09
Conopeum reticulum	5.04	x								5.18
Conopeum seurati[5]	9.1	x	x							2.20
Diplosolen obelia[3]	5.75			x		x				6.40
Einhornia crustulenta[5]	4.11	x	x			x				5.18
Electra pilosa[1,2,5]	8.58	x	x	x	x					2.20
Electra pilosa	8.58				x					4.32
Entalophoroecia deflexa[1,2]	4.5			x		x	x			6.40
Escharella immersa	5.66		x	x	x					1.89
Eucratea loricata[5]	7.74	x	x	x						1.89
Fenestrulina malusii[4]	2.84			x			x			4.18
*Fenestrulina rugula**	4.92	x	x							2.50
Flustra foliacea	7.9		x		x					3.51
*Hippadenella inerma**	5.02								x	4.48
Exidmonea atlantica[3]	3.77				x	x	x	x		6.18
*Inversiula nutrix**	2.64			x		x				6.40
Membraniporella nitida	6.25	x	x		x					2.20
Microporella ciliata	6.88		x		x					2.20
Plagioecia patina[1,3]	6.24			x		x	x			6.40
Scruparia chelata[5]	7.91	x	x			x				1.89
Tubulipora liliacea[3]	2.56				x	x	x	x		5.18
Tubulipora plumosa	1.03					x	x			6.61

Ultrastructure: 1 = wall-perpendicular prismatic; 2 = rod-like; 3 = wedge-shaped granular; 4 = acicular crystallites/planar spherulitic; 5 = rhombic semi-nacre; 6 = transverse fibrous platey; 7 = foliated; 8 = rod-like with tri-pointed axis. Underlined x indicates dominant fabric.

** = antarctic species; [1] (Williams 1990); [2] (Tavener-Smith & Williams 1972); [3] (Taylor & Weedon 2000); [4](Sandberg 1971); [5](Weedon & Taylor 2000); underlined species indicate those analysed by the author*

The fabrics and ultrastructure properties of each species are summarised in Table 6.1 alongside additional data from literature.

Antarctothoa antarctica (Fig. 6.3A) features a spirally growing, foliated fabric consisting of overlapping tablets of laminar calcite. Although these layers are very thin (100-200nm) they can be relatively large plates (over 10µm) with well defined leading edges. *Hippadenella inerma* (Fig. 6.3B) in contrast, features crystallite needles with a characteristic equiangular three-pointed star axis. These needles are of unknown length but the diameter of the axis is less than 1µm, with each star "arm" measuring approximately 400nm in length.

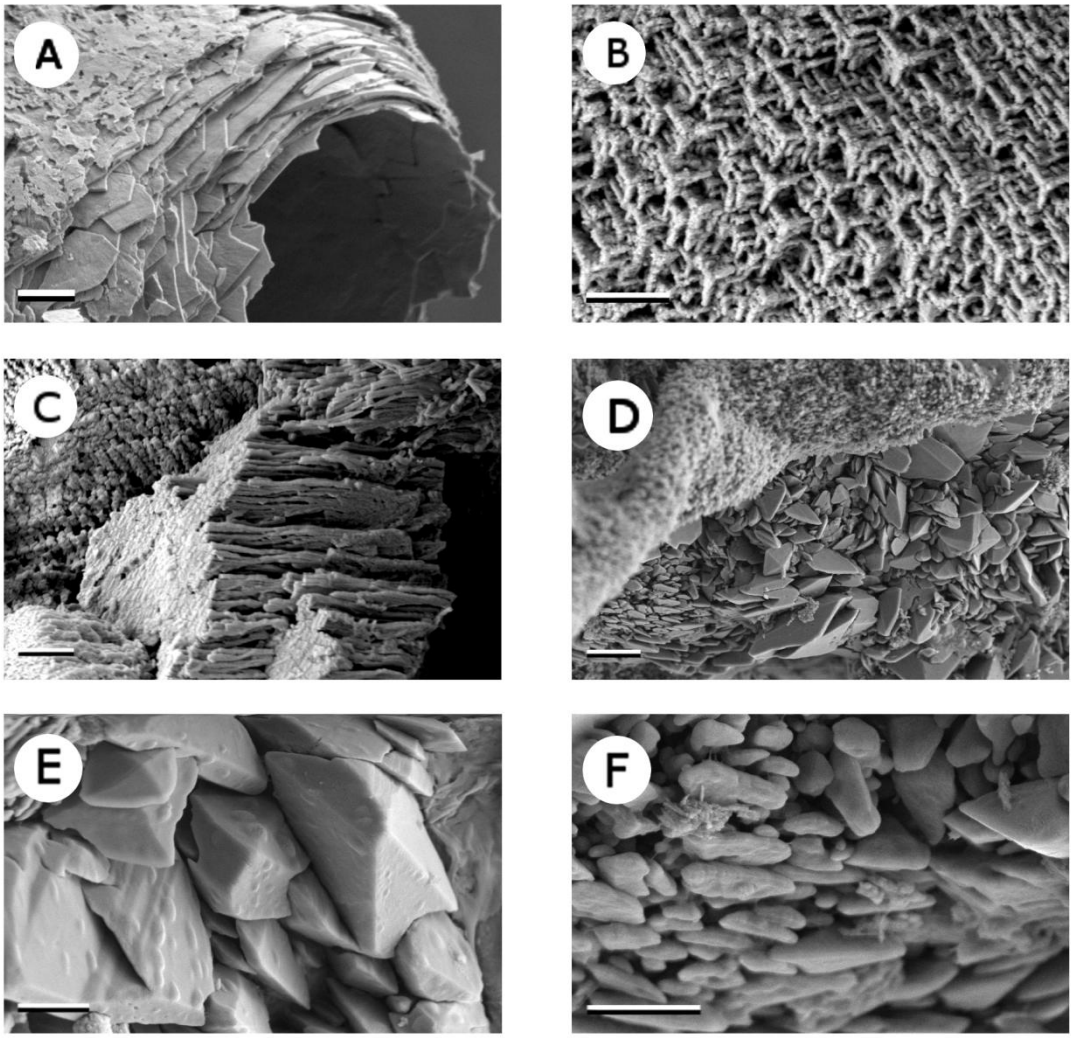

Figure 6.3: Skeletal ultra-structure of Antarctic species. A= Antarctothoa antarctica; foliate, overlapping laminar tablets, scale = 2µm, B= Hippadenella inerma crystallite needles with equiangular three-pointed star axis, scale = 1µm, C = Fenestrulina rugula crystallite needles, scale = 1µm, D = Inversiula nutrix showing two crystal structures, base fabric (shown magnified in E) and over-layer (magnified in F), scale = 2µm, E = I.nutrix semi-nacre tablets, scale = 1µm, F = I.nutrix wedge shaped crystallite, scale = 1µm.

Fenestrulina rugula (Fig. 6.3C) also features needles as its dominant crystal morphology although these appear more cylindrical in shape (approx diameter 100nm) and are an average of 4µm in length; they are categorised as rod-like by Weedon & Taylor (2000) definition.

Inversiula nutrix (Fig. 6.3D-F) features two distinct crystal morphologies (Fig. 6.3D) with the base, dominant fabric consisting of rhombic semi-nacre tablets 1-3µm in length (Fig. 6.3E). These feature distinct triangular sectors, fused at the centre of the tablet and closely resemble those described in bivalves by Taylor (1995). The secondary over-layer of granular fabric is made of a thin layer of wedge shaped crystallites (Fig. 6.3F) approx 200-400nm long, as described by Taylor & Weedon (2000). These crystallites are tightly packed and form an almost smooth outer surface.

Escharella immersa (Fig. 6.4A-B) features two distinct forms of ultrastructure – the zooid interior walls are formed from a densely packed fabric of calcite needles formed from small (<25nm) crystallites (Fig. 6.4A), while the frontal shield is formed from more blocky (~200-400nm) aragonite crystallites (Fig. 6.4B).

Microporella ciliata (Fig. 6.4C-D) features a dominant ultrastructure of irregular calcite laminar needles. Needles are very thin (~25-50nm) and long (~1µm) with varying width and occasional branching. Needles are tightly arranged into a dense fabric with orientation varying in the skeleton (Fig. 6.4D). Additionally large (600nm-1µm) cube shaped crystals (Fig. 6.4D) are observed at the growing edge of the zooid wall. It is unclear whether these are "planned" structures or the default calcite form for amorphous calcite which is yet to be organised through the growth process.

Membraniporella nitida (Fig. 6.4E-H) features three ultrastructure forms. The first and dominant form is very similar in both size and shape to the laminar "needles" observed in *Microporella ciliata* and is found densely packed in both spines (Fig. 6.4E) and avicularia (Fig. 6.4H) where alterations in crystal orientation are clearly observed. The second ultrastructure form is found predominantly in the zooid walls and has a strong, columnar form with columns >200nm in diameter and often exceeding 1µm in length. This form is also found in avicularia walls underneath a secondary layer of laminar "needles". Additional acicular crystallites were also found in the avicularia, deposited in the plane of the wall (Fig. 6.4H).

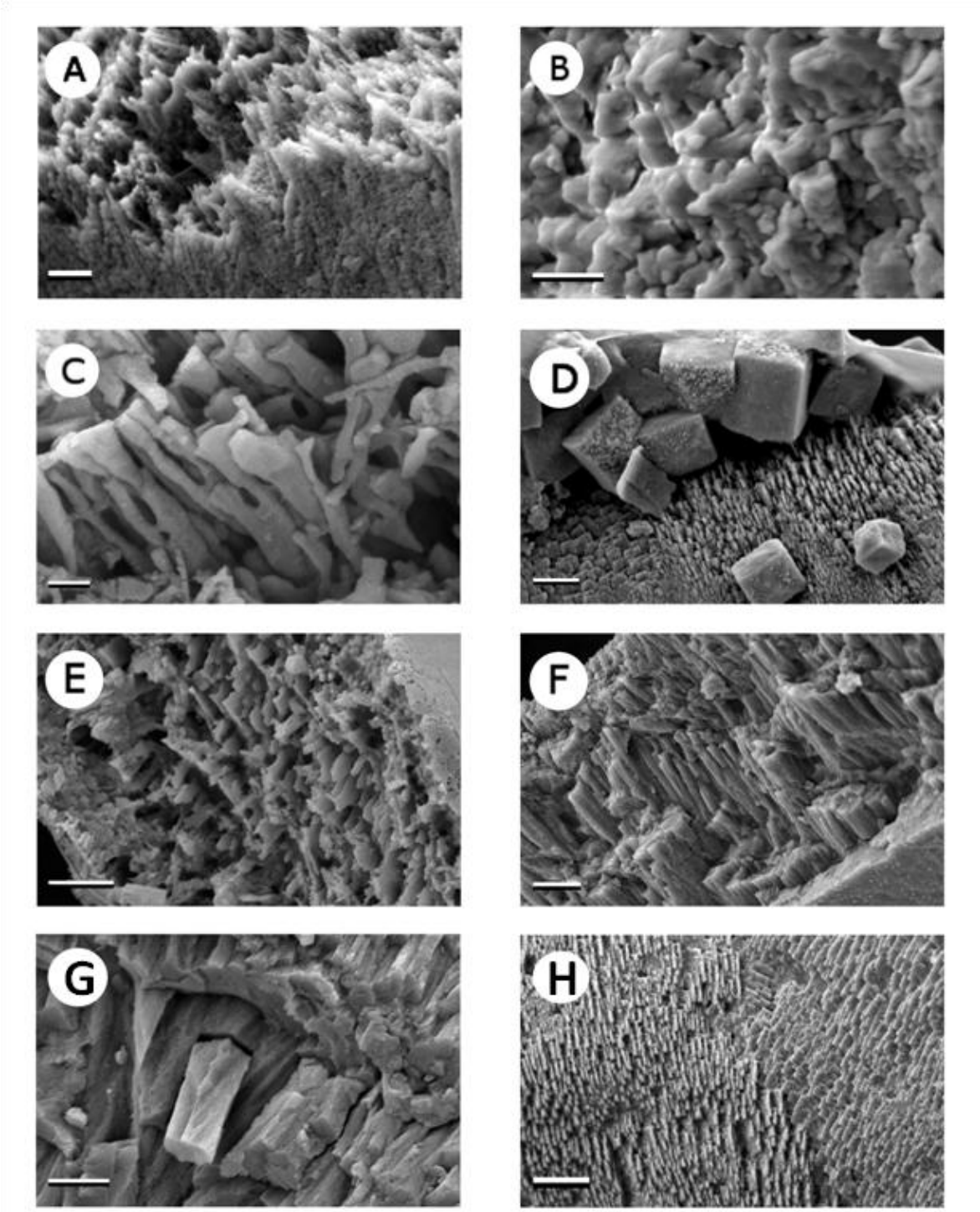

Figure 6.4: Skeletal ultra-structure of Orcadian species. A and B= Escharella immersa, A=crystallite needles (zooid wall), scale = 300nm, B = aragonite crystallites (frontal shield), scale = 400n. C and D = Microporella ciliata; C = irregular foliate needles (spine), scale = 200nm, D = mixed crystal forms (zooid wall), scale = 600nm. E- H = Membraniporella nitida, E = irregular foliate needles (spine), scale = 1µm, F = columnar structure (zooid wall), scale = 1 µm, G = columnar structure (avicularia), scale = 600nm, H= crystallite tablets (avicularia), scale = 1µm.

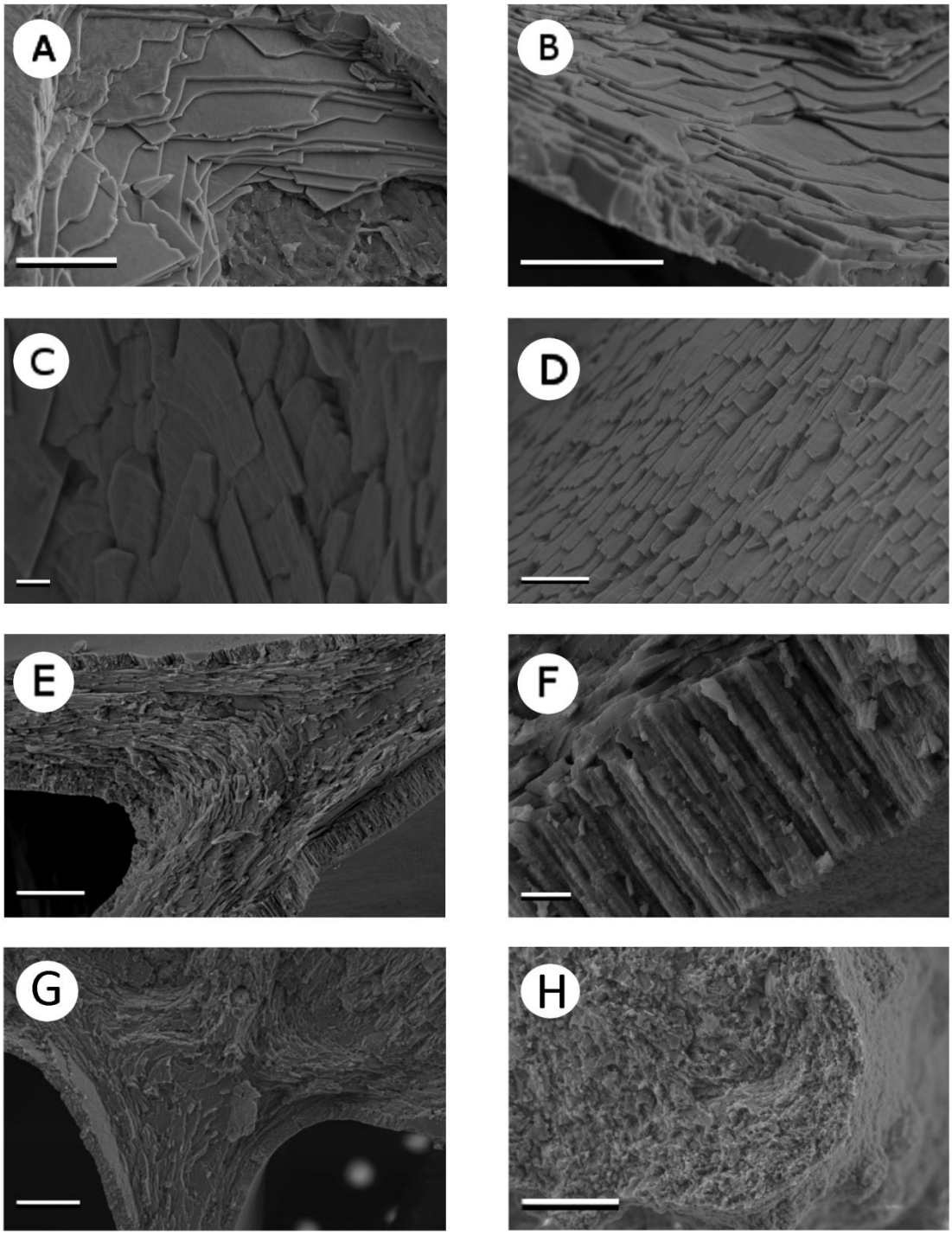

Figure 6.5: A-B= Celleporella hyalina; A = foliate, overlapping laminar tablets, scale = 5µm, B= crystallite needles with equiangular three-pointed star axis, scale = 5µm. C-D = Tubulipora plumosa; C= rhombic semi-nacre, scale = 1µm, D = transverse fibrous platey, scale = 5µm. E-H = Cellaria fistulosa; E = cross-section of internal septum showing internal transverse firous platey fabric and wall perpendicular prismatic columns (magnified in F , scale = 5µm, F = perpendicular prismatic columns, scale = 500nm, G = cross-section of top part of internal septum leading towards exterior wall of internode (top), scale = 10µm, H = exterior wall of internode showing a dense external fabric of wedge shaped granular crystallites over a scattering of acicular crystallites , scale = 5µm.

Celleporella hyalina (Fig. 6.5A-B) features a dominant ultrastructure of spirally deposited foliate overlapping laminar tablets. These flattened imbricated tablets are approximately 10 µm in length and are very thin (~50nm). Due to the complex wall structure (Fig. 6.5A), it is not possible to accurately determine the full extent of each individual crystal tablet. Predominantly right-handed screw dislocations are evident. The foliate laminar tablets are overlaid by a thin layer of dense granular fabric (Fig. 6.5B).

Tubulipora plumosa (Fig. 6.5C-D) primarily features a fabric of rhombic semi-nacre keeled laminae (2-4µm wide) bearing parallel growth striations at approximate 150nm intervals (Fig. 6.5C), a secondary fabric of narrower (1-1.5µm width), transverse fibrous plates is evident on the wall surface (Fig. 6.5D).

Cellaria fistulosa (Fig. 6.5E-H) features a highly complex ultrastructural arrangement. The dominant fabric is found in the centre of the axis and septums and consists of transverse fibrous plates, approximately 5µm long and 150-200 nm wide (Fig. 6.5E). Internal zooid walls are bounded by a thin (2.5 µm) layer of perpendicular prismatic columns, overlaid on the platey fabric (Fig. 6.5F). The exterior wall of the internode consists of a dense material of wedge shaped granular crystallites which bears regular tubercles of approximately 3 µm diameter; this fabric overlays a less well-defined course granulated layer of acicular crystallites which develops into the fibrous platey dominant fabric.

Fenestrulina malusii (Fig. 6.6A) hollow spine features three - four concentric rings of wedge shaped granular fabric 0.5-1 µm wide. Rings are clearly defined and separated by a join which terminates all crystals at that point. Although not imaged, a secondary fabric of transverse fibrous plates was observed in the zooid walls.

Flustra foliacea (Fig. 6.6B-D) zooid walls constructed of dense fabric of rod-like crystallites of approximately 500nm length and 25-50nm diameter, the dominant material in this species (Fig. 6.6C). Examination of the spines reveals up to six concentric rings of acicular crystallite needles, each ring is 750nm-1 µm wide and is clearly separated into a distinct layer (Fig. 6.6B). These rings form a predominantly hollow spine of approximately 25 µm diameter, with a central domed granular structure (~ 7 µm diameter) at the spine base (Fig. 6.6D).

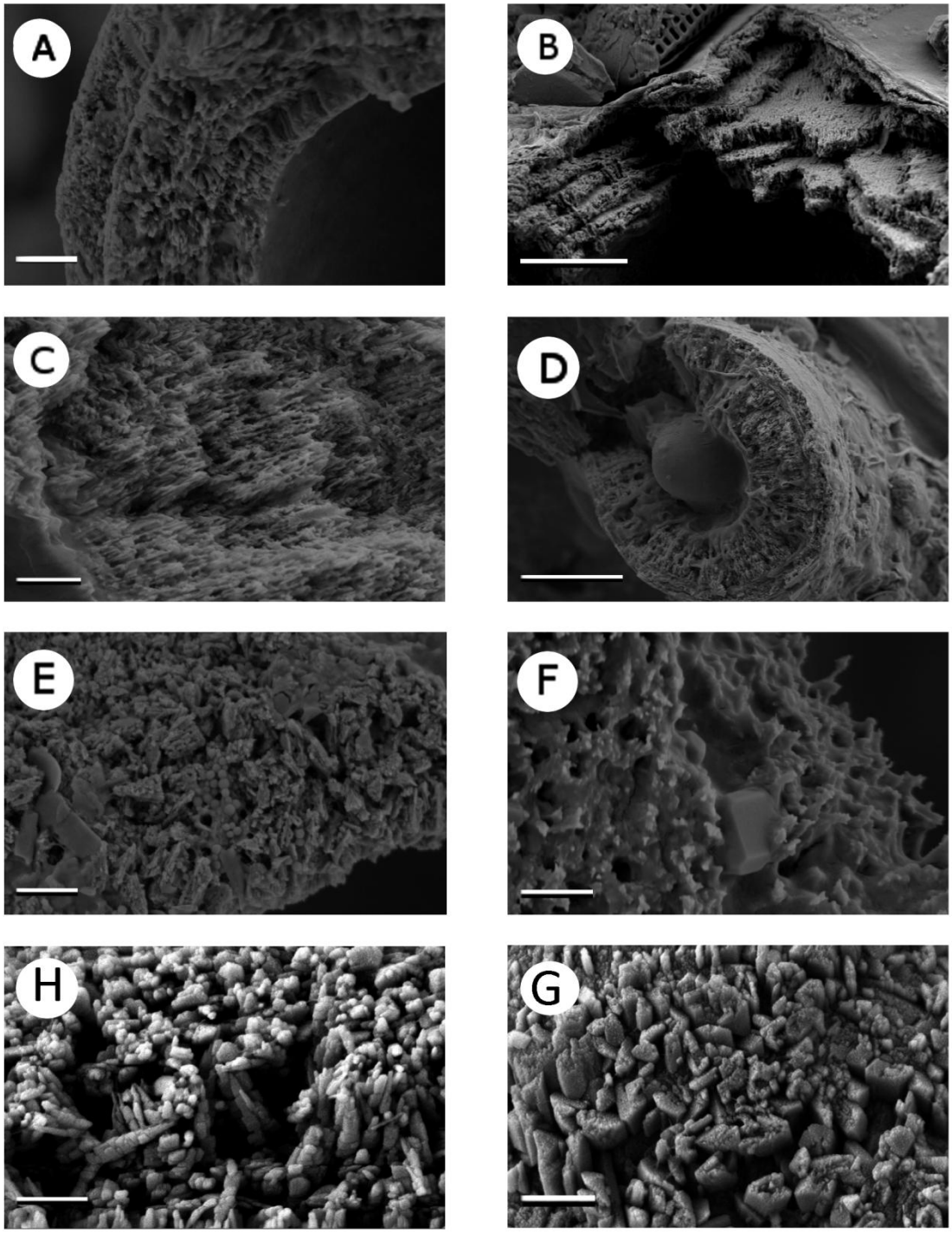

Figure 6.6: A= Fenestrulina malusii, spine; three concentric rings of wedge shaped granular fabric, scale = 1µm. B – D = Flustra foliacea; B= broken spine showing six concentric rings of acicular crystallite needles, scale = 5µm. C= zooid wall with densly packed rod-like crystallite needles, scale = 1µm, D = cross-section of spine showing concentric rings of crystallite needles surrounding a domed dense granular structure, scale = 10µm. E-F= Electra pilosa zooid wall; E =densely packed acicular crystals forming rod-like needles structures , scale =15µm, F = less defined crystals overlaid with granular fabric, scale = 1µm. G-H = Membranipora membranacea zooid wall; G = dense fabric of wedge shaped granular crystallites arranged in closely fitting needles, scale = 500nm, H = acicular planar spherulitic crystallites, scale = 500nm.

Electra pilosa (Fig. 6.6E-F) zooid wall is predominantly composed of acicular crystallites arranged into tightly packed rod-like needles (Fig. 6.6E). The exterior wall is less well defined with an overlay of dense granular fabric (Fig. 6.6F). Wall perpendicular prisms were occasionally observed.

Membranipora membranacea (Fig. 6.6G-H) zooid wall (top) is composed of a dense fabric of wedge shaped granular crystallites (50-100nm width) arranged in needles of between 0.5 and 1µm length and closely fitted together (Fig. 6.6G). This fabric forms shallow waves and troughs on the surface approximately 1 µm apart. The interior of the zooid wall was found to contain larger acicular planar rhombic crystallites (225nm x 100nm) with both right and left-hand screw dislocations.

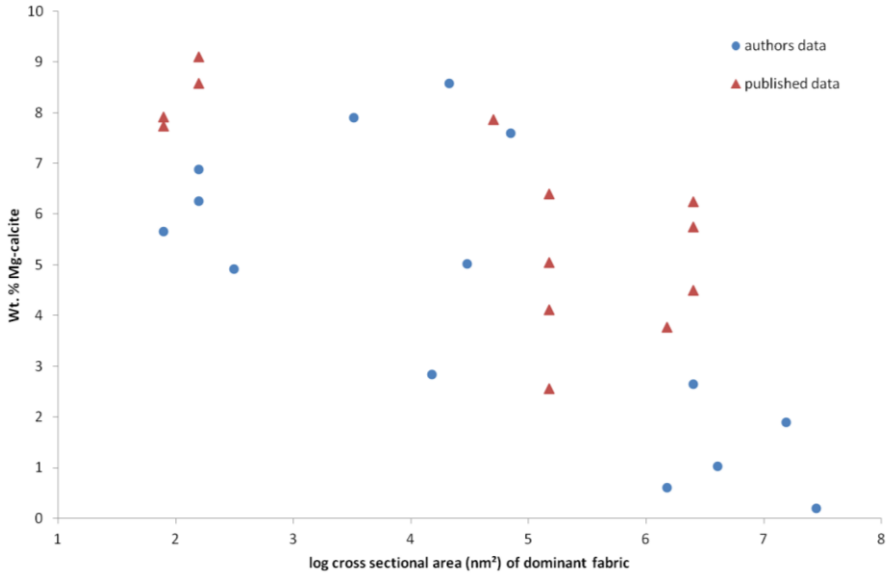

Figure 6.7: Scatter plot showing (log) cross-sectional area of dominant ultrastructure fabric crystals (nm2) against Wt% Mg-calcite. Author's data are indicated with blue circles, published data by red triangles. A quite strong negative correlation ($p<0.001$, $r_s=-0.699$) was found between crystal size of dominant fabric and wt% $MgCO_3$ in calcite. Wt% Mg-calcite refers to wt% $MgCO_3$ in calcite.

The author's images were examined alongside publication images (see Table 6.1) to determine the approximate cross-sectional area of the dominant ultrastructure fabric. Spearman's rank order correlation shows there to be a statistically significant negative correlation between (log) cross-sectional area of the dominant ultrastructure fabric (Tab. 6.1) and the mean species wt% $MgCO_3$ in calcite ($P<0.001$, Spearman's *rho*, $r_s = -0.699$) (Fig. 6.7). This correlation indicates quite a strong tendency for smaller crystals to feature higher wt% $MgCO_3$ in calcite.

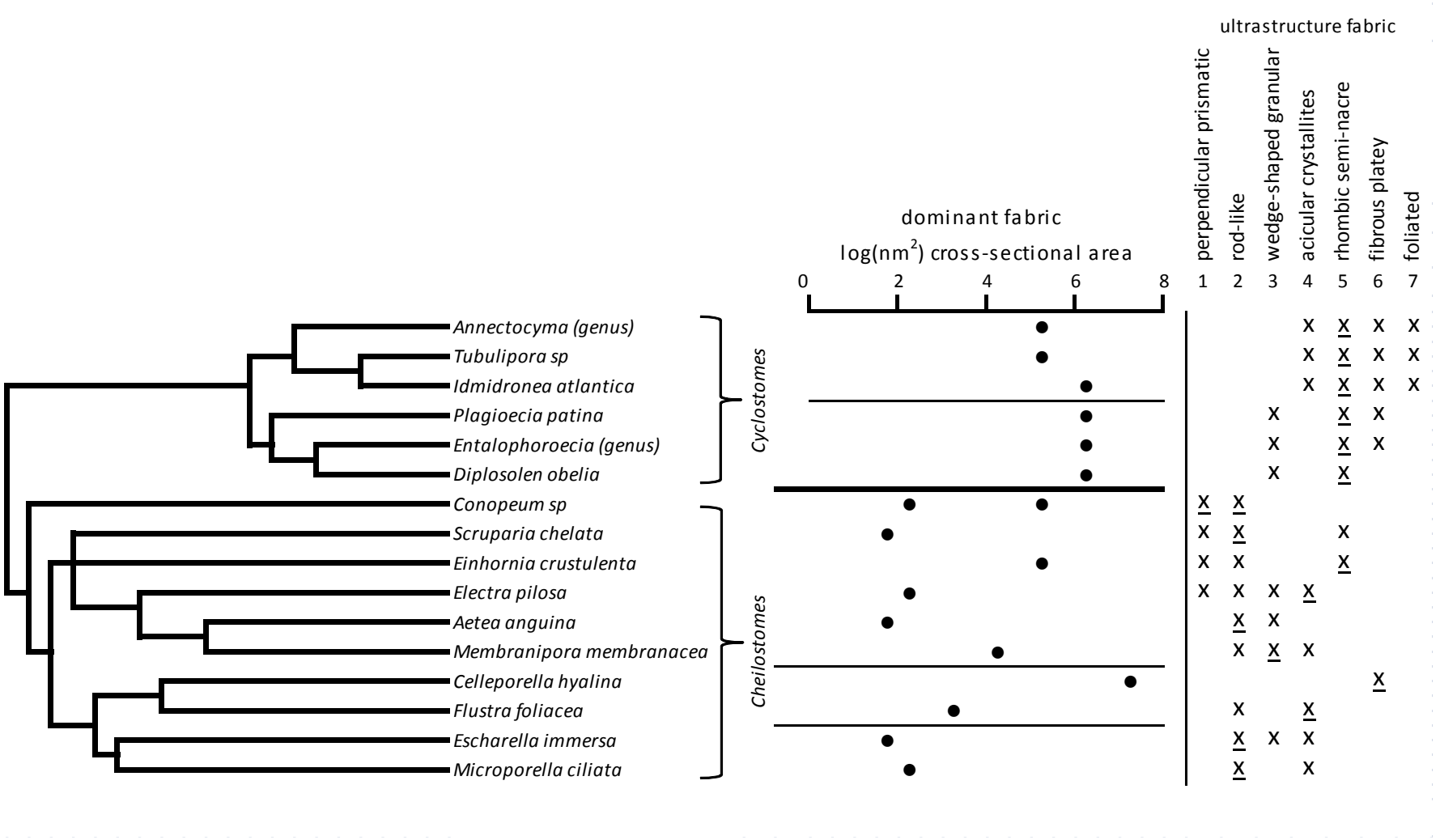

Figure 6.8: Chart showing the log cross-sectional area of the crystals in the dominant fabric and the types of ultrastructure fabric present. Species are organised phylogenetically (Waeschenbach et al. 2012a).

6.5.2 Spatial localisation of Mg-calcite and aragonite in bryozoan skeletons

Specimens of sixteen species were stained with titan yellow or Meigen's solution to investigate the localization of $MgCO_3$ (Titan Yellow stains $MgCO_3$ orange/red) and aragonite (Meigen's solution stains aragonite purple). This showed that in all calcitic species there was some degree of localization of $MgCO_3$ in skeletal features (Fig. 6.9 – 6.10).

F. rugula (Fig. 6.9A) exhibits a localization of $MgCO_3$ in ovicells, around the opening of pseudopores and around the apex of the umbo.

I. nutrix (Fig. 6.9B) shows distinctive localization of $MgCO_3$ in two clear concentric rings around the spine axes.

H. inerma (Fig. 6.9C) features $MgCO_3$ in ovicells, spines, avicularia rim, orifice rim/collar and around the apex of the umbo.

A. antarctica (Fig. 6.9D) features some localization of $MgCO_3$ in the ovicells and around the orifice rim.

Membraniporella nitida (Fig. 6.9E): $MgCO_3$ was localized around the avicularia opening, in ovicells, oral spines and the connecting walls between zooids. $MgCO_3$ was noticeably lacking from the costae and orifice rim.

Celleporella hyalina (Fig. 6.9F): Faint $MgCO_3$ localization was shown around the opening of the orifice and pseudopores.

Microporella ciliata (Fig. 6.9G): $MgCO_3$ was localized in ovicells, spines, and around the opening of avicularia and orifices. None was shown in the frontal shield.

Escharella immersa (Fig. 6.9H) has localization of $MgCO_3$ in the sub-oral lip, the lyrula and the spines. No $MgCO_3$ was seen in ovicells.

Cellaria fistulosa (Fig. 6.10A) has $MgCO_3$ localization orifice rim, at the connections between zooids and joints between internodes.

Chorizopora brogniarti (Fig. 6.10B) features $MgCO_3$ in ovicells, connecting walls between zooids and around the rims of avicularia and orifices.

Cryptosula pallasiana (Fig. 6.10C) has $MgCO_3$ around the orifice rim.

Electra pilosa (Fig. 6.10D) has localized $MgCO_3$ in the spines.

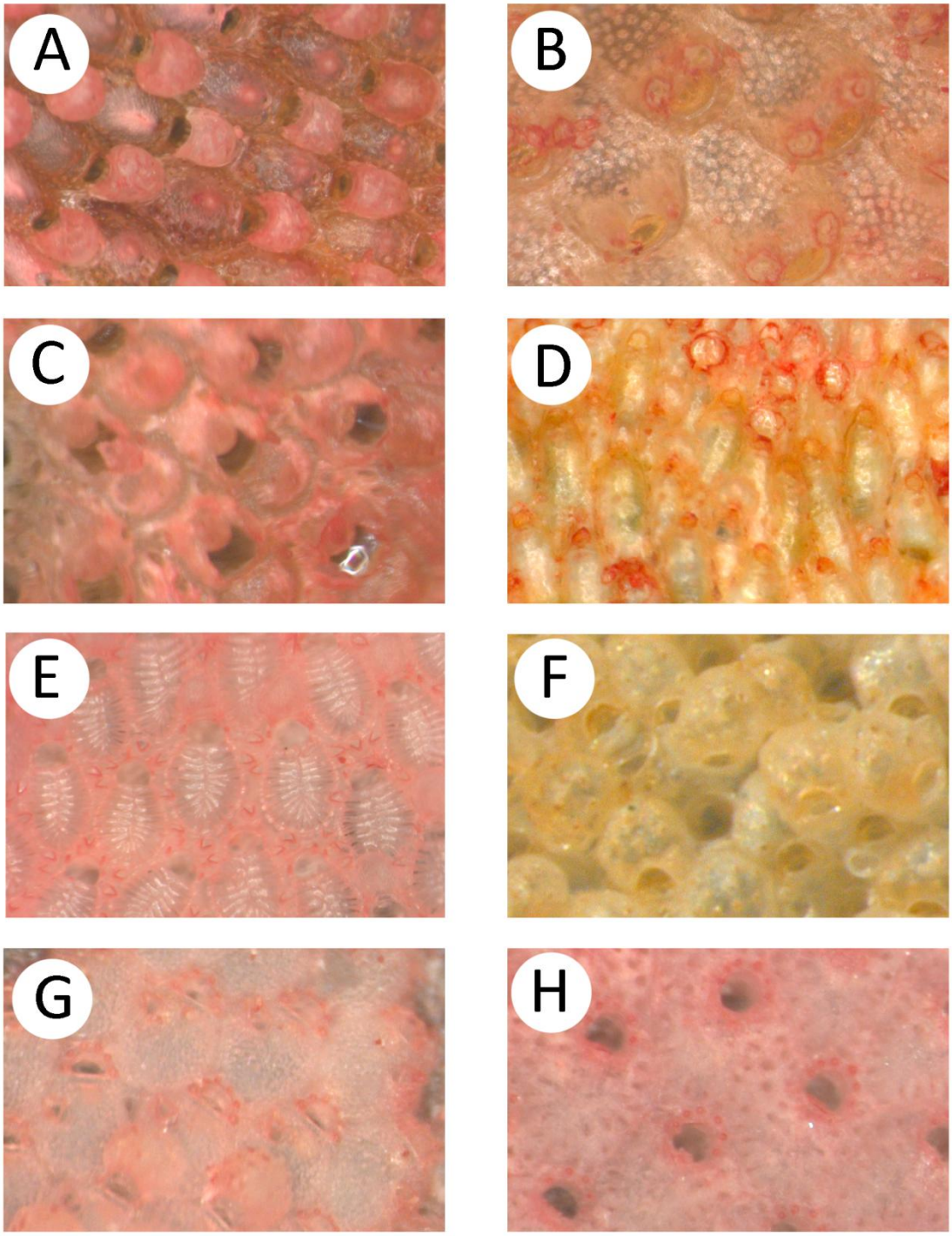

Figure 6.9: Titan Yellow stains $MgCO_3$ red/orange. A = Fenestrulina rugula; $MgCO_3$ localization in ovicells, around the opening of pseudopores and around the apex of the umbo; B = Inversiula nutrix: localization of $MgCO_3$ around the spine axes; C = Hippadenella inerma: $MgCO_3$ in ovicells, spines, avicularia rim, orifice rim/collar and around the apex of the umbo; D = Antarctothoa antarctica: $MgCO_3$ in the ovicells and around the orifice rim; E = Membraniporella nitida: $MgCO_3$ around the avicularia opening, in ovicells, oral spines and the connecting walls between zooids. $MgCO_3$ was lacking from the costae and orifice rim; F = Celleporella hyalina: Faint $MgCO_3$ localization around the opening of the orifice and pseudopores; G = Microporella ciliata: $MgCO_3$ in ovicells, spines, and around the opening of avicularia and orifices; H = Escharella immersa $MgCO_3$ in the sub-oral lip, the lyrula and the spines.

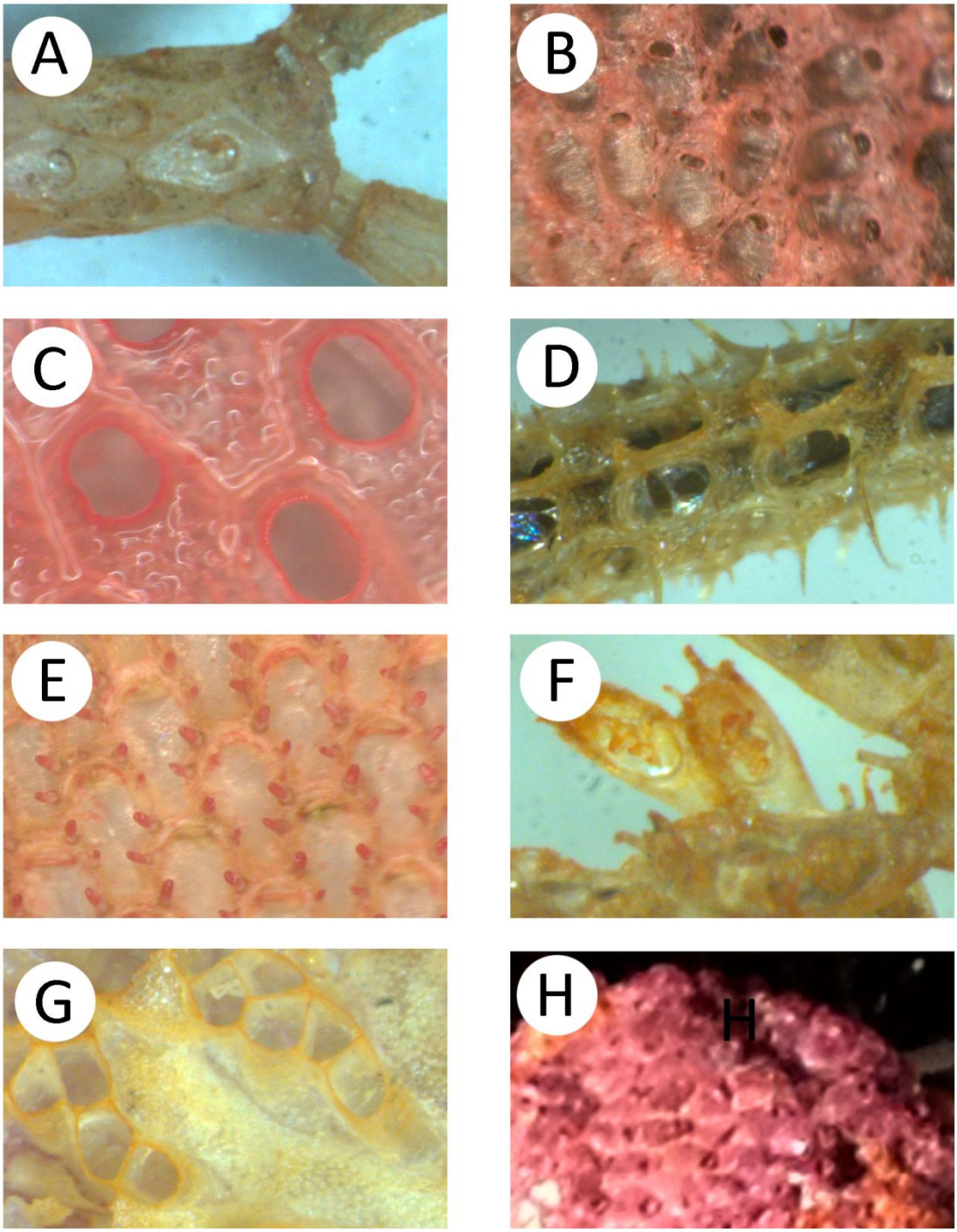

Figure 6.10: A-G Titan Yellow stains $MgCO_3$ red/orange., H = Meigen's stain colours aragonite purple. A = Cellaria fistulosa; $MgCO_3$ localization orifice rim, connections between zooids and internode joints; B = Chorizopora brogniarti: $MgCO_3$ in ovicells, connecting walls between zooids and rims of avicularia and orifices; C = Cryptosula pallasiana: $MgCO_3$ around orifice rim; D = Electra pilosa*: $MgCO_3$ in spines; E = Flustra foliacea: $MgCO_3$ in spines, orifice rim and ovicells; F = Scrupocellaria reptans: $MgCO_3$ in spines, scutum and orifice rim; G=Tubulipora plumosa: $MgCO_3$ localization around the rim of the tubular orifices; H = Anarthropora monodon, stained with Meigen's stain shows the skeleton to be entirely aragonitic.*

Flustra foliacea (Fig. 6.10E) features localized $MgCO_3$ in spines, around the operculum rim and in cap-like ovicells.

Scrupocellaria reptans (Fig. 6.10F) shows $MgCO_3$ in spines, scutum and around the orifice rim.

Tubulipora plumosa (Fig. 6.10G) has $MgCO_3$ localization around the rim of the tubular orifices.

Anarthropora monodon (Fig. 6.10H) is stained with Meigen's stain which shows the skeleton to be entirely aragonitic.

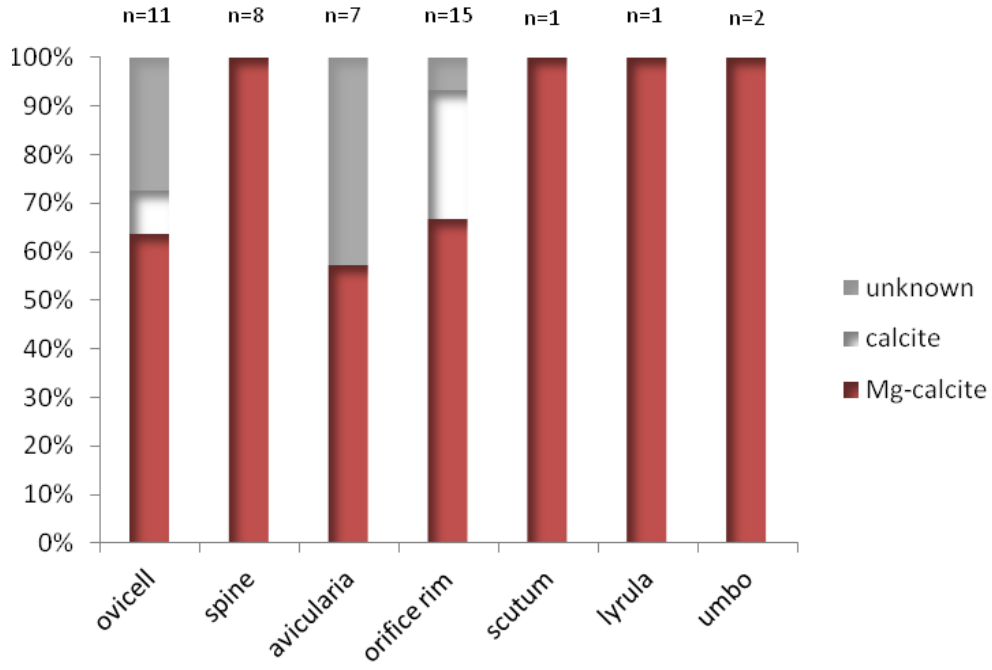

Figure 6.11: Bar graph showing percentage of species which show mineral localisation in different skeletal structures. Red shows Mg-calcite localisation, white calcite localisation (no Mg) and grey indicates unknown due to lack of feature or inconclusive staining.

Staining to highlight the localisation of $MgCO_3$ reveals that minerals are often concentrated in specific skeletal features (Fig. 6.11). All species which featured spines showed localisation of $MgCO_3$ in this structure (n=8). Localisation of $MgCO_3$ in ovicells and avicularia and orifice rims is less consistent between species although it was found in 55-65% of species featuring these skeletal structures. $MgCO_3$ was additionally found to be localised in scutums, lyrulas and umbos and at connecting walls between zooids. Skeletal features notably not containing $MgCO_3$ include costae and frontal shields and cyclostomatous gonozooids.

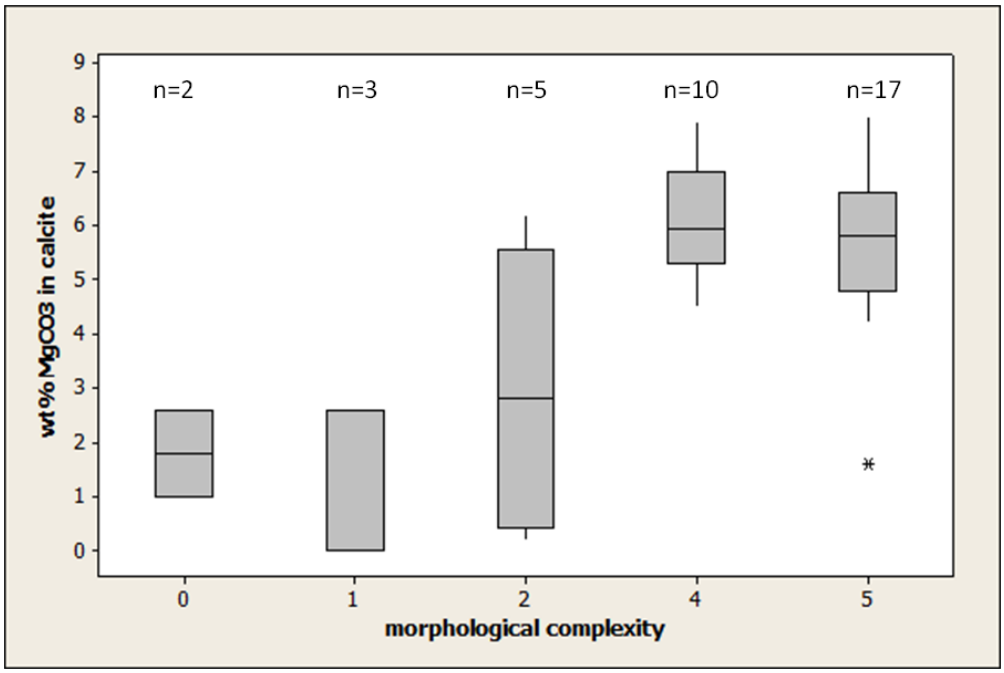

Figure 6.12: Boxplot of species wt% $MgCO_3$ in calcite against morphological complexity. Box indicates inter-quartile range, line indicates mean, tails indicate data range, asterisk marks statistical outlier (Callopora craticula).

6.5.3 Morphological complexity

Morphological complexity was assigned for 50 species for which mineralogy has confidently been determined from the analysis of multiple specimens. A grade of 0-5 was assigned to each species for morphological complexity. 0 indicates no ovicells/avicularia or spines; 1 = avicularia or spines; 2 = ovicells only; 3 = spines and avicularia only; 4 = ovicells and avicularia or spines; 5 = ovicells, avicularia and spines (Tab. 6.2).

There is a statistically significant difference between the mean wt% Mg-calcite for sub-tidal encrusting species grouped by morphological complexity (Tab. 6.2) (GLM ANOVA: $p<0.001$, $F=11.35$) (Fig. 6.12) Inclusion in this analysis is indicated by "Y" in the column "Fig?" in Table 6.2.

Encrusting species which feature spines have a statistically broader range of recorded wt% $MgCO_3$ in calcite than those without spines (Mann-Whitney U-test, $p=0.040$).

A strong and significant phylogenetic signal for morphological complexity (Fig. 6.13) was found (Blomberg's $K = 1.55$, $p<0.001$).

Table 6.2: Mineralogy and morphological complexity of species

Genus	Species	Fig ?	Wt. % calcite	Wt. % Mg-calcite	morphological complexity
Anarthropora	*monodon*	Y	0.0	0.0	1
Amphiblestrum	*flemingii*	Y	100.0	4.2	5
Antarctothoa	*antarctica**	Y	100	0.6	2
Bugula	*neritina*	N	100.0	6.8	2
Callopora	*craticula*	Y	100.0	1.6	5
Callopora	*lineata*	Y	95.6	5.5	5
Carbasea	*carbasea*	N	100.0	7.8	0
Cauloramphus	*spiniferum*	Y	94.9	5.3	5
Cellaria	*fistulosa*	N	100.0	4.5	4
Cellepora	*pumicosa*	Y	96.3	6.9	4
Celleporella	*hyalina*	Y	100.0	0.2	2
Chartella	*papyracea*	N	100.0	9.8	4
Chorizopora	*brogniarti*	Y	100.0	5.5	4
Cribrilina	*annulata*	Y	97.3	6.3	4
Cribrilina	*cryptooecium*	Y	100.0	4.5	4
Crisia	*eburnea*	N	100.0	3.9	0
Cryptosula	*pallasiana*	N	91.1	5.1	1
Dendrobeania	*murrayana*	Y	100.0	4.4	5
Electra	*pilosa*	N	78.9	8.8	1
Escharella	*immersa*	Y	87.5	5.3	4
Escharoides	*coccinea*	Y	74.9	5.8	5
Eucratea	*loricata*	N	98.4	6.2	0
Fenestrulina	*rugula**	Y	97.5	4.9	2
Fenestrulina	*malusii*	Y	88.5	2.8	2
Flustra	*foliacea*	N	100.0	7.9	4
Hippadenella	*inerma**	Y	100.0	5.0	5
Hippoporina	*pertusa*	Y	57.3	6.2	2
Idmidronia	*atlantica*	N	100.0	4.8	0
Inversiula	*nutrix**	Y	99.3	2.6	1
Membranipora	*membranacea*	Y	0.0	0.0	1
Membraniporella	*nitida*	Y	100.0	6.3	5
Microporella	*ciliata*	Y	77.3	6.9	5
Omalosecosa	*ramulosa*	N	97.0	6.2	4
Oschurkovia	*littoralis*	N	89.5	6.5	1
Parasmittina	*trispinosa*	Y	88.6	6.8	5
Pentapora	*fascialis*	Y	61.8	7.3	4
Porella	*concinna*	Y	73.9	8.0	5
Reteporella	*beaniana*	Y	100.0	6.3	5
Reteporella	*watersi*	Y	93.3	6.2	5
Securiflustra	*securifrons*	N	97.0	8.4	4
Schizomavella	*auriculata*	Y	31.6	6.4	5
Schizomavella	*linearis*	Y	20.0	7.2	5
Schizoporella	*japonica*	Y	64.3	5.3	4
Schizoporella	*patula*	Y	7.0	7.9	4
Schizoporella	*unicornis*	Y	45.5	5.8	4
Scrupocellaria	*reptans*	Y	94.8	5.1	5
Scrupocellaria	*scruposa*	Y	96.3	4.6	5
Stomachetosella	*cruenta*	Y	98.0	6.1	4
Tubulipora	*plumosa*	Y	100.0	1.0	0
Tubulipora	*liliacea*	Y	98.9	2.6	0

* indicates antarctic species

Species were assigned a morphological complexity (morph complex) grade of 0-5; 0= no ovicells/avicularia/spines, 1=spines or avicularia only, 2 = ovicells only, 3 = spines and avicularia only, 4 = ovicells and spines or avicularia, 5 = ovicells, spines and avicularia.

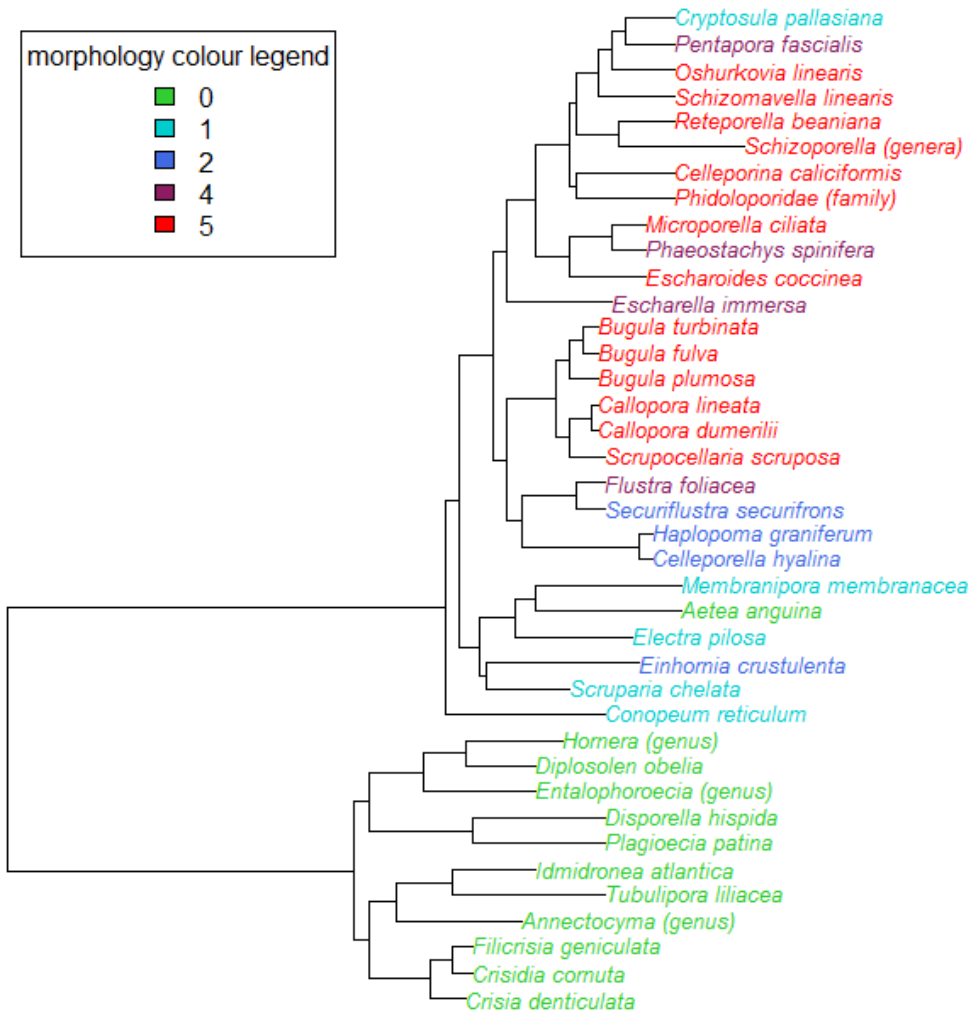

Figure 6.13: Phylogenetic trees modified from Waeschenbach et al (2012a). Branch length indicates number of substitutions per site. Species were assigned a morphological complexity grade of 0-5; 0= no ovicells/avicularia/spines, 1=spines or avicularia only, 2 = ovicells only, 3 = spines and avicularia only, 4 = ovicells and spines or avicularia, 5 = ovicells, spines and avicularia. Species names at the phylogeny tips are colour coded for morphological complexity.

The best fitting MCMCglmm model for wt% $MgCO_3$ in calcite was the model assessing the effects of phylogeny alone. In this case the ΔDIC between the phylogeny model and the next best fitting model, looking at the combined effects of phylogeny and morphology, was very small (2). In the model using both of the factors, phylogeny had the most significant effect on wt% $MgCO_3$ in calcite accounting for 57% of the total variance seen with morphology accounting for an additional 43% of the variability.

6.5.4 Colony morphology and ecological preferences

There is no statistically significant difference between the wt% $MgCO_3$ in calcite for encrusting species versus erect species (n=50). There is a statistically significant difference between the wt% $MgCO_3$ in calcite in erect species with flexible (mean = 7.5 wt% $MgCO_3$ in calcite, n=6), jointed (mean = 6.2 wt% $MgCO_3$ in calcite, n=5), or solid (mean = 4.9 wt% $MgCO_3$ in calcite, n=5) morphology (GLM ANOVA: p=0.018, F = 5.56). Encrusting species which inhabit solely hard substrates are found to feature significantly more aragonite than other calcifying bryozoans (Mann-Whitney U-test: p=0.002).

6.6 Discussion

This study aimed to increase the data available on the crystal ultrastructure, spatial distribution of minerals and morphological complexity of bryozoan skeletons and discuss findings in relation to mineralogy, phylogeny and mechanical properties.

6.6.1 Skeletal ultrastructure

The objective of this component of the study was to investigate and document the ultrastructure fabric in the skeletons of bryozoans. This was achieved through the examination of 14 bryozoan species. Ultrastructure fabrics were identified, measured and compared to previous publications in order to investigate phylogenetic and mineralogical patterns.

CYCLOSTOMATIDA, BUSK 1852

Only a single species, *Tubulipora plumosa*, was imaged in this study and found to have a dominant fabric of rhombic semi-nacre (Fig. 6.5C) and a secondary transverse fibrous platey fabric (Fig. 6.5D). This is largely in keeping with previous examination by Taylor & Weedon (2000), who recognised laminar fabrics overlaid with transverse fibres although they also recognised an additional two fabrics in the species – foliated and acicular crystallites. Our identification of the dominant laminar fabric as "rhombic semi-nacre" for this cyclostomatous species, differs slightly from Taylor & Weedon who describe the laminar fabric as "foliated", progressing through to "hexagonal semi-nacreous and pseudofoliated" material (Fig. 6.14). This difference may be due to the locality of the specimen area examined in our study which concentrated on the inner tube surface (transverse semi-platey) and a broken area of wall (rhombic semi-nacre) and did not include examination of fabrics through the full wall section. Our identification of our laminar fabric as rhombic semi-nacre is in keeping with identification and definition

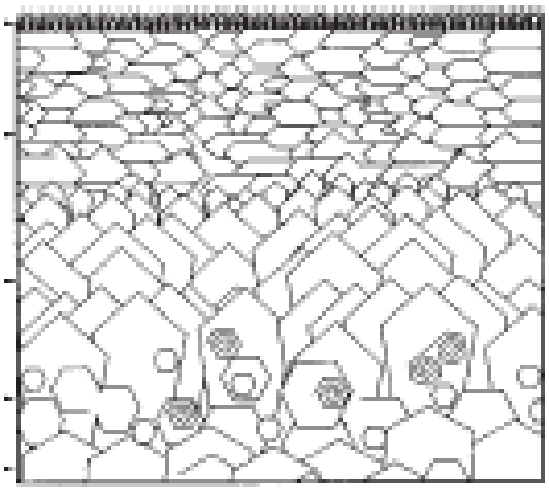

Figure 6.14: Fabric progression." fabric suite 1" reproduced from Taylor & Weedon (2000)

of a similar fabric from *Entalophora* (Tavener-Smith & Williams 1972; Williams 1990) (Figs. 6.15-6.17).

Figure 6.15: Rhombic semi-nacre fabric in Entalophora. Reproduced from Tavener-Smith & Williams (1972)

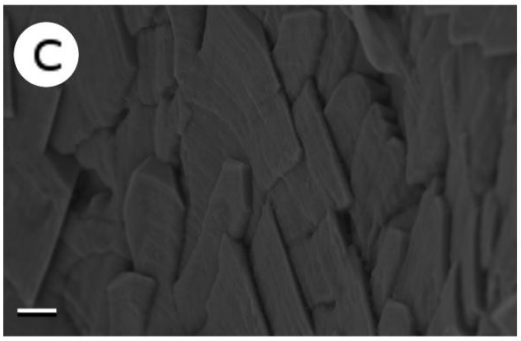

Figure 6.16: Rhombic semi-nacre fabric in Tubulipora plumosa. Reproduced from Figure 6.5

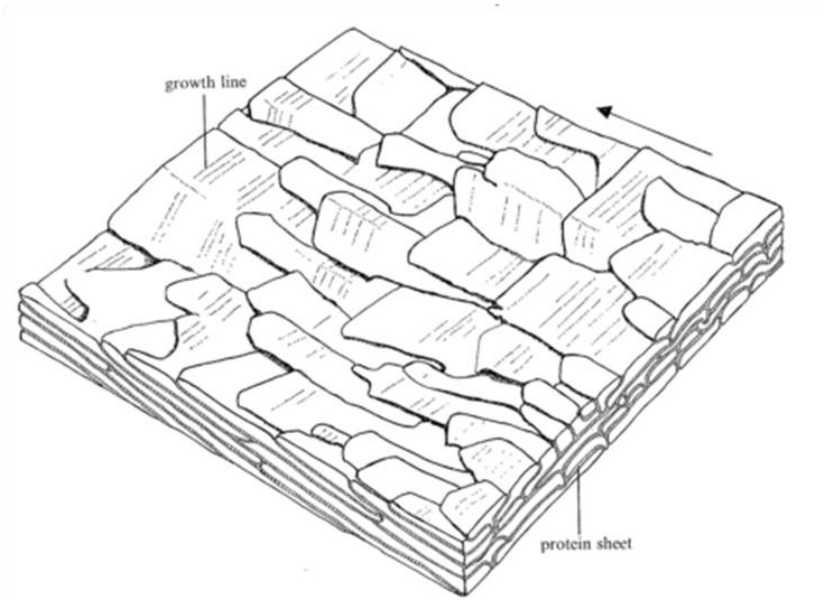

Figure 6.17: Diagram showing the relationship between fibres, some of them keeled, in rhombic semi-nacre in Entalophora. Reproduced from Tavener-Smith & Williams (1972)

This is also indicative of conserved fabrics between closely related species in the cyclostomes as has been previously proposed by Taylor & Weedon (2000). Taylor & Weedon (2000) produced a "phylogeny", actually a morphological cladogram, for cyclostomes based on ultrastructure and morphology alone and grouped *Tubulipora* close to *Idmidronea (*now *Exidmonea)*. Waeschenbach et al.'s (2012a) genetic phylogeny has proved this cladistic grouping correct and also grouped the Genus *Annectocyma* into the same clade. Analysis of ultrastructure fabrics and crystal size in this study (Fig. 7.8) found identical fabric suites between these three taxa, all dominated by rhombic semi-nacre and fitting the described fabric suite 1 by Taylor & Weedon (2000) (Fig. 6.13). A second phylogenetic clade containing the species *Plagioecia patina*, *Diplosolen obelia* and the Genus *Entalophoroecia*,(Waeschenbach et al. 2012a), is also shown to have the same dominant fabric, which appears to be common across all *Tubuliporina* species. In addition the species in this second clade all feature wedge-shaped granular fabric, over-laying the laminar layers on the wall surface (Fig. 6.18). The three species in this second clade show almost identical fabric suites between species and the fabric succession concurs with that described as fabric suite 2 by Taylor & Weedon (2000) which has a surface layer of granular fabric over foliated sheets and a base of rhombic semi-nacre (Fig. 6.18).

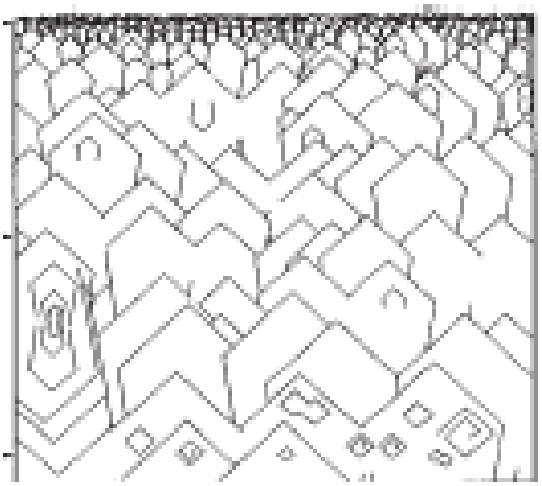

Figure 6.18: Fabric suite 2. Reproduced from Taylor & Weedon (2000)

The six cyclostomes species analysed in this study indicate a strong relationship between phylogeny and ultrastructure. No new fabrics or fabric suites have been identified in the cyclostomes beyond those identified in Taylor & Weedon (2000).

CHEILOSTOMATIDA, BUSK 1852

Super-family*: Membraniporoidea*, Busk 1854

Two species of the super-family, *Membraniporoidea*, were imaged in this study. In addition data was collated for a further three species from publications (Tavener-Smith & Williams 1972; Williams 1990; Taylor & Monks 1997; Weedon & Taylor 2000).

Electra pilosa was found to have a dominant fabric of acicular crystallites and this concurs with previous analyses (Bronstein & Prenant 1936; Tavener-Smith & Williams 1972; Williams 1990; Weedon & Taylor 2000). In previous studies an additional three fabrics were detected which were not observed in this study (Tab. 6.1) – this may be due to the very limited material that was analysed here. The delicate nature of the skeleton combined with the rigorous preparation protocol applied, meant that only a few fragments of wall were available for imaging. It is possible that additional fabrics, which are composed of smaller crystallites than the dominant fabric, were either dissolved or otherwise lost during preparation. Three additional British species from the Family, *Electridae*, were also analysed from data and images in Weedon & Taylor (2000): *Conopeum reticulum*; *C. seurati* and *Einhornia crustulenta*. Within this family there seems to be little in the way of conserved mineralogical or ultrastructure patterns. Wt% $MgCO_3$ in calcite ranges from 4.1 – 9.1 within the family and ultrastructures range from laminar to rod-like. Even within the Genus, *Conopeum*, wt% $MgCO_3$ in calcite ranges from 5.0 to 9.1 and dominant ultrastructures differ. This may be a reflection of the phylogenetic distance within this family with Waeschenbach et al. (2012a) placing both *Conopeum* and *Einhornea* "out on a limb" compared to the rest of the family. Another possibility is that this is a reflection of the diverse ecological niches these species inhabit as both *Conopeum reticulum* (5.0 wt% $MgCO_3$ in calcite) and *Einhornia crustulenta* (4.1 wt% $MgCO_3$ in calcite) favour brackish, estuarine conditions (Hayward & Ryland 1998) and have identical size crystals (5.18 log(nm^2)) forming their dominant ultrastructure fabric (Fig. 6.1). In contrast *Conopeum seurati* (9.1 wt% $MgCO_3$ in calcite) and *Electra pilosa* (8.6 wt% $MgCO_3$ in calcite) favour full salinity habitats and also have the same sized crystals (2.20 log (nm^2) forming their dominant ultrastructure. This may be indicative that a larger crystal with a lower Mg content is more energetically preferable to deposit under brackish conditions and that species may have adapted their ultra-morphology in order to inhabit this ecological niche.

Membranipora membranacea was found to have a dominant fabric of wedge shaped granular fabric with additional rod-like and acicular crystallites also observed. This concurs with fabric descriptions in previous studies (Tavener-Smith & Williams 1972; Ristedt 1977; Weedon & Taylor 2000; Gruhl 2009). Although Ristedt (1977) described *M. membranacea* as calcitic, it is now widely agreed that true *M. membranacea* is aragonitic (Taylor & Monks 1997). The southern hemisphere Genus, *Jellyella*, also falls within the

taxonomic Family, *Membraniporidae*, and is found to have a similar ultrastructure of predominantly rod-like and wedge-shaped granular fabrics (Taylor & Monks 1997; Weedon & Taylor 2000). Interestingly, however, despite the ultrastructural similarity, *M. membranacea* is aragonitic, while *Jellyella* species are calcitic; this is considered by some authors to be evidence of the derived nature of the aragonitic mineralogy of *M. membranacea* (Cheetham 1986; Weedon & Taylor 2000). It should be considered, however, that no phylogenetic data is yet available for *Jellyella*, and the actual phylogenetic relationship between this genus and *Membranipora* is not yet known.

Infra-order: *Ascophora*, Levinson 1909

The only species analysed within the Section, *Acanthostegomorpha*, is *Membraniporella nitida*. This species is found to have a predominantly rod-like fabric with additional wall-perpendicular prismatic and acicular crystallites observed. This seems to concur with previous observations by Tavener-Smith & Williams (1972) and Bronstein & Prenant (1936) although in viewing the prismatic fabric from above and at a lower magnification, Tavener-Smith & Williams (1972) describe the fabric as rhombic crystallites. No other acanthostegomorphs have been ultrastructurally analysed and neither has the phylogenetic placement of any of the *Acanthostegomorpha* been conducted (Waeschenbach et al. 2012) so it is not possible to compare this species to phylogenetically related taxa.

Two species from the Section, *Hippothoomorpha*, have been analysed; the British species *Celleporella hyalina* and the Antarctic species *Antarctothoa antarctica. Celleporella hyalina* has also been previously analysed by Tavener-Smith & Williams (1972) and the results presented in the current study concur with their report that the ultrastructural skeleton consists of distinct laminae (transverse platey fabric), overlaid with an external layer of wedge shaped granular fabric. Despite the large geographical distance between the origin of the two species, this fabric succession is identical to that seen in *Antarctothoa antarctica* with the large crystal size in the dominant fabric and the extremely low wt% $MgCO_3$ in calcite also conserved. Hippothoomorphs which have been phylogenetically analysed to date, have been found to be very genetically similar (Waeschenbach et al. 2012). In combination with the low Mg-calcite observed for the species, and the cretaceous origins of the family (Hippothoidae, FAD 86.3-89.8Ma (Smith et al. 2006)), this points towards a simple ancestral biomineralization and a highly conserved genotype in the

Hippothoomorpha. Although *Antarctothoa* has not been genetically analysed to date, it is predicted that the genus would also fall within the tight *Hippothoomorpha* clade identified by Waeschenbach et al. (2012).

Four species from the Section, *Lepraliomorpha*, have been analysed: three from the family, *Microporellidae* and one from the Family, *Buffonelloididae*. The *Microporellidae* species; (*Fenestrulina malusii*, *Microporella ciliata* and the Antarctic *F. rugula)* and the *Buffonelloididae* species (*Hippadenella inerma* from Antarctica), feature a range of $MgCO_3$ in calcite from 2.84-6.88 wt% and may be calcitic or bimineralic. They also have no obvious patterns in ultrastructure fabric type related to genus or geographical origin. *F. malusii* has been previously analysed by Sandberg (1971; 1977) and the dominant wedge-shaped granular ultrastructure was confirmed in this study. Phylogenetic information is only available for one of our analysed species, *M. ciliata*, but phylogenetic data on other lepraliomorphs places them within the same clade but with a high genetic distance between most species (Waeschenbach et al. 2012a). It is likely that ultrastructure and mineralogy have diverged in our study species since the common ancestor with resultant differences in mineralogy and ultrastructure.

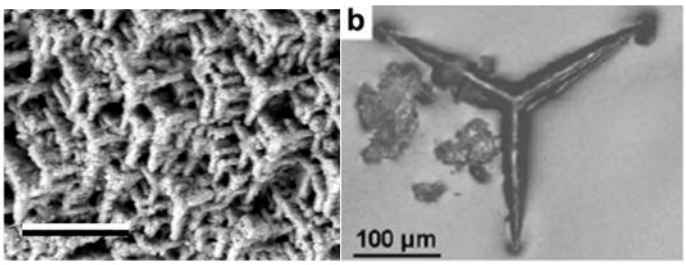

Figure 6.19: left) Rod-like tri-pointed axis fabric from Hippadenella inerma. scale = 1µm, right) Single crystal sponge spicule with tri-pointed axis. Reproduced from Chen et al (2012)

H. inerma features an unusual ultrastructure fabric, not previously reported in bryozoans, which we describe as rod-like with a tri-pointed axis (Fig. 6.20 left). This fabric is very similar in shape, if not size, to the single-crystal calcite spicules observed in sponges (Fig. 6.20 right) and sea-urchin larvae (Chen et al. 2012). In some invertebrates these spicules and rods are formed from Amorphous Calcium Carbonate (ACC) which crystallises in protein "moulds" (Stolarski & Mazur 2005). Although the evidence of ACC has not yet been found in the Bryozoa, spicule–like rods and needles are common, and a "moulded fabric" catch-all has been described by Taylor & Weedon (2000) to encompass such

structures. Meibom (2004) has found that Mg is used in stabilizing ACC and indicates that "high magnesium concentration might in part be the trace element signature of transient ACC". If the tri-pointed axis rods found in *H. inerma* are formed from ACC then this may explain why *H. inerma* features an IMC skeleton of 5.02 wt% $MgCO_3$ in calcite, which is unusually high for a Polar species (Smith et al. 2006; Taylor et al. 2009).

Infra-order: *Flustrina*, Smitt 1868

Two species from the infraorder, *Flustrina*, have been analysed; *Flustra foliacea* and *Cellaria fistulosa*. The calcareous skeleton of *F. foliacea* is constructed from rod-like and acicular crystallites. Early studies by Levinson (1909) and Bronstein & Prenant (1936) investigated skeletal microstructures using thin-section techniques; however it is thought that this study represents the first ultrastructure images of this species. No ultrastructure studies have been conducted on species which are known to be closely phylogenetically related to *F. foliacea* and although *C. fistulosa* falls within the same Infra-order, they are from different families and are morphologically and mineralogically dissimilar. *C. fistulosa* is the only British species to feature two distinct phases of $MgCO_3$ in calcite and this is reflected in the clear succession of ultrastructures in its skeleton. This will be discussed in detail in Section 6.6.5 as a combined ultrastructure and spatial distribution case-study.

Overall in this study cheilostomes show less of a phylogenetic link to ultrastructure fabrics and fabric suites than cyclostomes and this may be attributed to the greater genetic distance of species from common ancestors than in the cyclostomes (Fig. 6.8). Only a small proportion of cheilostomes have been ultrastructurally imaged so far however, and even fewer have phylogenetic data available; there is plenty of scope for elucidation of this pattern through future experimentation.

Comparison of $MgCO_3$ content of calcite against (log) cross-sectional area of the dominant crystal form reveals quite a strong negative correlation (Spearman's rank order correlation: $p<0.001$, Spearman's *rho*, $r_s = -0.699$) with smaller crystals tending to feature higher Mg-calcite. There are two deposition related possibilities as to why this may occur: 1) Involvement of organic material in the deposition process; 2) Utilisation of ACC as a crystal precursor in the deposition process.

1) Tavener-Smith & Williams (1972) show that all skeletal deposition occurs in association with an organic component; the periostracum, epithelial layer or protein filaments or layers.

The study of shell deposition in the Mollusca is the most advanced among marine invertebrates due to the commercial importance of calcium carbonate in this group. Within the molluscs the association between shell deposition and organic components has also been documented by many authors (e.g. Dauphin & Denis 2000; Nudelman et al. 2006; Furuhashi et al. 2010) who record that control of mineral formation is entirely exerted by the macromolecules of the organic matrix, especially specialized proteins (e.g. Marin & Luquet 2004; Addadi et al. 2006; Marie et al. 2008; Furuhashi et al. 2010). The association between organic material and calcium carbonates has also been well documented in other groups e.g. echinoderms (Feng et al. 2011), Sabellids (Vinn et al. 2008), corals (Tambutté et al. 2006) and crayfish (Inoue et al. 2008; Shechter et al. 2008; Glazer et al. 2010). The $MgCO_3$ content of calcite deposited in close association with organic material has been shown to be controlled through protein type and structure in sea-urchins (Feng 2011), with *in vitro* studies providing evidence that electronegative groups produce particularly high Mg incorporation (Han et al. 2013).

2) The cause of this trend between crystal size and wt% $MgCO_3$ in calcite may be associated with the relationship between Mg and ACC. ACC has been shown to be a precursor in calcitic biocrystals of sea-urchin spines, some crustaceans and molluscan larval shells (Stolarski & Mazur 2005) resulting in fine single crystal spicules, rods and aragonite needles. Although the evidence of ACC has not yet been found in Bryozoa, spicule–like rods and needles are common. Meibom et al. (2004) found that Mg is used in stabilizing ACC and indicates that "high magnesium concentration might in part be the trace element signature of transient ACC". The speed of crystallization of ACC into calcite has also been shown to be related to final $MgCO_3$ content of calcite and crystal shape (Kitano et al. 1969; Han et al. 2013). The calcification process in Bryozoa is currently poorly understood and needs a lot more investigation; however it is conceivable that ACC is involved in the formation of small, spicule-like rods and needles in Bryozoa and it may be the stabilization of this transient phase which has resulted in high signatures of Mg in these smaller crystals.

The relationship between crystal size and Mg-calcite may also provide reasoning for the mechanical properties of the different minerals, especially the properties of solubility and strength. From the literature we know that lower wt% $MgCO_3$ in calcite is less soluble than higher wt% $MgCO_3$ in calcite (Bischoff et al. 1987). The correlation between crystal size

and wt% $MgCO_3$ in calcite may help to explain this pattern of solubility. The rate of dissolution of a substance can be calculated using the Noyes-Whitney equation which shows the rate of dissolution as dependent upon the surface area of the interface between the dissolving substance (the skeleton) and the solute (seawater); a greater surface area results in a higher rate of dissolution (Dokoumetzidis & Macheras 2006). LMC plates or tablets, although larger, provide a lower interface surface area and less complexity for a set volume than IMC needles – this would indicate that we would expect the IMC needles to be more dissolution susceptible than LMC, a prediction which is reinforced by observations in the literature (Arvidson et al. 2003; Morse et al. 2007; Chen et al. 2012). Solubility of calcite in biological systems is however complicated by factors such as the variability of seawater composition (the solute), the lack of heterogeneity of crystal forms and the involvement of biological systems (Arvidson et al. 2003) and so the data here should not be considered as conclusive.

The smaller volume of IMC needles also allows for more dense packing of the crystals and makes the resultant fabric denser, harder and more flexible than LMC (Chen et al. 2012). Conversely the regularly stacked tiles often seen in LMC are stacked along the c axis of the crystals making the fabric more susceptible to sheering along this fracture plane (Chen et al. 2012). Although the process has not yet been studied in Bryozoa it is usually necessary for invertebrates to counteract this sheering tendency by incorporating organic "glues" and imperfections between tiles (Chen et al. 2012). This process may have been documented by Tavener-Smith & Williams (1972) as they describe that organic material and calcium carbonate is simultaneously extruded within an epithelial fold to form laminae with inter-laminar protein sheets as shown in Figure 6.21. Nacreous tablet formation is most commonly made from aragonite in marine creatures, as in the case of most Mollusca, and bryozoans are unusual in constructing this crystal form from calcite (Weedon & Taylor 2000; Addadi et al. 2006).

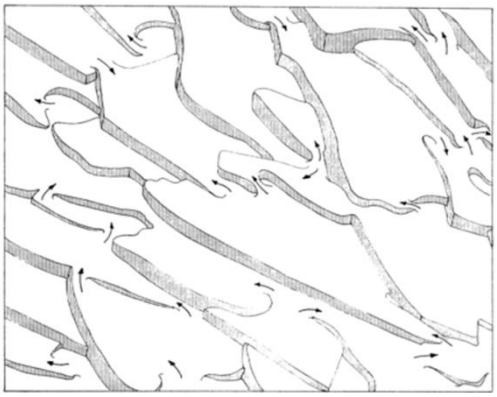

Figure 6.20: Diagram showing overlapping laminae in the cyclostome, Crisidia. Arrows indicate unbroken connections in the interlaminar protein sheets from one level to the

6.6.2 Spatial localisation of $MgCO_3$ and aragonite in skeletal features

Previous studies have predominantly looked at the localisation of calcite versus aragonite in bimineralic species and have found aragonite to be preferentially deposited in frontal shields (e.g. Taylor et al. 2007; Smith & Girvan 2010), and in the bases of free-living bryozoans (Taylor et al. 2009) and usually absent from complex skeletal features such as ovicells, avicularia and orifice rims (Taylor et al. 2007), which were instead found to be constructed from calcite. The localisation of aragonite has not been examined in this study, however it will be investigated in Chapter 7. There have only been a few studies looking at the localisation of $MgCO_3$ in calcite, and these have all been conducted at the colony scale (Smith & Lawton 2010; Smith & Girvan 2010; Loxton et al. 2012). These have all documented increased $MgCO_3$ content in calcite around the base of erect colonies. There have been no previous studies looking at localisation of $MgCO_3$ in the calcite of fine skeletal structures.

In the staining experiments in this Chapter $MgCO_3$ was found to be commonly localised in ovicells, spines, avicularia, and orifice rims. All species (n=8) with spines, showed localisation of $MgCO_3$ in this feature. Additional localisation has been found in the scutum, lyrula and umbos of all species which featured this construct. All of the examined species showed localisation of $MgCO_3$ and there were commonalities in the spatial organisation between many species and phylogenetic clades. Both cyclostomes and most cheilostomes showed localisation of $MgCO_3$ in the calcite surrounding the orifice (or tube) opening. As calcification in these two Orders is thought to have evolved separately (Taylor et al. 2009), then this may be indicative of independent evolution of this mineral organisation.

The reason for the differential Titan Yellow staining of certain skeletal structures could potentially be explained by three theories:

1) Apparent localisation of $MgCO_3$ in distinct skeletal structures could be an artefact caused by differential "shielding" of some structures over others, resulting in differential staining with Titan Yellow. Shielding could be caused by the organic "cuticle" or aragonite. Taylor et al. (2009) discussed a bimineralic species with aragonite localisation on the frontal shield, leaving the avicularia, ovicells and orifice rims as the only exposed calcite. Clear localisation of $MgCO_3$ in the entirely calcitic species, *Antarctothoa antarctica, Cellaria fistulosa, Celleporella hyalina, Chorizopora brogniarti, Cryptosula pallasiana,*

Flustra foliacea, Hippadenella inerma, Mmembraniporella nitida and *Tubulipora plumosa* suggests that this is a highly unlikely explanation.

2) Differential incorporation of $MgCO_3$ in the calcite of certain skeletal structures may be a by-product of the deposition method caused by either i) growth rate or ii) the involvement of a high proportion of organic component. The rate of skeletal deposition has been suggested as an explanation for differences among species in wt% $MgCO_3$ in calcite (Smith 2007; Barnes et al. 2007; Kuklinski & Taylor 2008; Taylor et al. 2009). It is possible that some of the localisation of HMC in seasonal features occurs as a result of the faster rate of skeletal disposition when building structures such as ovicells or spines. The $MgCO_3$ content of calcite can also be influenced by the extent of association with organic material(Feng 2011; Han et al. 2013). It is probable that some of these more complex skeletal features are built around a complex organic matrix and may therefore have a higher organic component than wall and frontal shield material. Although both growth rates and organic components could potentially be contributing factors, these do not, however, explain the variation in Mg-calcite observed within some of these structures. Examples of this can be seen in Figure 6.9E where the concentration of Mg-calcite around the avicularia openings can be observed but not in the rest of the entirely calcitic avicularia and in Figure 6.9B where parts of the spine base show more $MgCO_3$ in calcite than others.

3) Previous studies into biomineralization show that the lower the Mg within calcite the more energetically favourable it is to build (Smith et al. 2006; Taylor et al. 2009). However in some circumstances, less energetically favourable HMC and aragonite is localized in specific regions of the skeleton despite the energetic cost. In these cases it could therefore be assumed that the use of these materials in these locations conveys a competitive advantage to the colony. One competitive advantage may be in the form of increased strength. $MgCO_3$ has been reported to give mechanical strength when localized in spicules in octocorals (Mann 2001) and calcareous sponges (Feng 2011), and has been localised in the teeth of urchins where it has been described as producing "amazing mechanical toughness and hardness"(Yurong & Limin 2010). This property of increased strength could be an explanation for the concentration of Mg around the orifice and avicularia rims of Bryozoa (Figs. 6.9-10). HMC has also been shown to decrease brittleness, and therefore the risk of snapping, when localised in the central axis and around the base of urchin spines

(Loste 2003; Borzęcka-Prokop et al. 2007; Loxton et al. 2012). Bryozoan spines also feature a higher proportion of $MgCO_3$ incorporation than surrounding calcite and this may be evidence for localisation of Mg for improved mechanical properties. Quantitative research into the mechanical properties of different skeletal features would provide evidence of the use of $MgCO_3$ for mechanical and ecological advantage in some bryozoan species.

6.6.3 Morphological complexity

The 50 species examined have varying levels of morphological complexity (Tab. 6.2). There is a statistically significant difference between the mean wt% $MgCO_3$ in calcite of sub-tidal encrusting species grouped by morphological complexity (Fig. 6.12) (GLM ANOVA: $p<0.001$, $F=11.35$). In cheilostomes it has been suggested (Taylor et al. 2009; Smith & Girvan 2010) that evolutionary/phylogenetic patterns of mineralogy are not driven by ocean chemistry or FAD alone but are also related to the increased morphological complexity which started to arise with adaptive radiation. This origination of morphological complexity is evidenced by the MCMCglmm modelling conducted in this study which shows a strong statistically significant relationship between phylogeny and morphological complexity (Blomberg's $K = 1.55$, $p<0.001$). Those species with a greater phylogenetic distance from the common ancestor have a tendency towards greater morphological complexity (Fig. 6.13). This quick analysis only took into account the presence and absence of morphological characters of ovicells, avicularia and spines as a rough grading of morphological complexity. Nonetheless the data showed a strong pattern and a wider analysis with more species and more characters, including the aragonitic characters such as secondary calcification, would be hoped to show this relationship even more decisively.

Examples of morphological complexity and the suggestion of a relationship to skeleton chemistry can be seen in the staining experiments presented in Section 6.5.2. These indicate that complex skeleton features usually have a higher proportion of $MgCO_3$ in calcite, as shown in ovicells, spines and avicularia, or aragonite, as secondary calcification of frontal shields. This relationship between morphology and skeleton chemistry is further evidenced by the MCMCglmm modelling for Mg-calcite where the model assessing the effects of

both phylogeny and morphology found that morphological complexity explained 43% of the variability of $MgCO_3$ in calcite between species.

The seasonal, transient and fragile nature of many of these skeletal features could also be an explanation for the higher range of variability within species and families with complex skeletons. Supporting this theory is evidence that younger families such as *Bugulidae, Bitectiporidae*, *Smittinidae* and *Candidae* all feature highly complex skeletons and also feature amongst the mineralogically most variable of skeletons (see Chapter 3).

6.6.4 Colony morphology and ecological preference

A group with a seemingly ecologically-driven mineralogy are species which form encrusting colonies on solid substrates. These species (e.g. *Cryptosula pallasiana, Escharoides coccinea*) feature significantly more aragonite than other bryozoans which suggests that the mechanical properties of aragonite are advantageous in this habitat. Smith et al. (2006) speculate that an abrasive lifestyle in shifting substrates may require some species to use the stronger, denser and less brittle polymorph, aragonite, regardless of the increased energy cost. In this particular circumstance Smith et al. (2006) are referring to free-living bryozoans living amongst sandgrains, however it could be viable that the same reasoning be applied to species living amongst shifting pebbles and other small, hard substrates. An unusual species, which builds its skeleton from pure aragonite, is *Anarthropora monodon.* The first measurement of mineralogy for this species was confirmed by both duplicate XRD analyses (Chapter 3) and staining using Meigen's stain (Fig. 7.10H). Both species of the genus, *Anarthropora,* are deep water species (Wass & Yoo 2006) with the examined specimens of *A. monodon* collected in 1996 between Shetland and Faroe at an average depth of 550m (range 246m-896m). The Faroe-Shetland channel is an environment of strong flow and swift currents(Scottish Government 2011) and *A. monodon* is found exclusively on small pebbles and gravel (Hayward & Ryland 1999), an abrasive substrate. The combination of cold temperatures, deep-water circulation patterns, low pH and correspondingly low Aragonite Compensation Depth (ACD) of the waters of the Faroe-Shetland channel, an Arctic-Atlantic boundary environment, allow aragonite formation at depths which would not be possible in tropical seas (Berger 1978; Steger & Smith 2005). In this challenging habitat, where a strong skeleton is necessary, *A. monodon* utilises aragonite to occupy this niche.

The competitive advantage of strength may also be the driver for many species to form secondary calcification – the majority of which is formed from aragonite, with occasional examples using HMC (Taylor et al. 2009). These species predominantly encrust hard substrates, although additionally some secondary calcifying species form erect foliose colony forms (e.g. *Reteporella beaniana* and *R. watersi*). It is surmised that the increased aragonite and/or $MgCO_3$ in older (basal) parts of the colony, with its extra hardness and decreased brittleness, gives mechanical advantages over calcite (Taylor et al. 2009; Smith & Lawton 2010; Smith & Girvan 2010). An early study by Cheetham & Thomsen (1981) investigated the relationship between colony morphology and mechanical properties in cheilostomatous Bryozoa. Unfortunately the majority of the samples they examined were either fossilised or heavily bleached, and therefore the mechanical properties may have been compromised by diagenesis (Zuschin et al. 2003) or through the preparation procedure (see Chapter 2). Cheetham & Thomsen (1981) did, however, observe that the strongest skeleton was *Cystisella sacatta* (one of the few species for which they examined fresh material). Cheetham & Thomsen (1981) expressed surprise that a calcitic erect Polar species would prove to have a stronger skeleton than aragonitic species. Three decades later XRD studies have shown that despite its high latitude origins *C. sacatta* is constructed from high magnesium calcite (Loxton et al. 2012), providing additional evidence of the mechanical advantage provided by this material.

There are also species which either form a flexible erect colony (e.g. *Flustra foliacea, Carbasea carbasea, Chartella papyracea, Securiflustra securifrons*) or which encrust on flexible substrates such as kelp (e.g. *Electra pilosa*). Erect flexible species, such as *F. foliacea*, are most commonly found in areas of rapid current flow and incorporate substantial amounts of organic material to help with their flexibility. In addition these species often feature high levels of $MgCO_3$ incorporation in calcite (e.g. *F. foliacea* = 7.9 wt% $MgCO_3$ in calcite, *Chartella papyracea* = 9.8 wt% $MgCO_3$ in calcite, *Securiflustra securifrons* = 8.4 wt% $MgCO_3$ in calcite). Although LMC is less soluble and economically cheaper to produce than HMC it also has more perfect cleavage and is more brittle. Increasing incorporation of Mg into calcite increases its flexibility, an advantage for a species which need to be able to bend in the current.

A statistically significant difference was found in the wt % $MgCO_3$ in calcite among erect species with flexible, jointed or solid colony forms; flexible species feature the highest proportion of $MgCO_3$ in calcite and solid species the lowest.

6.6.5 Cellaria: a case study

Cellaria is the only known genera in Scotland which consistently produces dual phase calcite. The dual phase behaviour of this genus was also observed by Smith et al. (2006) in two species, *Cellaria immersa* and *C. tenuirostris* and by Bone & James (1993) in *C. pilosa*. The mineral distribution of *C.sinuosa* was examined in more depth in the thorough publications by Shäfer & Bader (2005; 2008); this study adds an additional two previously unanalysed species, *C. fistulosa and C. salicornioides*, to this dataset. *Cellaria* is predominantly found in habitats with high water flow. In order to occupy this challenging ecological niche it is necessary for the colony to bend in the current without snapping. This is aided by strong chitinous joints between internodes and multi-stranded chitinous rhizoids which attach the colony to the substrate but it may also be the purpose of the distinct dual-phase skeleton.

Staining and previous publications (Bader & Schäfer 2005; Schäfer & Bader 2008) localize the first, and usually dominant, of these phases, LMC/IMC, in the central axis of internodes and the second, IMC/HMC, often subdominant phase predominantly around the chitinous joints between internodes and in the outer surface of internodes. Staining of *C. fistuloa* in this study also shows selective localisation of $MgCO_3$ around the joints (Fig. 6.10A). Taylor-Smith & Williams (1972) also noted that the articulation surfaces of jointed colonies of *Crisia eburnea* feature finely granular ultrastructure, a morphology that has been found to usually be associated with higher incorporation of $MgCO_3$ in calcite (Fig. 6.7). This would need to be confirmed through staining experiments but may suggest that the localisation of $MgCO_3$ around the articulation surfaces of jointed colonies has evolved independently in both cheilostomes and cyclostomes (Taylor et al. 2009) and is evidence of the mechanical advantage of this localisation.

Cross-sectional analysis of *Cellaria fistulosa* shows a highly organised ultrastructural organisation from laminar fibrous plates in the central axis (primary fabric), through perpendicular prismatic columns (secondary fabric) to fine granular crystallites (tertiary

fabric) at the exterior wall of the internode (Fig. 6.5 E-H). This ultrastructural determination concurs with a thorough investigation conducted by Tavener-Smith & Williams (1972) (Fig. 6.22).

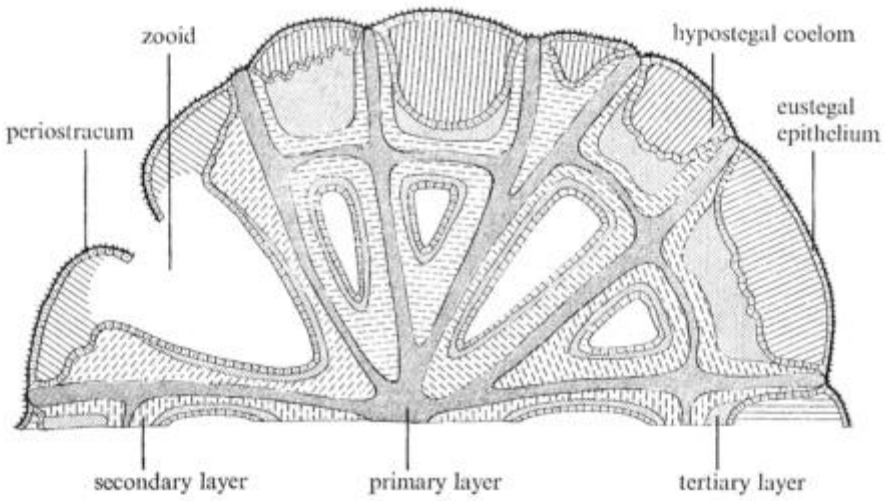

Figure 6.21: Diagram showing the arrangement of secretory epithelium in relation to the skeleton of Cellaria as seen in transverse section. Reproduced from Tavener-Smith & Williams (1972)

Staining confirms that the laminar layer is constructed from low magnesium calcite with the higher proportions of $MgCO_3$ incorporated in the calcitic perpendicular prismatic columns and granular fabric.

Comparing the colony form within the genus, *Cellaria*, *C. fistulosa* has the shortest internodes (4-5mm) of the three species examined but is one of the tallest colony forms (5-6cm) (Fig. 6.15) and the ratio of joints to internodes is correspondingly highest. In the numerous joints, flexibility would give a mechanical advantage and provides an explanation for the localisation of IMC (7.6 wt% $MgCO_3$ in calcite) in this position. These flexible joints enable *C. fistulosa* to make the "bulk" axis of the internodes from the most energetically favourable, but least flexible form of calcite, low Mg-calcite, (1.9 wt% $MgCO_3$ in calcite). *C. sinuosa*, in contrast, has longer internodes (4-10mm) which are also much wider than the other species (0.4-1.6mm) (Fig. 6.23). The greater diameter of the internodes means that the "bulk" axis material is present in greater proportions to the second phase HMC. Additionally the longer length of the internodes and, therefore, greater distance between joints, means that they may benefit from some flexibility. This, in turn, could offer a potential explanation for the IMC first phase (4.91 wt% $MgCO_3$ in calcite).

The joints will be under increased pressure from the greater mass[1] of the internodes and so high Mg-calcite (9.8 wt% $MgCO_3$ in calcite) is utilized to increase strength and flexibility. *C. salicornioides* is the most delicate of the three Scottish species with long (5-10mm) thin (0.2-0.5mm) internodes (Fig. 6.23). The greater length of the internodes compared to the other species would favour a IMC in the axis to prevent the risk of snapping (4.16 wt% $MgCO_3$ in calcite), while the generally low mass of the internodes would allow for a relatively moderate incorporation of Mg around the joints (6.65 wt% $MgCO_3$ in calcite).

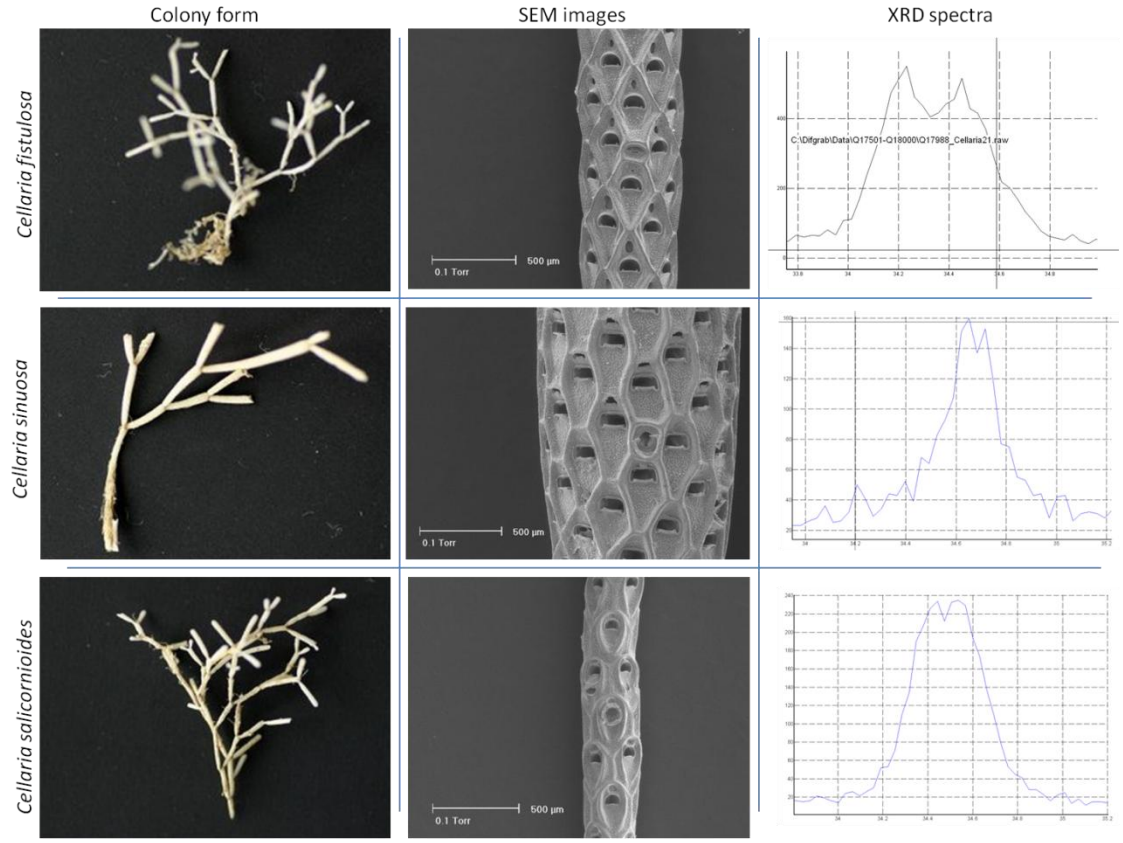

Figure 6.22: Diagram showing different colony morphology and internode diameter between Scottish species of Cellaria. XRD spectra show the characteristic "ragged" peaks indicating the presence of dual Mg-calcite. Colony morphology and SEM images taken from eol.org (Species 2013),

If we apply the same reasoning to mineralogical and morphological data from publications and include a further three species, *C. immersa*, *C. tenuirostris* and *C. pilosa* (Hayward & Thorpe 1989; Bone & James 1993; Smith et al. 1998; Smith & Garden 2012), then we find that there is a statistically significant relationship (regression R^2= 62.53%, f=7.33, p=0.050)

[1] Maximum volume of internodes is calculated assuming cylindrical volume, ($\pi r^2 h$) where r = maximum radius of internode and h = maximum length of internodes.

between volume[1] (and implied mass) of internodes and the concentration of $MgCO_3$ in the second phase calcite (Fig. 6.24).

Together the organisation of $MgCO_3$ and ultrastructures, and the relationship between mineralogy and colony morphology provide evidence that mineralogy and the associated ultrastructures within *Cellaria* are organised to optimise the mechanics of the colony, providing ecological advantage that enables the genus to occupy the challenging fast-flow niche.

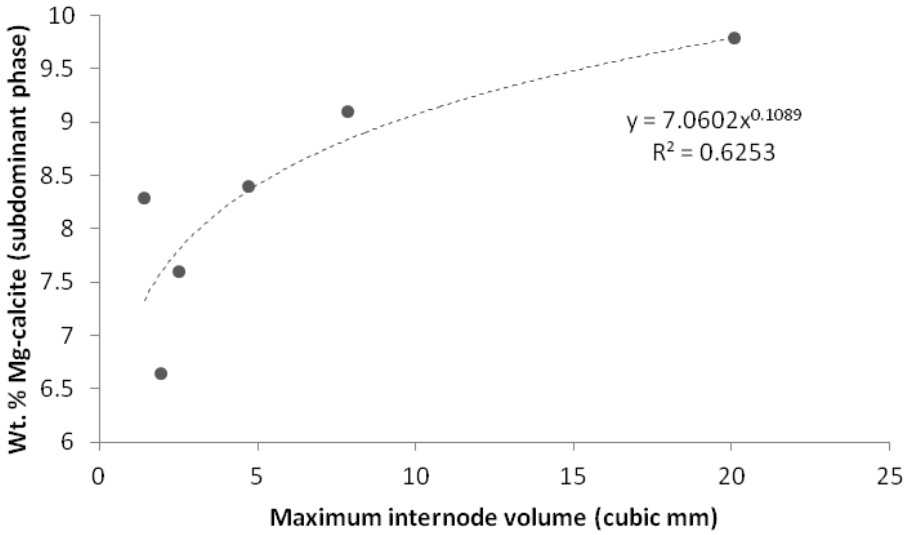

Figure 6.23: Scatterplot showing relationship between internode volume and the Wt% Mg-calcite in the subdominant phase of Cellaria (regression: R^2= 62.53%, f=7.33, p=0.05). The subdominant, IMC/HMC, phase in Cellaria is primarily located around internode joints. Wt. % Mg-calcite refers to wt% $MgCO_3$ in calcite.

6.6.6 Mineralogy and morphology in a wider scientific context

The techniques and results presented in this study show relationships between skeletal mineralogy and morphology at the ultra, micro and macro scale in bryozoans. This raises the possibility for the use of imaging and staining as alternatives to the destructive mineralogy techniques more commonly used (e.g. XRD, microprobe, ICP-AES). More species would need to be analysed however, and more replicates conducted in order to

[1] Maximum volume of internodes is calculated assuming cylindrical volume, ($\pi r^2 h$) where r = maximum radius of internode and h = maximum length of internodes.

calibrate this relationship before morphology could be used as an effective proxy for the quantification of $MgCO_3$ in calcite.

Methodologies could also be further developed in the future with improvements including the combined use of Meigen's stain, EDX and SEM to image aragonite localisation at the ultrastructure level. Meigen's stain causes colour change by attaching cobalt to aragonitic crystals. It is feasible that this could be used in conjunction with EDX (which shows the spatial localization of elements, and could select for cobalt) in order to identify aragonite crystals under ultra-SEM. Meigen's stain has been used in conjunction with SEM in molluscan shells to successfully differentiate between aragonite and calcite (Suzuki et al. 1993) and the application of EDX could potentially advance this technique.

The evidence presented in this Chapter points towards $MgCO_3$ in calcite being differentially deposited in bryozoans skeletons for mechanical advantage. This is in keeping with studies conducted on other taxa (e.g. Mann 2001; Loste 2003; Borzęcka-Prokop et al. 2007; Yurong & Limin 2010; Feng 2011). Bryozoans feature mineralogically complex skeletons and increasing our understanding of the mechanics of these structures will add more data to the growing body of knowledge on biomaterials. Studies on biomaterials are already inspiring the growing field of biomimetics and biologically inspired design of materials (Chen et al. 2012). Examples of biologically inspired design include Mollusca nacre which has inspired design of military vehicle armour and toughened ceramic laminates (Chen et al. 2012). In order to improve our knowledge of the mechanics of mineralogy in bryozoans a mechanical study has been initiated with engineers to quantify the properties afforded by different mineralogies and the mechanics of localization in *Cellaria*.

The evidence presented in this study for spatial localization of $MgCO_3$ in bryozoans has potential implications with regards to ocean acidification (OA). It has been widely acknowledged that some calcium carbonate materials are more susceptible to dissolution than others (e.g. Smith et al. 1992; Dokoumetzidis & Macheras 2006; Morse et al. 2007; Mitchell et al. 2010), and Mg-calcite is more susceptible to dissolution than pure calcite (Anderson & Crerer 1993; Morse et al. 2007). Ocean acidification is decreasing the pH and carbonate ion content of oceans and is predicted to cause increasing dissolution of calcium carbonate materials (Orr et al. 2005). The localisation of higher concentrations of $MgCO_3$

in specific parts of bryozoan skeletons suggests that some morphological features and functions may suffer increased dissolution under ocean acidification conditions. The localisation of $MgCO_3$ in predominantly defensive and reproductive features may mean that morphologically complex species may be less well represented in marine communities in the future. The heightened utilisation of $MgCO_3$ in flexible erect colonies, and the localisation of aragonite and $MgCO_3$ for mechanical support in other erect colony forms, may give rise to selection against these colony forms under OA conditions and subsequent under-utilisation of the challenging fast-flow niches they occupy.

Smith & Lawton (2010) suggested the possibility of differential dissolution with regard to aragonite. However, they also discussed that dissolution kinetics alone may not be the only determining factor with regard to OA consequences, as organic components can act to decrease dissolution susceptibility. In this case the high organic component in erect flexible species in particular may help to negate the effects of OA on this otherwise susceptible group. Future dissolution experiments would help to identify whether there is an actual risk of differential dissolution under predicted ocean acidification conditions.

6.7 Summary and Conclusions

Mineralogy and morphology at the ultra, micro and macro scale are found to be closely interlinked with additional relationships proven with phylogeny and ecological preference of species. The results of the different experiments show that:

- Skeletal ultrastructure type and crystal size is linked to $MgCO_3$ content in calcite. Higher $MgCO_3$ crystals are smaller and often morphologically similar to aragonite.
- Skeletal ultrastructures found in study species adhere to those previously proposed by Weedon & Taylor (2000). An additional calcitic ultrastructure of rod-like with a tri-pointed axis is described.
- Skeletal fabrics and fabric suites show evidence of phylogenetic conservation of ultrastructure among species; this pattern is stronger in cyclostomes than cheilostomes.
- All species analysed show differential deposition of $MgCO_3$ in skeletal features, many of which are transient features related to reproduction or defence.
- Morphological complexity of skeletons is related to both $MgCO_3$ composition and phylogeny with $MgCO_3$ content increasing in more genetically evolved, morphologically complex species.
- Some evidence is provided for the utilisation and/or localisation of $MgCO_3$ and aragonite in bryozoan skeletons for mechanical advantage. Study species are shown to utilize the mechanical properties of aragonite and $MgCO_3$ to allow them to colonise abrasive and fast-flowing habitats.

The observed relationship between mineralogy and morphology raises concerns about the future of bryozoan species with complex skeletons under ocean acidification conditions. Localisation of dissolution susceptible fabrics in skeletal features may lead to selection against morphologically complex species and those which utilise aragonite and $MgCO_3$ to occupy challenging ecological niches. Further study is recommended to elucidate the mechanical properties associated with the differential mineralogy of bryozoan skeletons and the potential effects of ocean acidification on vulnerable species.

Chapter 7: A case-study on the skeletal mineralogy of the non-native bryozoan *Schizoporella japonica* Ortmann (1890)

7.1 Abstract

In early 2011, the non-native bryozoan *Schizoporella japonica* was found in the UK; its first recorded occurrence in Europe. This study aimed to establish the extent of *S. japonica*'s distribution in Scotland, and investigate its skeletal mineralogy and morphological variability, comparing it to the native species, *S. unicornis*.

To document the distribution of *S. japonica* in Scotland, 57 marinas were surveyed. Specimens were collected and environmental variables recorded and a two year sampling time series was also conducted at two Orkney marinas. X-ray diffraction was used to quantify the wt% $MgCO_3$ in calcite and wt% aragonite present in the skeletons of 77 *S. japonica* and 13 *S. unicornis* specimens. *S. japonica* was further examined to characterise the skeletal ultrastructure. Avicularia and ovicell counts were conducted on samples of *S. unicornis* and *S. japonica* to determine seasonal morphological variability. Morphological and mineralogical variability were examined alongside environmental measures in order to elucidate patterns and major contributors to differences between the two species.

The results of this study show that *S. japonica* has a highly complex and variable skeletal mineralogy and morphology. It features a high occurrence of multi-ovicells which are unique to the genus. Complex skeletal features show differential deposition of $MgCO_3$ and aragonite for mechanical advantage. Evidence suggests that seasonal variations in wt% $MgCO_3$ in calcite are biologically controlled and linked to ovicell production.

The combination of morphological and mineralogical adaptability, prolific breeding and cold-adapted stenothermal preferences make *S. japonica* an effective competitor in fouling communities and give it the potential to cause both ecological and commercial impact in Scotland. Commercially it has the potential to affect the aquaculture, shipping and marine renewable energy industries. Ecologically, if it is able to colonise the intertidal, it could increase its rate of spread around the UK and cause community and biotope changes.

Further research on *S. japonica* is recommended to understand its breeding cycle, model its likely future movements and predict potential ecological impacts.

7.2 Introduction

7.2.1 Invasive and non-native marine fouling species

For millennia, the natural geographical and biological barriers in the oceans have provided levels of isolation essential for unique species and ecosystems to evolve (International Union for the Conservation of Nature 2000). However, since the advent of ship travel people have been inadvertently or deliberately carrying marine organisms into new habitats, where they can establish themselves as alien/ non-native or invasive species (Bax et al. 2003; Hulme 2009). The prevalence of ship travel has dramatically increased in the last few hundred years (Bax et al. 2003), and it is estimated that at any given time, 10,000 different species are being accidentally carried in ballast water between bio-geographic regions (Carlton 1999). Globalisation and growth in trade and tourism, provide more opportunities than ever before for species to be spread (International Union for the Conservation of Nature 2000). Ballast water is just one of an ever increasing list of marine vectors which are provided by commercial shipping, aquaculture and fisheries, drilling platforms, canals, recreational boating, marine sport/dive equipment and floating debris (Bax et al. 2003). Gollasch (2006) examined the origin of non-native species in European coastal waters and estimated that 39% were transported by ships, either in ballast water (22%) or hull fouling (17%), 16% were introduced with mariculture, 9% were intentionally released and 24.5% entered through the Suez Canal. Upon arrival in a new geographic region many non-native species are absorbed into the background flora and fauna (Bax et al. 2003), and are simply referred to as alien or non-native species (International Union for the Conservation of Nature 2000). Occasionally non-native species can have a positive impact on their new home, by providing increased settlement areas for native species (Bax et al. 2003), or as new commercially valuable species for aquaculture (Occhipinti-Ambrogi 2007). Some non-native species become established in a natural ecosystem, changing that ecosystem or threatening native biodiversity; these are categorised as invasive non-native species (International Union for the Conservation of Nature 2000). Invasive non-native species are expensive globally, both in terms of direct measureable economic impacts, and indirect ecological costs (International Union for the Conservation of Nature 2000). The Scottish Government estimates the annual cost of invasive non-native species (terrestrial and

marine) in Great Britain to be up to £2 billion, of which an estimated £200 million is accrued in Scotland (The Scottish Government 2014). The principal legislation in place to prevent non-native arrival and spread in Scotland is the Wildlife and Countryside Act (UK Parliament 1981) , which was amended in 2011 with the Wildlife and Natural Environment (Scotland) Act (The Scottish Government 2011).

In the marine sector bryozoans, sea-squirts, sponges, mussels, barnacles and other fouling organisms constitute the majority of non-native species (Eno et al. 1997), as they are able to attach to boat hulls, ballast tanks, solid ballast and aquaculture imports. Until recently the number of non-native bryozoans recorded in the UK was considered to be underestimated, as insufficient data was available to establish them as native or non-native (Eno et al. 1997). Identification and documenting accurate distribution is often problematic for non-native bryozoan species due to their plasticity and cryptic morphology. Recent genetic analyses by Mackie et al (2012) highlighted that specimens of non-native *Watersipora* from around the world, consist of a complex of six clades, unveiling some previously unrecognised cryptic invasions. A publication by Minchin et al. (2013) recognised five non-native bryozoan species in the UK including *Tricellaria inopinata* d'Hondt & Occhipinti Ambrogi, 1985 (Dyrynda et al. 2006), *Bugula neritina* (Mackie et al. 2010), *B. simplex* (Ryland et al. 2011) , *B. stolonifera* Ryland, 1960 (Ryland et al. 2011) and *Watersipora subtorquata* (d'Orbigny, 1852) (Ryland 2009; Mackie et al. 2012). Of these species, only one has been recorded in Scotland with two reports of *B. neritina* from South-West Scotland (Ryland et al. 2011). To date bryozoan non-natives in the UK have all been species with warm-water preferences, restricting their encroachment into Scottish waters.

7.2.2 Schizoporella japonica Ortmann (1890)

Schizoporella japonica was first described by Ortmann in 1890 from Japan. It has since established non-native populations along much of the West coast of North America (Ryland et al. 2014). The first documentation of this non-native species in North America was in 1938 from Los Angeles and it has since made its way northwards as far as Alaska (Dick et al. 2005). Recent reports for *S. japonica* show that it is well established in the West coast of Canada and North America (Clarke Murray et al. 2011; Treibergs 2012). *S. japonica* has not previously been reported in Europe.

The first reports of *S. japonica* in Europe were from Holyhead marina, July 2010, and by the author in Stromness marina in spring 2011 (Ryland et al. 2014). It is likely that the arrival of *S. japonica* in the UK pre-dates this by some months, possibly years, as at the time of discovery it was found to be well established with high surface coverage, particularly in Stromness marina. The discovery of *S. japonica* in Holyhead marina occurred after a first round eradication program for the invasive sea-squirt, *Didemnum vexillum* Kott, 2002 (Ryland et al. 2014). *S. japonica* was not recorded during the in depth surveys conducted in 2008 (Griffith et al. 2009). Following the eradication program, an in- depth monitoring program was implemented and during this colonies of an unknown bryozoan were observed. Images from winter 2010, following the chemical eradication attempt, show a heavy coverage of *S. japonica* under-laying colonies of *D. vexillum* (Fig. 7.1). This suggests that *S. japonica* probably established a stronghold shortly after the chemical eradication attempt in winter-autumn-spring 2009/2010, possibly aided by the removal of competitor foulers (Ryland et al. 2014).

7.1: Image of pontoon in Holyhead marina taken in winter 2010/2011. Orange crust is Schizoporella japonica with overgrowth of Didemnum vexillum in centre of picture. Image courtesy of Rohan Holt.

It is reported by Powell (1970) that S. *japonica* was first introduced to North America from Japan via Pacific Oyster, *Crassostrea gigas* (Thunberg, 1793), stock imported for mariculture from 1932. Aquaculture of the Pacific Oyster has the highest value and production of all shellfish cultured in Scotland (Scottish Government 2011), and it is possible that *S. japonica* was introduced with seeding stock, as in North America. Scottish areas with the most shellfish aquaculture include Strathclyde (oysters) and Shetland (mussels) and aquaculture takes place in all parts of Scotland (Scottish Government 2011).

Import of oyster seed into Scotland is currently restricted in order to limit spread of disease (*Ostreid herpes* virus) (Scott et al. 2010) and only two hatcheries supply all Scottish oyster farms; these are based in Morecambe Bay, Cumbria and Guernsey (Scott et al. 2010). Neither of these regions host known *S. japonica* populations, therefore it is unlikely that imported oyster seed is the source of *S. japonica* in Scotland.

An alternative vector for the movement of marine non-native species is on the hulls and in the ballast water of marine vessels and pleasure craft (Clarke Murray et al. 2011). Small recreational yachts can travel long distances, and their slow speed, compared to commercial shipping vessels, makes them ideal vectors for the transport of non-native species (Minchin et al. 2013). Scottish non-native species which have been documented as being transported by pleasure craft include the marine algae *Codium fragile* spp. *Tomentosoides* Van Goor (P.C. Silva) and the skeleton shrimp, *Caprella mutica* Schurin, 1935 (Frey et al. 2009). Furthermore, the British non-native bryozoans *W. subtorquata* and *B. neritina* have been shown to be tolerant to anti-fouling paints and transported on ship hulls (Ryland 2009; Ryland et al. 2011; Mackie et al. 2012). Phylogenetic studies by Waeschenbach et al (2012) have shown *S. dunkeri* to be within the same clade as *W. subtorquata* and we may speculate that *Schizoporella* species might have a similar resistance to anti-foulants as *W. subtorquata*. Future controlled experiments would be required to prove or disprove this possibility. A study by Clarke Murray et al. (2011) identified *S. japonica* on the hulls of 9% of the 491 pleasure craft examined in British Columbia and concluded that pleasure craft are a key vector in the transport of *S. japonica* in Canada and that regular movement of craft did not prevent them from being fouled. A potential transport route from Canada to Scotland via pleasure crafts could be the "North Route" Trans-Atlantic crossing. This crossing takes vessels from Newfoundland, Canada to Shetland/Orkney via Labrador, Iceland and the Faeroes (Royal Yacht Association 2008). Examination of marinas along this North Route would provide further information as to whether this is a potential ingress route for *S. japonica* into Scotland.

S. japonica is a good model species for study because the genus, *Schizoporella,* is known to produce bimineralic skeletons and a highly plastic morphology (Smith et al. 2006; Taylor et al. 2009). Orkney is chosen as the site for seasonality and comparison studies as it has two marinas "infected" with *S. japonica* within 20km of each other, and an intertidal site with the native, *S. unicornis,* less than 5km from Stromness marina. When established, *S.*

japonica grows rapidly and profusely, providing high quantities of material for each time point. This study also provides the opportunity to advance knowledge of the ecology of this unusual non-native species, adding data which could be used in the future to predict its movements and economical and ecological impact in the UK and abroad.

7.3 Aims and Objectives

7.3.1 Summary of purpose of study

The skeletal mineralogy of the non-native bryozoan, *Schizoporella japonica* has not previously been investigated. The aim of this study is to determine the distribution, mineralogy and morphological variability of the species in the UK and compare this to the native species, *S. unicornis*. Results will be discussed in relation to spatial, seasonal and morphological patterns of mineralogy and the potential implications of these on future distribution and competitive advantage.

7.3.2 Objectives of study

1. Investigate and document the distribution of *S. japonica* in Scottish marinas.
2. Determine the mean wt % $MgCO_3$ in calcite and wt% aragonite and the range of variability for *S. japonica* and *S. unicornis*.
3. Characterise the crystal ultrastructure and spatial localisation of $MgCO_3$ and aragonite in the skeletal features of *S. japonica*.
4. Compare the mineralogy of samples collected from twelve sites in the UK and Japan and discuss alongside environmental conditions.
5. Create a time-series of environmental, morphological and mineralogical data for *S. japonica* at Stromness marina and *S. unicornis* from the nearby intertidal. Interrogate this to investigate the influence of seasonality and morphological plasticity on skeletal mineralogy and compare between the two species.
6. Discuss the potential ecological and commercial implications of the above.

7.4 Materials and Methods

7.4.1 Sample collection

This study is based on the mineralogical and morphological examination of 77 specimens of the non-native bryozoan, *Schizoporella japonica.* Results are compared to 13 specimens of the native bryozoans, *S. unicornis.* All specimens were collected from either the intertidal (*S. unicornis*) or from marinas (*S. japonica*). Details of specimen processing methods can be found in Section 3.4.1

7.4.2 Analysis techniques

IMAGING

Where required to confirm identification SEM was undertaken according to methodology detailed in Section 3.4.1. Ultra-SEM and staining was undertaken according to the methodology detailed in Section 6.4.2.

7.4.3 Data sources, processing and presentation

Plates were prepared using GNU Image Manipulation Program (GIMP) (Kimball & Mattis 2012).

7.4.4 Statistics and data analysis

Wt% $MgCO_3$ in calcite and wt% aragonite data was tested for normality using Anderson-Darling normality tests and homogeneity of variance using Levine's test of equal variance. Where the criteria for parametric testing were satisfied data was analysed using a generalised linear model (GLM) ANOVA. Where the criteria for parametric testing were not satisfied non-parametric Mann-Whitney U-tests were used. P-values for all analyses were calculated; this is a measure of the strength of the evidence and is based on the traditional probability of error of 0.05. Only when the p-value is less than 0.05 is the evidence considered statistically significant. Statistics and data analysis were conducted using MINITAB release 14 software (2004) and R programming environment (R Core Team 2013).

7.5 Results

7.5.1 UK distribution of Schizoporella japonica

57 marinas were assessed in Scotland for the presence/absence of *S. japonica* between summer 2011 and winter 2013; this represents 94% of the coastal marinas in the country. *S. japonica* was found to be present in ten marinas: - Kirkwall, Stromness and Westray in the Orkney Islands; Burravoe and Lerwick in Shetland; Portavadie and Croabh on the West coast; Peterhead and on the East coast and Tobermory in the Hebridean Islands (Fig. 7.2). In addition samples were found by Rohan Holt (Countryside Council for Wales) in Holyhead marina, Wales.

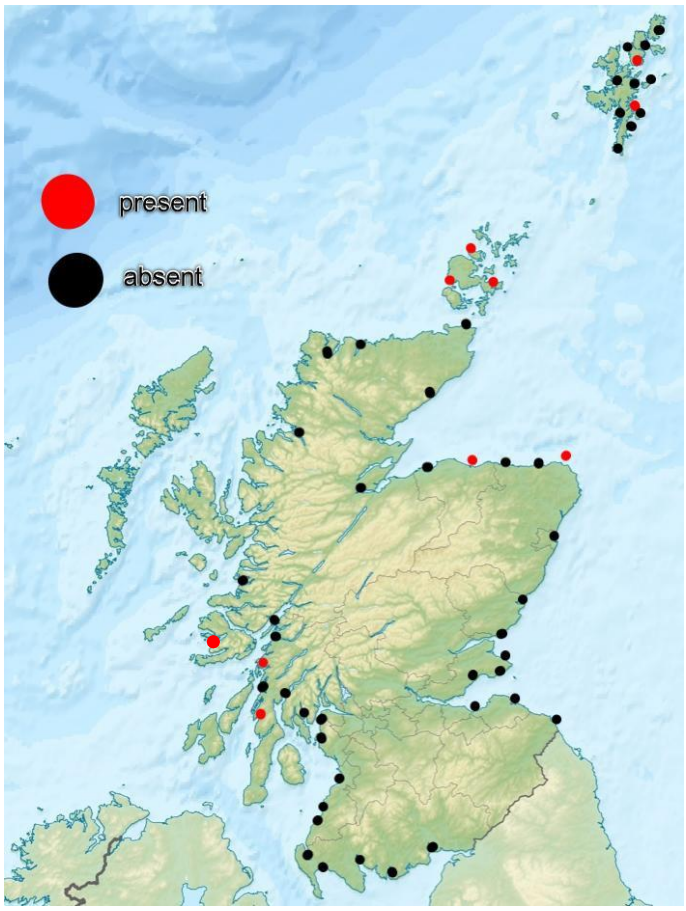

Figure 7.2: Map of Scotland showing presence and absence of Schizoporella japonica at marinas. Red dots represent presence, black dots represent absence

In the UK, *S. japonica* has been found as far North as 60.5ºN and with a southern extent to 53.3ºS. All marinas where *S. japonica* has been found are full salinity habitats (>30 p.s.u). *S. japonica* was found to be established in Lerwick marina, Shetland in September 2012 but entirely absent in December 2012.

7.5.2 Mineralogy of Schizoporella in the UK.

Analysis was conducted on 77 specimens of *S. japonica*, and 13 specimens of *S. unicornis*, for quantification of wt% $MgCO_3$ in calcite and wt% aragonite. *S. japonica* was found to be bimineralic (mean of 40.55 wt% aragonite) and intermediate Mg-calcite (IMC) with a mean of 5.98 wt% $MgCO_3$ in calcite. The native species, *S. unicornis*, was found to be bimineralic (mean of 26.03 wt% aragonite) and have a high Mg-calcite (HMC) skeleton with a mean of 8.01 wt% $MgCO_3$ in calcite (Fig.s 7.3 & 7.4).

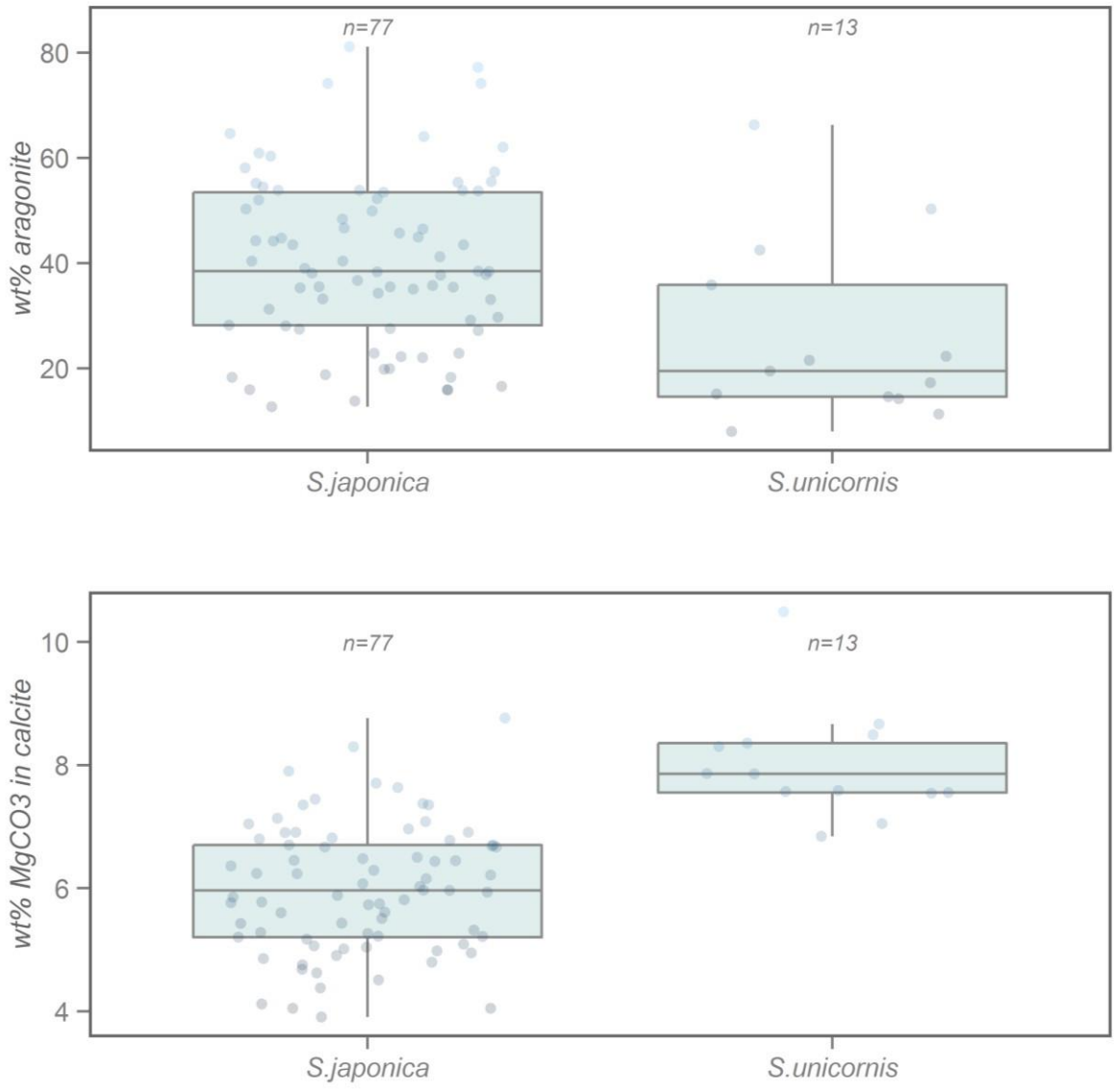

Figure 7.3: Boxplots for Schizoporella japonica and Schizoporella unicornis. Top plot shows wt% aragonite, bottom plot shows wt% $MgCO_3$ in calcite. Box represents interquartile range, horizontal line shows mean, tails show range of data. Data points are indicated with blue circles. Sample number is indicated (n)

Carbon analysis of *S. japonica* showed that the species had a mean of 28.9% organic carbon and 71.1% inorganic carbon in its skeleton.

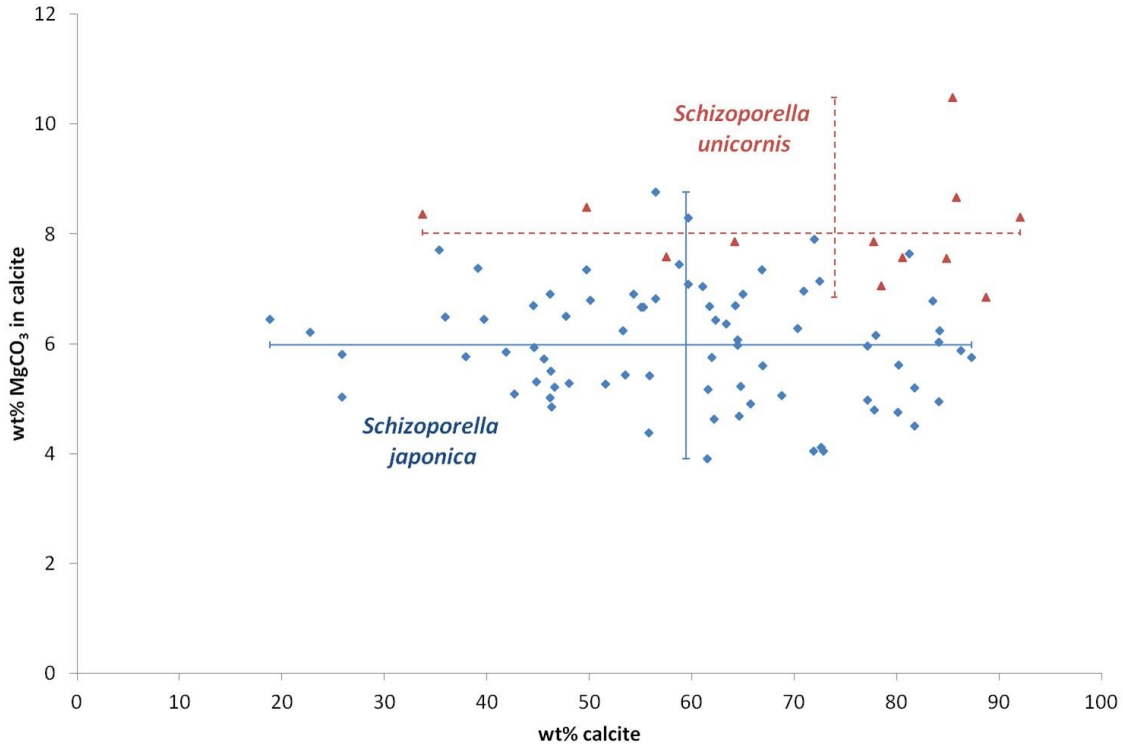

Figure 7.4: Scatter plot showing the range of wt% $MgCO_3$ in calcite and wt% calcite occupied by each species. Red triangles indicate Schizoporella unicornis; blue diamonds indicate Schizoporella japonica.

7.5.3 Spatial patterns in mineralogy of S. japonica

Mineralogy of 77 samples of *S. japonica* from 12 different geographical locations was analysed. Samples were collected at different times of year. Japanese samples were not collected fresh but were air-dried specimens donated for the purpose of this experiment.

- There is no statistical difference in wt% $MgCO_3$ in calcite for samples collected from different sites (Fig. 7.5).
- There is a statistical difference in wt% aragonite for samples collected from different sites (Kruskall-Wallis ANOVA: P = 0.020, df = 11, χ^2 = 22.59) (Fig. 7.5). *Posthoc* Mann-Whitney U tests shows wt% aragonite for Oshuro, Japan is the most statistically distinct from other sites (statistically significantly different to 5/11 sites), followed by Kirkwall (statistically significantly different to 3/11 sites),

Portavadie (statistically significantly different to 2/11 sites with 7 additional sites nearing significance) and Peterhead (statistically significantly different to 2/11 sites with 6 additional sites nearing significance). Croabh, Lerwick, Portaknockie and Westray marina all showed no statistical difference in wt% aragonite to any other sites (Tab. 7.1).

Table 7.1: Results of Mann-Whitney posthoc analysis comparing wt% aragonite between sites. Bold crosses indicate no significant difference between sites; ticks indicate significant difference between sites; crosses and underlined p-values with · indicates nearing significance.

	Kirkwall	Westray	Burravoe	Lerwick	Portaknockie	Peterhead	Portavadie	Croabh	Holyhead	Daiko	Oshuro
Kirkwall	✘	✘	✘	✘	✘	✘	✓ p=0.016	✘	✘	✘	✓ p=0.022
Westray		✘	✘	✘	✘	✓ p=0.049	✓ p=0.012	✘	✘ p=0.059·	✘	✓ p=0.005
Burravoe			✘	✘	✘	✘ p=0.081·	✘ p=0.081·	✘	✘	✘	✘
Lerwick				✘	✘	✘ p=0.081·	✘ p=0.081·	✘	✘	✘	✓ p=0.050
Portaknockie					✘	✘ p=0.081·	✘ p=0.081·	✘	✘	✘	✘
Peterhead						✘	✘	✘	✘	✘	✘
Portavadie							✘ p=0.081·	✘	✘ p=0.081·	✘ p=0.081·	✓ p=0.052
Croabh								✘ p=0.081·	✘ p=0.081·	✘ p=0.081·	✘
Holyhead									✘	✘	✘
Daiko										✘ p=0.081·	✘
Oshuro											✓ p=0.050

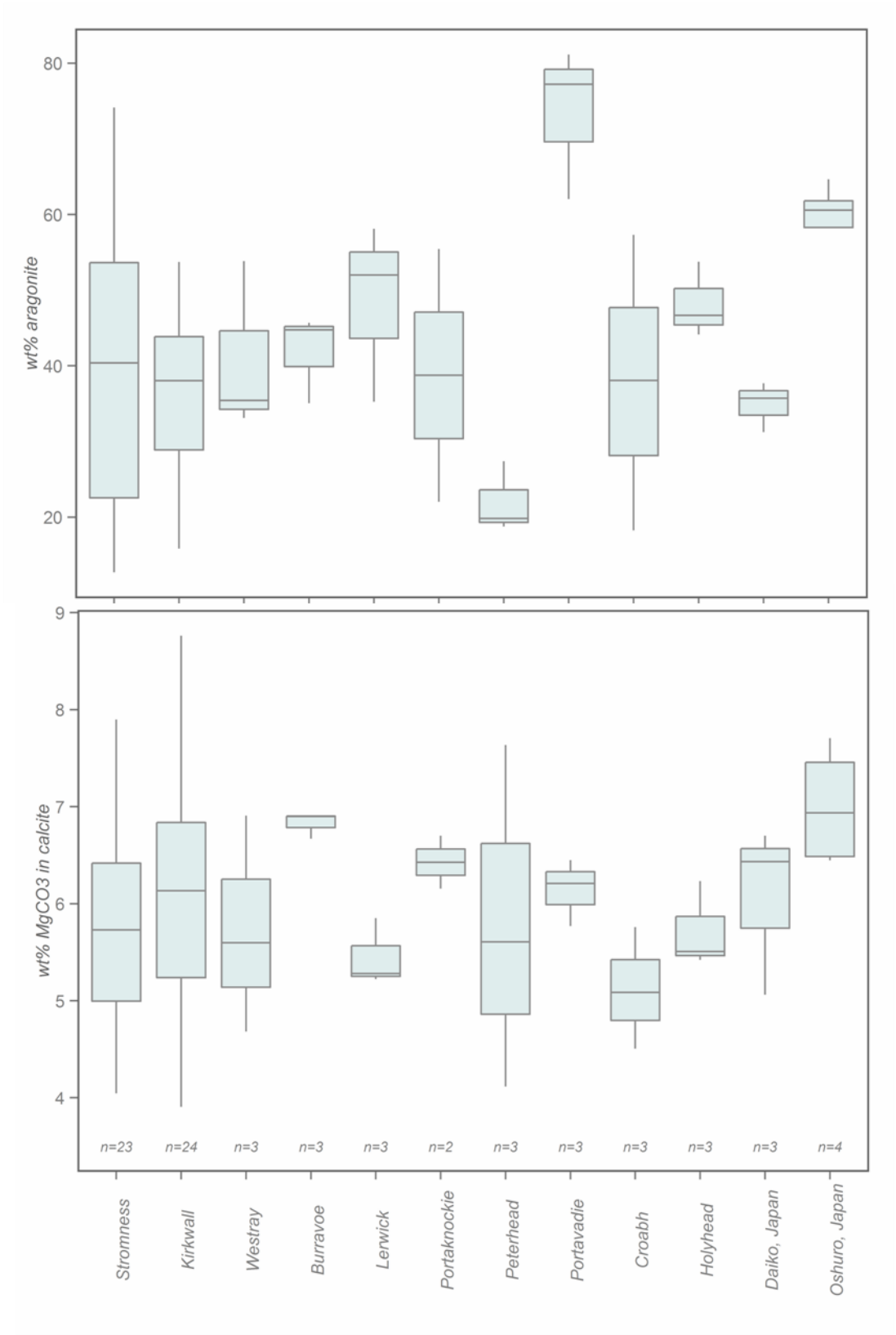

Figure 7.5: Boxplots showing data for different sites. wt% MgCO3 in calcite (bottom) and wt% aragonite (top).

There is no statistical difference in wt% $MgCO_3$ in calcite, or wt% aragonite, for samples of *S. japonica* from Kirkwall and Stromness marinas collected on the same dates between 2011 and 2013. There is no statistical difference (GLM ANOVA) between the sea-water temperature at Kirkwall marina and Stromness marina over the time period Sept' 2011 to March 2013 (Fig. 7.6).

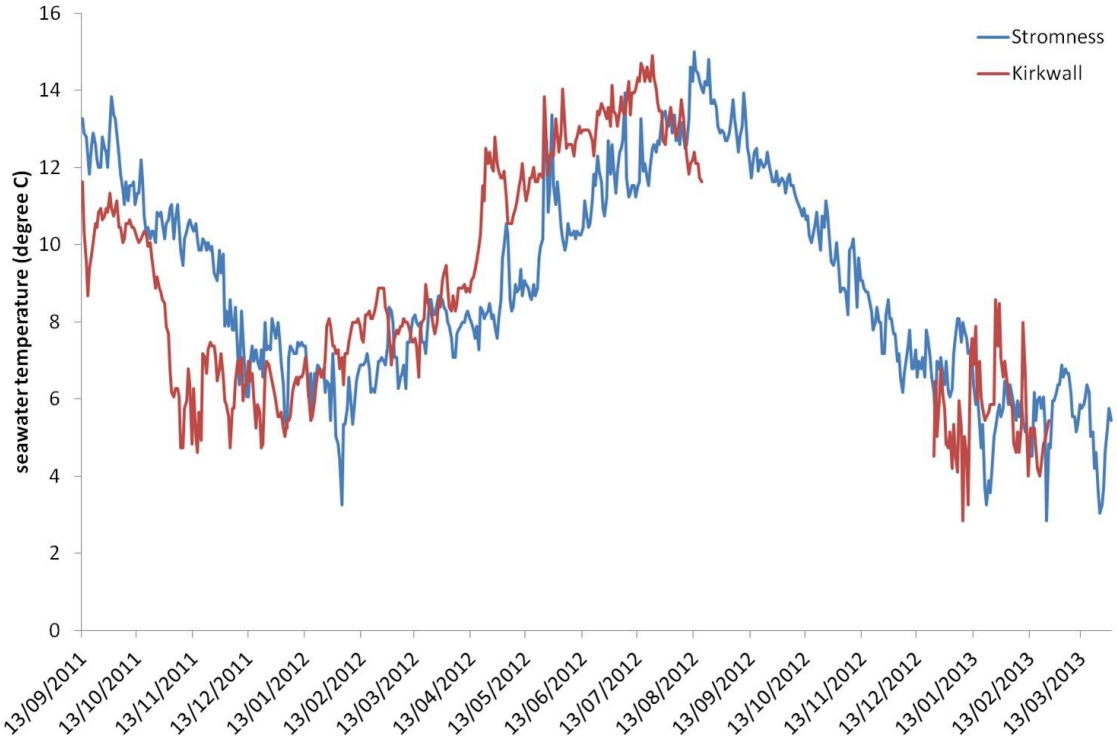

Figure 7.6: seawater temperature between Sept 2011 and March 2013 for Stromness and Kirkwall marina. Missing data between Aug-Dec 2012 in Kirkwall marina was due to a lost logger.

7.5.4 Seasonal patterns of variation in mineralogy of S. japonica

Analysis of Stromness samples of *S. japonica* alongside environmental data (collected between Aug 2012 and March 2013) shows no statistically significant correlation between wt% $MgCO_3$ in calcite or wt % aragonite and the environmental variables of salinity, pH, total alkalinity (TA) or concentrations of HCO_3^{2-}, pCO_2, nitrate, phosphate, silicate, $\Omega_{calcite}$, $\Omega_{aragonite}$, of dissolved organic carbon (DIC). See Appendix D for more details of environmental variables at Stromness marina.

Spearman's rank order correlation shows there to be no statistically significant correlation between wt% aragonite and seawater temperature (Fig. 7.7), however, a statistically significant negative correlation between wt% $MgCO_3$ in calcite and seawater temperature

was detected (p=0.012, Spearman's *rho*, r_s = -0.488) (Fig. 7.7). This correlation indicates a weak tendency for higher wt% Mg-calcite at lower temperatures in Stromness marina.

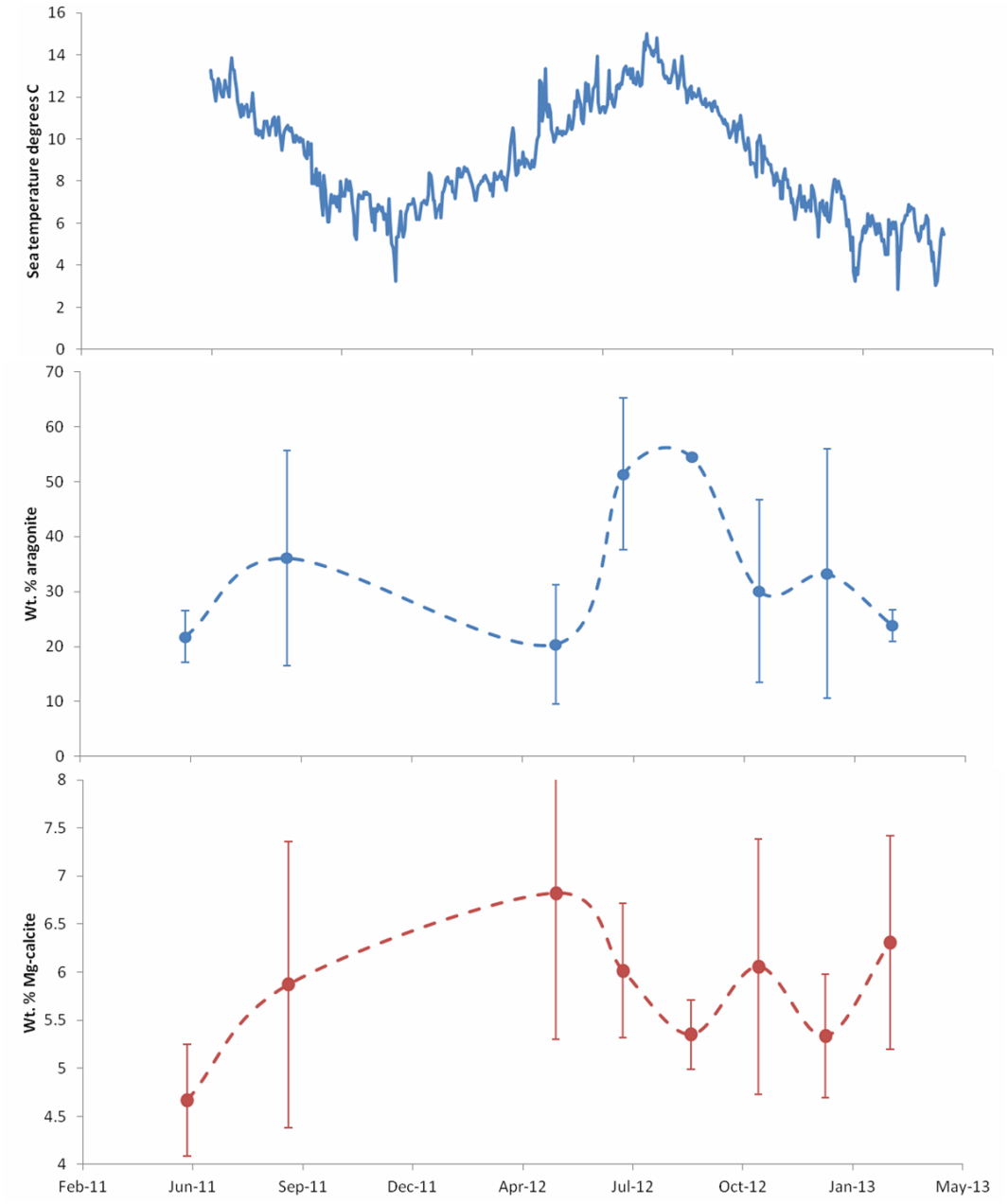

Figure 7.7: Stromness marina time-series of seawater temperature (top), mean wt% MgCO3 in calcite (red, bottom) and mean wt% aragonite (blue, centre) between Feb 2011 and May 2013. Range is indicated with vertical bars. Wt% Mg-calcite refers to wt% $MgCO_3$ in calcite.

If temperature data for all sites is included (where temperature data is available) (Fig. 7.8) in the analysis (n=71) then we find an extremely weak negative correlation between wt% $MgCO_3$ in calcite and temperature is exhibited across all sites (p=0.001, Spearman's *rho*, r_s = -0.375). No correlation between wt% aragonite and temperature is detected in all

site data. There is no statistically significant correlation between either wt% $MgCO_3$ in calcite or wt% aragonite and temperature for the native species, *S. unicornis* (n=13).

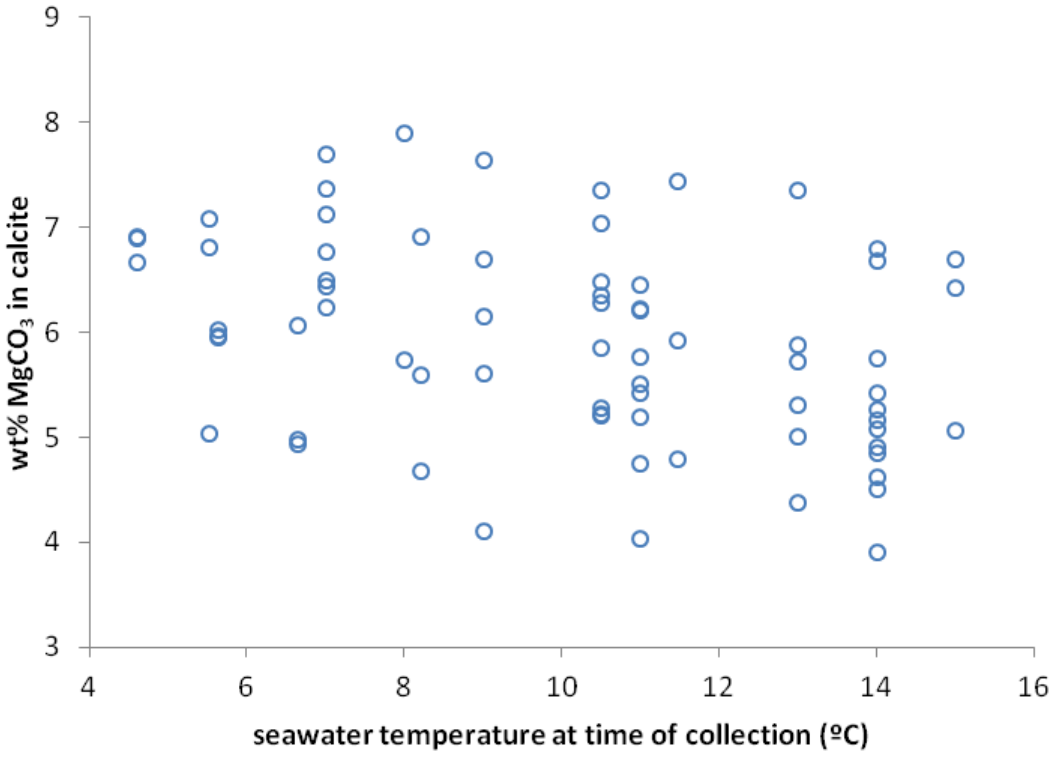

Figure 7.8: Scatterplot showing wt% $MgCO_3$ in calcite plotted against seawater temperature. Data is included for all sites where temperature was measured at the time of collection.

7.5.5 Morphology of S. japonica

ULTRASTRUCTURE

The base fabric and walls of the zooid (Fig. 7.9D-E) are constructed of large, platey crystals of rhombic semi-nacre, with crystals 500nm - 1µm across and sheered both in the plane of the wall and at right-angles. The frontal shield (Fig. 7.9A-C) is constructed of aragonite acicular crystallites which form a fan array initially and are constrained into a compound fabric in later stages. Acicular crystallites are 150nm wide and up to 3.5µm in length. Avicularia are constructed of rod-like fabric (100-150nm diameter, 1.5µm in length) (Fig. 7.9F–G). The surface of the ovicells (Fig. 7.9H) was found to be constructed from a fine, densely packed surface of granular crystallites (50-100nm diameter).

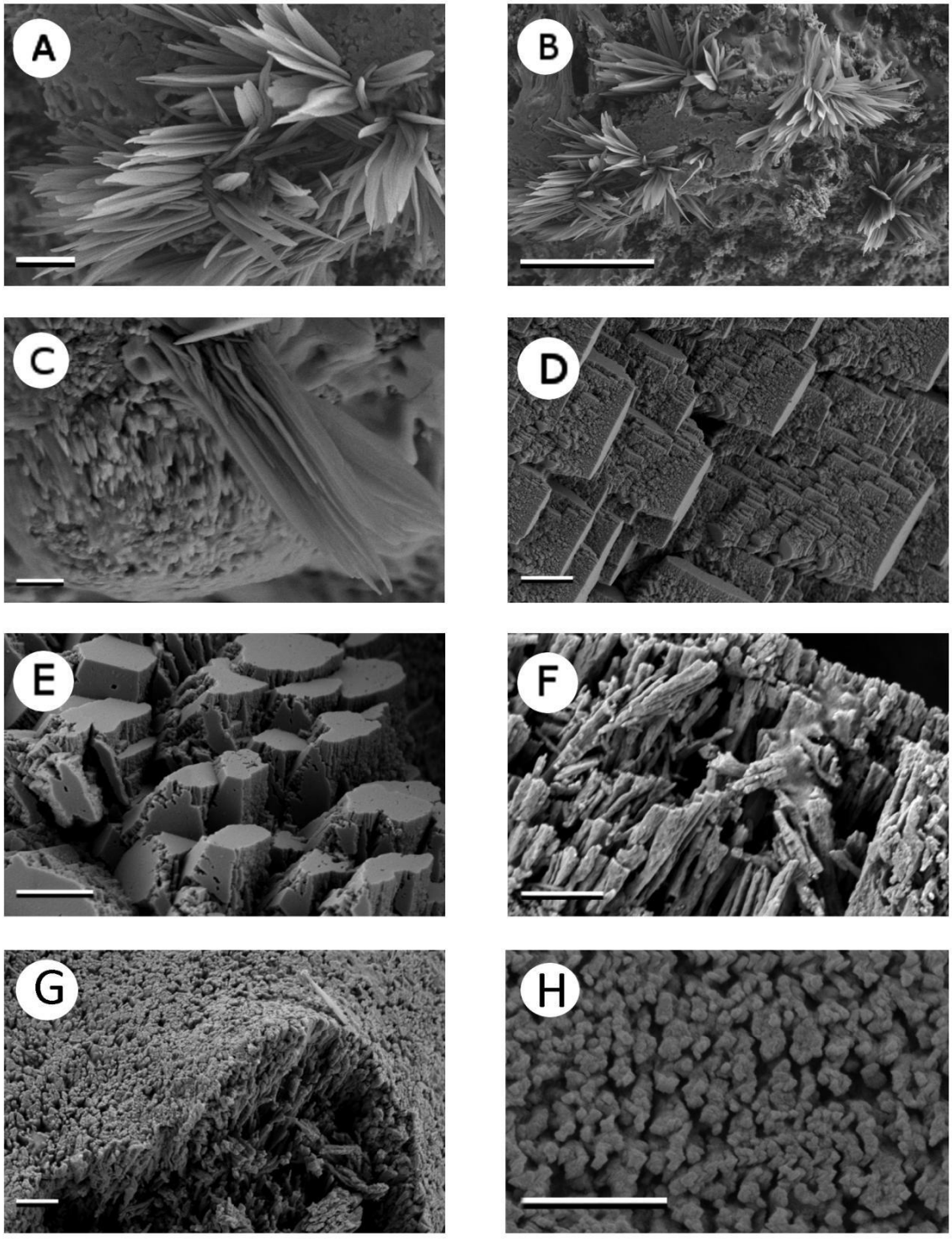

Figure 7.9: Ultrastructure of Schizoporella japonica; A-C frontal shield ultrastructure showing aragonite acicular needles in fan array (A and B), and forming a compound fabric (C): A scale = 1µm, B scale = 5µm, C = 500nm. D & E bases and wall material showing large, laminar crystals of rhombic semi-nacre, scale = 1µm. F & G avicularia rim constructed of rod- like fabric. Scale = 1 µm. H ovicells surface of granular crystallites, scale = 1µm.

SPATIAL LOCALISATION OF MINERALS

Aragonite was found to be localized in the frontal shield (Fig. 7.10B) and in reinforcing buttresses attaching ovicells to the frontal shield (Fig. 7.10C). It was notably absent from avicularia, ovicells (Fig. 7.10A) and the ancestrula (Fig. 7.10D). $MgCO_3$ was found to be particularly concentrated around the avicularia openings and ovicells (Fig. 7.10E-F). $MgCO_3$ was not particularly concentrated in closure plates (Fig. 7.10F, bottom left).

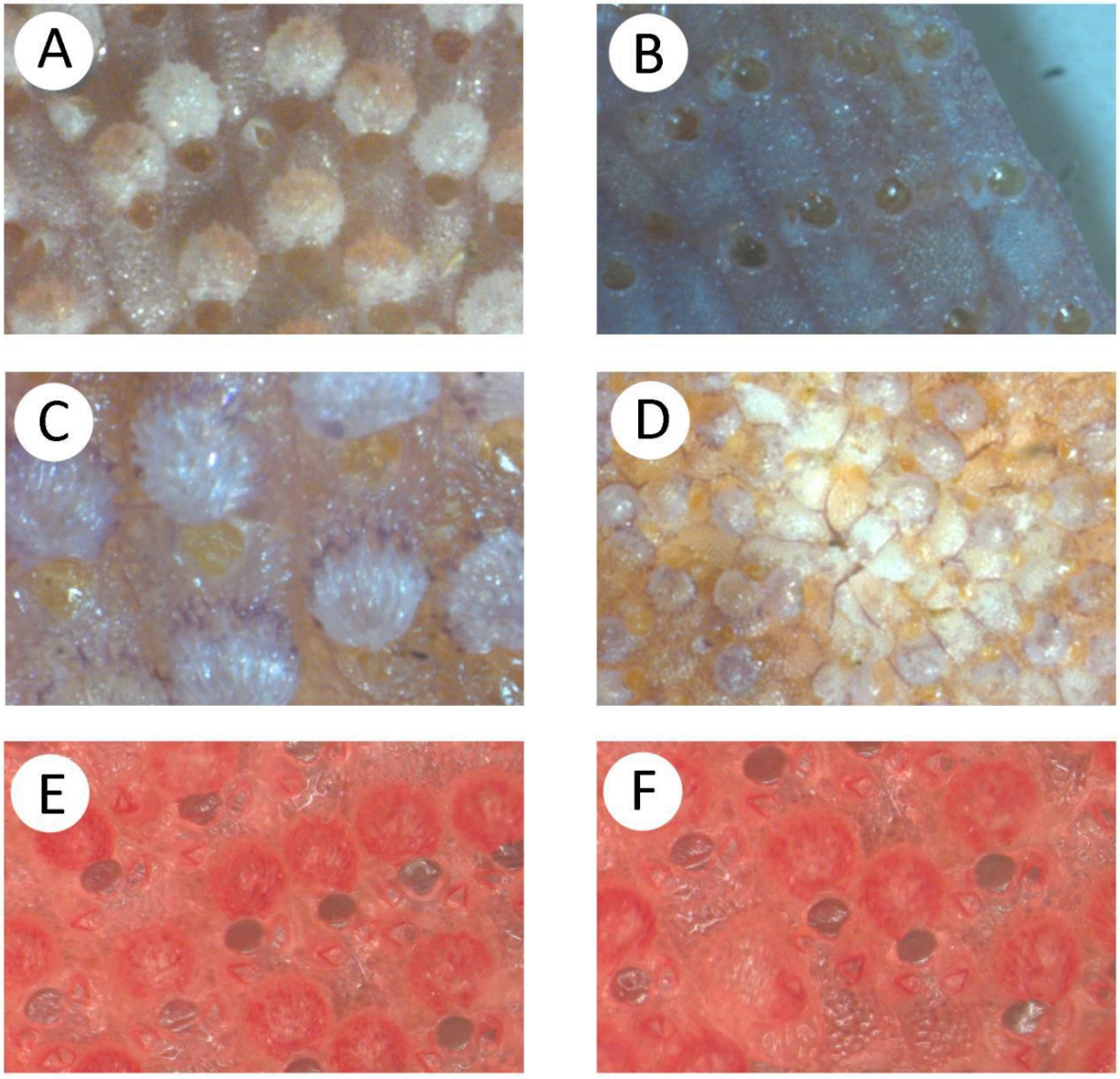

Figure 7.10: Schizoporella japonica. Stained with Meigen's stain (A-D), showing aragonite in purple, and Titan Yellow (E-F), staining Mg-calcite red.

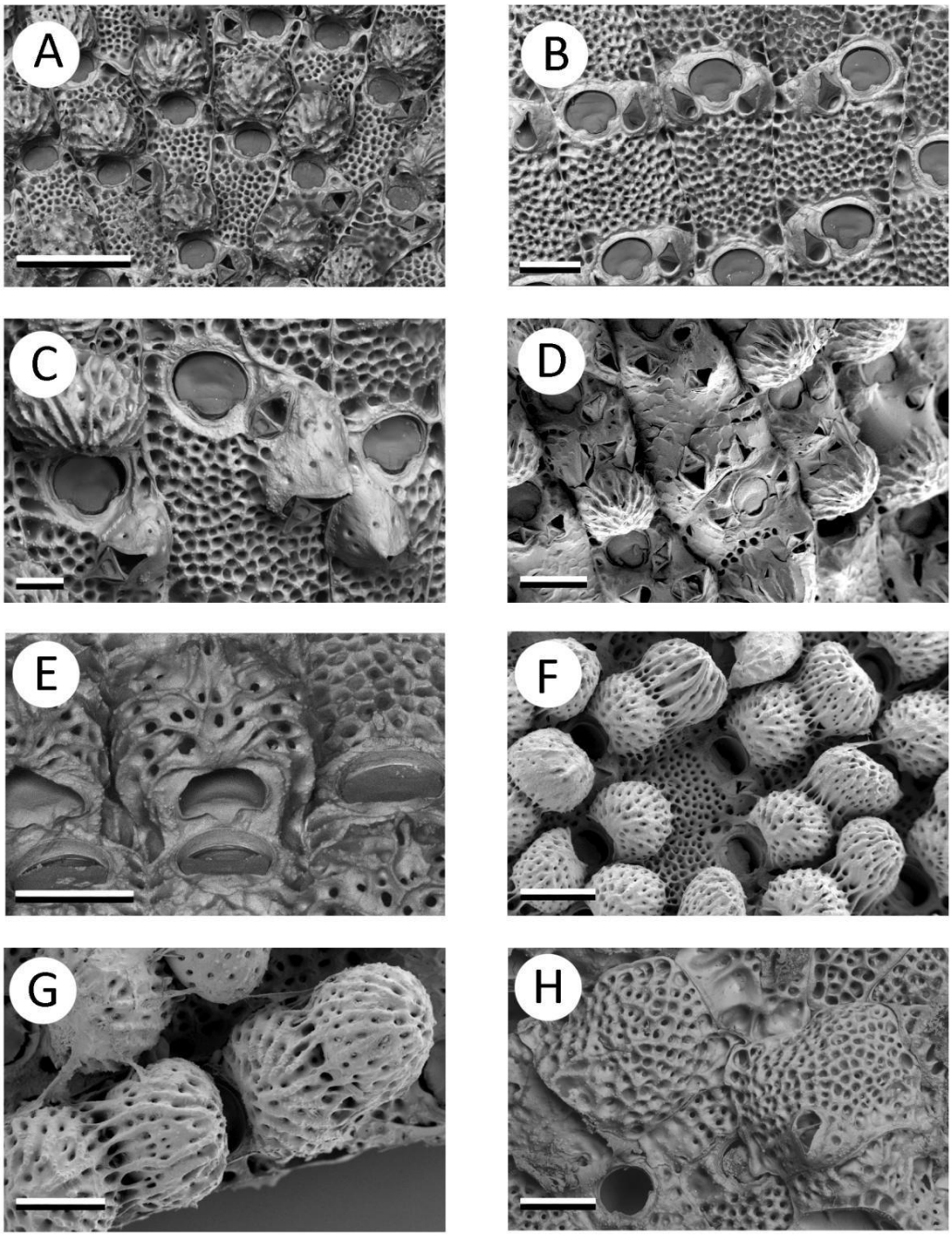

Figure 7.11: Schizoporella japonica; Samples from Stromness marina, Orkney; bleached (A-C, E,H); lightly bleached (F,G); and from Portavadie marina, Scotland; unbleached (D): (A) array of ovicellate zooids; (B) array of zooids featuring single and paired oral avicularia; (C) zooids (left and right) with a single large frontal avicularium and autozooid (centre) with both oral avicularium and a large frontal avicularium; (D) array of zooids with multiple mixed avicularia, up to four per zooid; (E) angled zooid (centre) showing perforate ovicell and ovicell opening; (F) array of ovicellate zooids showing single and double ovicells; (G) close up of multi ovicellate zooid (centre) showing secondary ovicell on frontal shield facing primary ovicell and third ovicell stacked on primary ovicell; (H) zooids (left and right) with perforate closure plates covering orifice. Scale bars: 500 µm (A); 300µm (F); 200µm (B,D,E,G,H); 100µm (C).

S. japonica features a high degree of morphological plasticity (Fig. 7.11) with two types of avicularia (Fig. 7.11C) and differing occurrence of both avicularia and ovicells. The highest occurrence of avicularia was observed at Portavadie marina where up to five avicularia were observed on a single zooid (Fig. 7.11D). Multi-ovicells were also observed in *S. japonica* (Fig 7.11 F-G) with up to five ovicells recorded on a single zooid; the most common positioning of secondary ovicells being on the frontal shield with the ovicell opening facing the orifice (Fig. 7.11F). Ovicells were also observed "stacked" on top of the primary ovicells (Fig. 7.11G) in towers up to three ovicells high and side-by-side on the frontal shield facing the orifice. Specimens from Stromness, Kirkwall and Portavadie marinas were examined and multi-ovicells were observed at all three sites. Avicularia and ovicell counts were conducted on 13 specimens of *S. japonica* from Stromness marina from five timepoints.

Across all timepoints specimens were found to have a mean of 23.47 ovicells/100 zoids with peak ovicell presence (48.9/100 zooids) occurring in the Spring. Specimens had a mean of 33.51 avicularia/100 zooids with the highest counts of avicularia occurring in the Summer (58 avicularia/100 zooids). For *S. japonica* the two timepoints with the highest number of ovicells/100 zooids (March and May) also featured the lowest avicularia counts. For *S. japonica* there is a statistically significant positive relationship between the number of ovicells/ 100 zooids and the wt% $MgCO_3$ in calcite (Linear regression: $p=0.01$, $R^2 = 91.5\%$) (Fig. 7.12).

Avicularia and ovicell counts were conducted on 12 specimens of the native species, *S. unicornis,* from an intertidal site near Stromness. No multi-ovicells were observed in the specimens and a maximum of two oral avicularia were recorded per zooid. Across all timepoints specimens were found to have a mean of 11.71 ovicells/100 zoids with peak ovicell presence (25.21/100 zooids) occurring in the Autumn. Specimens had a mean of 131.15 avicularia/100 zooids with the highest counts of avicularia occurring in the Autumn (186.87 avicularia/100 zooids). For *S. unicornis* the two timepoints with the highest number of ovicells/100 zooids (September and November) also featured the highest avicularia counts. For *S. unicornis* there is no statistically significant relationship between the number of ovicells/ 100 zooids and the wt% $MgCO_3$ in calcite (Fig. 7.13).

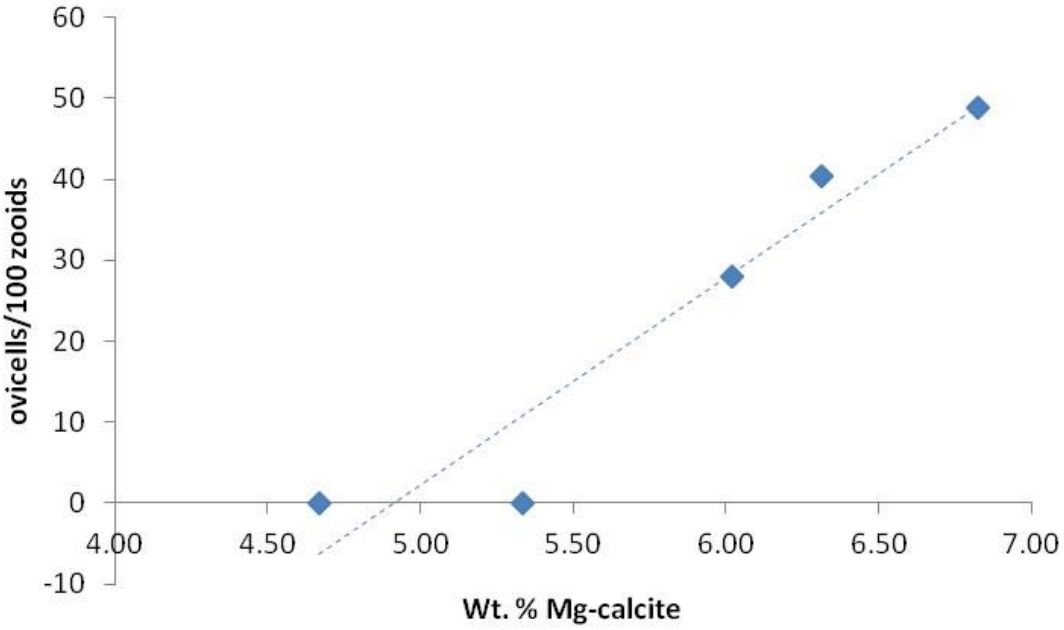

Figure 7.12: Scatterplot showing the mean wt% $MgCO_3$ in calcite plotted against the mean number of ovicells/100 zooid for samples of Schizoporella japonica from five timepoints from Stromness marina. Dotted line shows statistically significant regressional relationship between ovicells counts and wt % $MgCO_3$ in calcite (p=0.001, R^2 = 91.5%). Wt% Mg-calcite refers to wt% $MgCO_3$ in calcite.

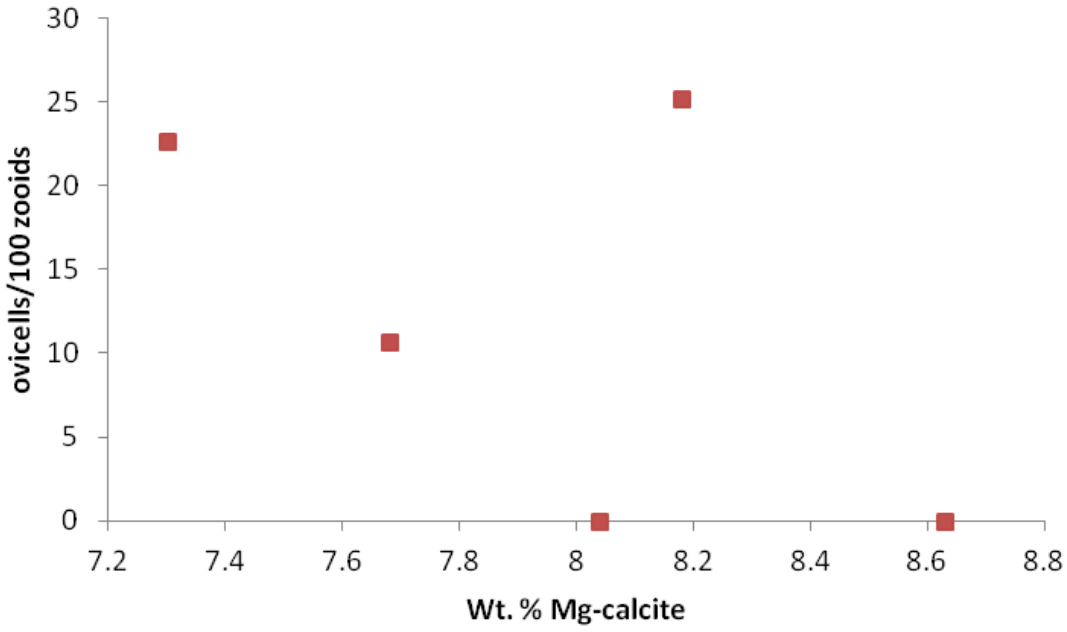

Figure 7.13: Scatterplot showing the mean wt% $MgCO_3$ in calcite plotted against the mean number of ovicells/100 zooid for samples of Schizoporella unicornis from five timepoints from Stromness intertidal. Wt% Mg-calcite refers to wt% $MgCO_3$ in calcite.

7.6 Discussion

This study presents the first analysis of the skeletal mineralogy of *Schizoporella japonica* and aimed to determine the distribution, mineralogy and morphological variability of the species in the UK and compare this to the native species, *S. unicornis*. Results are discussed in relation to spatial, seasonal and morphological patterns of mineralogy and potential implications on future distribution and competitive advantage.

7.6.1 Scottish distribution of S. japonica

S. japonica was identified from ten marinas in Scotland and a single marina in Wales. Within Scotland it is not limited to a single region but is found on the East coast, West coast, Hebridean islands and in Orkney and Shetland. Movement of *S. japonica* within Scotland is likely attributable to the movement of recreational and small trade vessels based on the following evidence: 1) Colonisation has only occurred in marinas or on vessels/marine equipment which have spent considerable time in or next to marinas. 2) "Infected" sites are on popular leisure-craft routes (Fig. 7.14).

Not all marinas examined in Scotland were colonised. This may simply be due to lack of exposure or could, in part, be attributable to the different environmental characteristics of the marinas. *S. japonica* was only found in full salinity marinas (>30 psu). This is in accordance with a study by Treibergs (2012) which found that along an estuarine gradient of 6 sites in Oregon, USA, settlement by *S. japonica* only occurred at the three most oceanic study sites (mean > 32psu). It should be noted, however, that identification of *Schizoporella* study species in Treiberg's MSc. thesis (2012) was not confirmed using SEM and could potentially therefore not be *S. japonica* as claimed. Further evidence of a salinity preference in *S. japonica* may be the observed change in colonisation in Lerwick marina, Shetland. In September 2012 *S. japonica* was found heavily colonised at Lerwick marina, whilst in December 2012 it was entirely absent. Environmental measurements in December 2012 detected a highly stratified freshwater layer overlaying the saltwater beneath. It is not known if this stratification was exceptional or a common occurrence in the wetter autumn/winter months. It is suggested that this stratified freshwater layer created an intolerable habitat for *S. japonica*, resulting on its "dying off". This theory would, however, need to be ratified in structured salinity tolerance experiments.

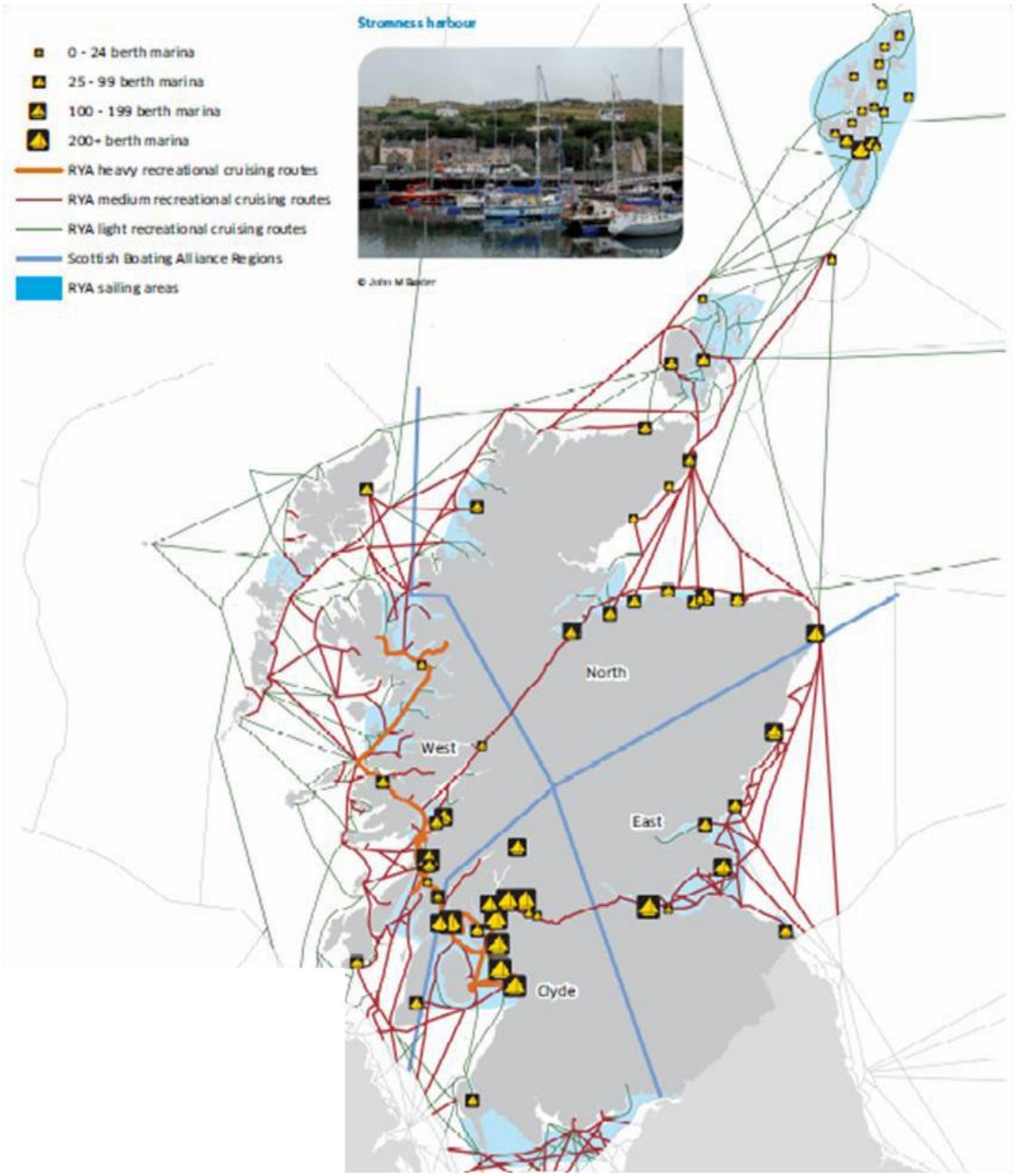

Figure 7.14: Recreational cruising routes and their relative popularity in Scotland. Reproduced from Scotland's Marine Atlas (Scottish Government 2011)

Further work on the environmental preferences of *S. japonica* and the application of this data into modelling algorithms such as BIOCLIM or MAXENT would allow ecological niche modelling and potentially help predict future movements of the species through Scotland and further afield.

7.6.2 Comparative mineralogy of S. japonica and S. unicornis

S. japonica was found to be bimineralic (mean 40.55 wt% aragonite) with an IMC (mean 5.98 wt% $MgCO_3$ in calcite) skeleton. *S. unicornis*, in contrast, had less aragonite (mean 26.03 wt% aragonite) and HMC (mean 8.01 wt% $MgCO_3$ in calcite). Both of these measurements are within the previously known mineralogy range of the genus, as presented in Chapter 3, Section 3.5.2. CHN analysis of *S. japonica* showed a ratio of 29:71 organic:inorganic carbon. Previous mineralogical analyses of *S. unicornis* was reported by Smith et al. (2006) to range between 0.6 – 7.8 wt% $MgCO_3$ in calcite. *S. unicornis* has been widely reported in bryozoan mineralogical publications for its mineralogical plasticity (Clarke & Wheeler 1922; Lowenstam 1954; Schopf & Manheim 1967; Carver & Rucker 1969; Poluzzi & Sartori 1973; Smith et al. 2006) with authors using and recommending it as an ideal species for studying the effects of change in environmental correlations with mineralogy (Lowenstam 1954) and potential palaeoenvironmental interpretation (Smith et al. 2006). Both recent publications and work for this Chapter, however, has highlighted that many records and museum specimens previously identified as *S. unicornis* are actually misidentified *S. japonica*, *S.errata*, *S.dunkeri* or other *Schizoporella* species (Hayward & Ryland 1995; Tompsett et al. 2009; Clarke Murray et al. 2011). Tompsett et al.'s (2009) re-description of the species observes that out of 25 examined papers where the species is named, only four (16%) present correct identifications. With this taxonomic doubt cast on past records of *S. unicornis* (including past mineralogical analyses) caution should be exercised regarding the reported wide geographical distribution of 84 latitudinal degrees and the plastic range in mineralogy. Revisiting the published mineralogical analyses and applying Tompsett et al.'s (2009)guidance based on geographical location would discredit the following "*S. unicornis*" analyses: Lowenstam's (1954) analyses from Bermuda, which are more likely to be misidentified *S. errata*; Clarke & Wheeler (1917, 1922) analyses from Massachusetts and Florida, which are more likely to be misidentified *S. errata* and *S. japonica* respectively; and Rucker & Carvers (1969) 15 analyses from Massachusetts, which are more likely to be misidentified *S. japonica*. Mineralogical studies of *S. unicornis* are analyses by Taylor et al (2009) and Poluzzi & Sartori (1973, 1974) which report 6.65 and 7.69 wt% $MgCO_3$ in calcite respectively; within the range of samples analysed in the current study. Mineralogical analyses conducted in the current study show that although *S. japonica* and *S. unicornis* have a comparable wide range of variability in

aragonite content (68.45wt% and 58.27wt% respectively). *S. unicornis* has a slightly lower range of $MgCO_3$ in calcite (3.65wt%) compared to *S. japonica* (3.99wt%). This is most likely attributable to the wider range of geographical location *S. japonica* was collected from, while *S. unicornis* was collected from a single site. If we limit the included *S. japonica* samples to those from Stromness only then the ranges for the two species become more closely aligned (*S. unicornis:* range wt% $MgCO_3$ in calcite = 3.44; *S. japonica:* range wt% $MgCO_3$ in calcite = 3.85).

The difference in wt% $MgCO_3$ in calcite between the species may be related to the differential ranges of the species, with *S. unicornis* featuring a Lusitanean/Boreal distribution (Tompsett et al. 2009) while, from what we have found so far, *S. japonica* seems to favour a more Boreal distribution. It is possible that the different ranges of wt % $MgCO_3$ in calcite between the species could provide a feature to aid taxonomic distinction in the future, however more analysis of specimens with confirmed identification would be needed in order to calibrate the robustness of this feature.

7.6.3 Ultrastructure and spatial localisation in the skeleton of S. japonica

S. japonica features four ultrastructure fabrics, one of which is aragonitic (acicular crystallites) and three calcitic (rhombic semi-nacre, rod-like and granular). The aragonitic needles are unlike the fabrics previously described for cheilostome bryozoans by Weedon & Taylor (2000) although they do resemble the acicular crystals pictured by Sandberg (1971) in the bimineralic species *Parasmittina californica* (Robertson, 1908). The fabric is also consistent with the aragonitic crystals seen in coral septa (e.g. Clode & Marshall 2003; Stolarski & Mazur 2005). The acicular crystals construct the frontal shield which is in accordance with the localisation of aragonite as revealed by staining (Fig. 7.10A-C).

The three calcitic fabrics are very distinct and are localised in different parts of the zooid. The rhombic semi-nacre makes up the base and walls of the zooid and staining indicates this to contain less $MgCO_3$ than the surrounding calcite. Chapter 6 shows that species with a dominant fabric of rhombic semi-nacre crystals are predominantly cyclostomes, although the bimineralic species *Cellepora pumicosa* (Pallas, 1766) and *Inversiula nutrix* also feature rhombic semi-nacre in their base and walls. The long, flat crystal structure, which sheers in a plane with the settlement surface, may help explain the "flakiness" of the colony on smooth surfaces. Field observations show that removal of colonies from buoys and boat

surfaces can easily be achieved with little, if any, breakage of the colony. Rod-like fabric is used to construct the avicularia rim and this is very similar to fabrics seen in the spines of *Fenestrulina malusii* and *Flustra foliacea*. $MgCO_3$ is shown to be localised in the calcitic rim of the avicularia, indicating that this fabric is IMC; this concurs with data from Chapter

6. The highest concentration of $MgCO_3$ is localised in the calcitic ovicells, which are made up of the smallest granular crystals, further reinforcing the pattern of increasing $MgCO_3$ in smaller calcitic crystals as described in Chapter 6.

The localisation of $MgCO_3$ and aragonite in certain skeletal features adds further data to the growing evidence that mineral localisation conveys a mechanical advantage. Strong, hard aragonite is localised in the frontal shield, as has been previously reported by Smith & Girvan (2010), and to attach the ovicells to the frontal shield, an application of aragonite localisation which has not previously been recorded in bryozoans. $MgCO_3$ was shown to be localised in ovicells and avicularia rims, as was reported for other species in Chapter 6. And the fabrics of small, densely packed crystals would indicate a strong fabric in these important defensive and reproductive structures. The presence of different crystal types and differential staining of calcite in the skeleton indicates that *S. japonica* may feature two or more phases of Mg-calcite although these are not obviously apparent on the XRD spectra.

7.6.4 Spatial patterns of mineralogy for S. japonica

Analysis of samples from 12 different sites revealed no statistically significant difference in wt% $MgCO_3$ in calcite among different sites. This is most likely due to the different seasons (and years) of collection and unknown age of the colonies leading to a wide range of measurements. A more controlled collection of samples from different sites, conducted at the same time of year, may yet reveal differences between sites. A statistically significant difference in wt% aragonite was detected among sites although this also may be attributable to the different ages and secondary calcification of colonies, rather than sites. Portavadie marina featured a particularly high wt% aragonite compared to the other sites and one potential explanation may be increased threat of damage at this site. During collection it was noted that specimens were heavily covered in the invasive skeleton shrimp, *Caprella mutica*, a grazer of the diatoms which coat fouling communities (Cook et al. 2009). The high degree of "scraping" by *C. mutica* may be a trigger for *S. japonica* to produce a thicker layer of protective aragonitic secondary calcification than at other sites.

Further research and observations would be required, however, to investigate this possible connection.

7.6.5 Seasonal patterns of mineralogy and morphology

The native and non-native species differ in morphological variability through the seasons with *S. japonica* featuring more ovicells, including multi-ovicelled zooids; peak production occurs in spring, when seawater conditions are coldest. *S. unicornis* is seen to produce most ovicells in the autumn, when sea temperatures are at their warmest. This observation aligns with previously published data on the reproduction times for S. unicornis (Todd & Turner 1986). Multi-ovicell behaviour has previously been observed in bryozoans in publications by Hincks (1880), Powell (1970) and Ross & McCain (1976) on Schizoporella. Both Powell and Ross & McCain discuss multi-ovicells in a bryozoan they names as S. unicornis, an identification we now know to probably be an incorrect identification of *S. japonica* (Tompsett 2010). Powell (1970) asserts that multi-ovicell behaviour is an abnormality caused by pollution and presents a relocation experiment showing multi-ovicells were induced in "normal" colonies within 7-9 days of relocation to a site close to tar-treated oyster cages. In contrast Ross & McCain assert that multi-ovicell production is a normal behavior which occurs in controlled, clean aquarium conditions. Multi-ovicell production was also noted by Hincks (1880) as a "monstrosity" observed on a deep-water specimen of a Boreal/Arctic species he identified as *S. ansata*, later assumed by Powell (1970) to be *S. dunkeri*, although this seems unlikely given the more Lusitanian distribution of *S. dunkeri* (Tompsett 2010). Hincks's (1880) description of the ovicell arrangement in this deep-water *Schizoporella* species is identical to that which occurs in *S. japonica*.

It is possible that trace pollution is the trigger for multi-ovicell production in the UK, as proposed by Powell (1970) for North American specimens. An increase in multi-ovicells was observed in *S. japonica* specimens from Stromness marina in March 2012, when dredging and construction work was taking place nearby, potentially causing an increase in pollutants in the water column. It is not possible to distinguish, however, whether multi-ovicell production is a stress response to pollution or other factors such as increased turbidity or predation. It is also not known whether such a stress response could be an energetically expensive "mistake" or whether this is a colonies' "last gasp" attempt to reproduce before it dies. It seems more likely, however, that this is not a stress response but

is a normal response at his time of year (peak breeding season), allowing the species to rapidly colonise newly vacated substrate caused by the winter die-off of other species. It should be noted that Powell (1970) observed "unusually well developed" multi-ovicells on specimens from Japan, the native habitat of *S. japonica*, indicating that this is a common response in the genus. Hincks's (1880) observations of multi-ovicells in *Schizoporella* were based on a specimen collected from deep-water, before oil transportation or refinery became common in the UK. This suggests that multi-ovicell production is unlikely to be a response to oil pollution but may be a natural occurrence in the genus *Schizoporella* as suggested by Ross & McCain (1976). Future research could ascertain whether the additional ovicells on multi-ovicell zooids contain viable larvae and whether this behaviour can be induced under variable temperature, turbidity, predation pressure, pollutant concentration or occurs under benign conditions. Multi-ovicellate behaviour appears to be unique to this genus, and possibly to the species *S. japonica*, and offers an interesting and potentially fruitful area for future research.

S. unicornis features more consistent avicularia production than *S. japonica* with between 1-2 avicularia per zooid throughout the year. *S. japonica*, in contrast, has between 0-5 avicularia per zooid and at times of peak ovicell production, avicularia production is seen to be reduced, an observation also documented by Powell (1970). Spine production in bryozoans has been shown to be a defensive trait, induced by cues from predators in some species (Yoshioka 1982; Iyengar & Harvell 2002). Avicularia are also a defensive feature and specimens of *S. japonica* from Portavadie marina featured the highest occurrence of avicularia with up to five avicularia per zooid (Fig. 7.10D). As discussed previously, specimens from Portavadie were also heavily exposed to grazing pressure from *C. mutica*. It is possible that the production of high volumes of defensive avicularia is being triggered by the presence of *C. mutica*. It would be interesting to test this hypothesis by testing if avicularia production could be induced through controlled exposure of *S. japonica* to *C. mutica* in an aquarium experiment.

Mineralogy is also highly variable in both *S. japonica* and *S. unicornis*. The only statistically significant link between mineralogy and seasonal variation in environmental conditions for *S. japonica*, however, is a negative correlation between temperature and wt% $MgCO_3$ in calcite. This negative correlation is weakly statistically significant if data from Stromness marina alone is analysed, increasing in significance when data from the 11

additional sites is included. In contrast there is no statistically significant correlation between mineralogy and seasonal variation in environmental conditions for the native, *S. unicornis*. The negative correlation between wt% $MgCO_3$ in calcite and temperature is contrary to the expected positive correlation which is commonly used as a proxy for seawater temperature in marine invertebrates (Lowenstam 1954). Thermodynamic studies have shown that, where temperature is the only influencing factor, Mg incorporation within calcite can be expected to increase by approximately 3% per 1º C (Rosenthal et al. 1997). The temperature difference between seasons in this study is over ten degrees and so, if mineralogy of is under environmental control, we would expect an increase in $MgCO_3$ in calcite of approximately 30% in both *S. japonica* and *S. unicornis*. Instead in *S. japonica* we observe a decrease in skeletal Mg-calcite and in *S. unicornis* we see no significant increase or decrease. In these two species, therefore, $MgCO_3$ in calcite is probably attributable to biological control of skeletal mineralogy. Two potential biological explanations for seasonal variation in $MgCO_3$ in calcite for *S. japonica* may be: 1) the ecological preferences of *S. japonica* and 2) Seasonal morphological variability.

Identifying the preferred ecological niche of *S. japonica* globally is problematic. Although *S. japonica* has been reported from Northern Japan to Hong Kong (Lui et al. 2001), Alaska to California (Dick et al. 2005) and even from Sydney in Australia (Ross & McCain 1976), the confused taxonomy of the genus means that the majority of these reports may be unreliable. In its native habitat, Japan, *S. japonica* is found in water temperatures of approximately 6-22ºC, with its distribution limited to North Japan (south to mid Honshu) (Dick et al. 2005) where temperatures are cooler. In North American sites it has been found to thrive from Alaska to Vancouver and is particularly well established in Alaska where temperatures rarely exceed 10ºC (Ruiz et al. 2006). In this study, most ovicells were found during periods of lower temperature in the spring months and when considered alongside its generally Boreal distribution this suggests *S. japonica* is a stenothermal species with a preference for cooler temperatures. At warmer temperatures, such as those encountered in northern hemisphere late Summer and Autumn, *S. japonica* may be encountering some physiological and metabolic stress (Portner et al. 2000). This could result in reducing the energy available for reproduction and skeleton construction. As $MgCO_3$ is more energetically expensive to build than calcite, during times of metabolic stress we could therefore expect less $MgCO_3$ to be deposited. As sea temperature decreases *S. japonica*

deposits increased Mg-calcite in its skeleton and increases ovicells production and this could be considered evidence of the stable metabolic state of this species in cool conditions. Further collection of data on growth rates and breeding times, analysed alongside improved distribution data would enable testing of this hypothesis. An alternative explanation is that seasonal changes in mineralogy are less related to sea water temperature but are instead a "side-effect" of morphological changes with seasons. Counts have shown that during cooler months more ovicells are present in colonies. Section 7.6.3 demonstrates that these ovicells are constructed from calcite with a higher wt% $MgCO_3$ that the rest of the skeleton and it therefore follows that a colony with more ovicells would feature a higher mean wt% $MgCO_3$ in calcite. The increase in ovicells during colder months is probably, in turn, a result of a stenothermal preference of *S. japonica* for cold water and thus it could be considered that the increasing wt % $MgCO_3$ in skeletal calcite is a combined result of ecological preferences and morphology. The native *S. unicornis*, in contrast, has increased reproduction during autumn, when seawater temperatures are highest. Considered alongside its wide Lusitanean/Boreal distribution this suggests that *S. unicornis* is a eurythermal species, metabolically comfortable at the full range of seasonal temperature variation which occurs in Orkney. The lack of influence of metabolic stress on skeletal deposition could explain why there is no statistically significant correlation between mineralogy and sea-water temperature for this species.

7.6.6 Potential ecological and commercial implications of S. japonica

The stenothermal ecology of *S. japonica* could help explain the species success as a non-native fouler. *S. japonica* reproduces most vigorously during the coldest months of the year, a time when much of the native British fouling fauna is dying-back or static (Griffith et al. 2009) and competition for space is minimal. Winter is also the time when boats etc. are more likely to be moored for longer periods of time, allowing establishment of colonies. Non-native fouling costs the British economy an estimated £1.7 billion per year (Scottish Government 2011) and commercially *S. japonica* has potential to be both costly and inconvenient to Scottish industry. The areas where *S. japonica* is well established, the West Coast, Orkney and Shetland, are important areas for aquaculture. So far, no impacts on aquaculture have been reported, however heavy *S. japonica* fouling has been found on boats used to service fish farms in Orkney and it is likely that fish farm cages may be "infected". The extent of *S. japonica* colonisation at fish-farms is not yet known and it is

not yet understood whether consumption of *S. japonica* by farmed fish could cause health impacts; future research in this area would provide useful information to the aquaculture industry. Shetland produces more rope-farmed mussels than any other region in Scotland (Scott et al. 2010) and there is potential for *S. japonica* to cause inconvenience to this sector by fouling mussels and ropes. Marina observations have shown, however, that *S. japonica* only settles to approximately 50cm depth and so the majority of the mussel ropes would remain "out of reach" for colonisation and it is likely that impact on the industry would be minimal.

In Orkney *S. japonica* can already be found fouling pleasure craft (Fig. 7.15A), small commercial vessels (Fig. 7.15C) and marine renewable energy devices (Fig. 7.15B).

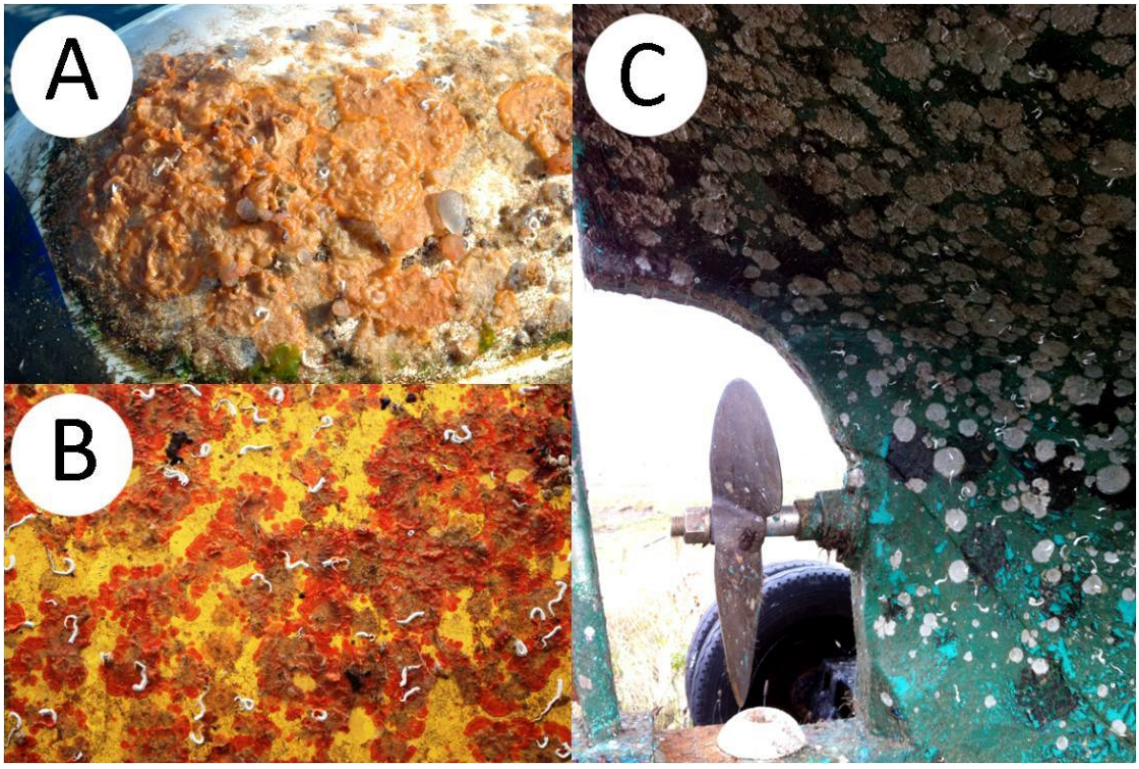

Figure 7.15: Schizoporella japonica fouling of A) yacht buoy in Stromness marina (Dec 2013); B) SR250 prototype tidal turbine device (Dec 2012), image taken by A. Want; C) small fishing vessel in dry-dock, usually moored in Westray marina (Oct 2012)

The fouling of pleasure-craft and small commercial vessels by *S. japonica* is likely to increase the rate of spread of the species throughout Scotland and further afield. Although usually static in marinas during the winter months, many vessels from Orkney and Shetland travel between islands and mainland in Scotland, East to Norway, South to England and West across the North Passage to the United States, and this is most likely the key vector

for this species. *S. japonica* is unlikely to cause any lasting damage to the vessels, however, as we have observed that it is quick to die and flake off after removal from the water or exposure to freshwater. The fouling of marine renewable devices could be a more costly and potentially ecologically damaging problem. The waters around Orkney, Shetland and the Pentland Firth are the location of a high percentage of Europe's tidal and wave power (Scottish Government 2011); as such it is a hotspot of development of renewable devices. Wave devices, in particular, are potentially susceptible to heavy colonisation from *S. japonica*. These devices bob on the surface of the sea, with the underwater portion of the devices, such as the Pelamis P2-002, mostly within the 50cm "reach" of *S. japonica*. The devices are anchored in a static position, and are serviced by boats mooring alongside. In periods of high swell the devices are towed into Orkney marinas or harbours where they are often moored for prolonged periods of time. During both servicing and mooring there is opportunity for *S. japonica* colonisation and the likelihood of this is heightened as currently renewable development companies are not applying any anti-foulant to wave or tidal devices. Heavy colonisation of wave devices in particular could have both commercial and ecological implications. Mechanically the devices are complex (Fig. 7.16B & D), with both the Oyster 800 (Aquamarine Power 2014) and P2-002 (Pelamis Wave Power 2014), the leading wave device designs in Orkney at the moment, dependent on moving joints to function (Fig 7.16B and D). *S. japonica*, with its high aragonite content, forms a gritty surface when crushed; this could cause abrasion or damage in these joints and potentially impede the function of the devices.

An additional risk from fouling of wave devices is that *S. japonica* may be able to use the devices as "stepping stones" in order to colonise the intertidal zone. In both its native Japan and in North America, *S. japonica* primarily inhabits the intertidal region (Dick et al. 2005; Tompsett et al. 2009; Tompsett 2010), however, as yet, it has not been found in the intertidal in Britain. Recent modelling on pelagic larval dispersal and renewable energy devices on the West coast of Scotland by Adams et al. (2014) identified the potential of marine renewable energy devices to act as "stepping-stones", extending the range of native species and potentially increasing the reach of non-natives. A well colonised device, anchored close to shore, could conceivably be a source of *S. japonica* larvae into the intertidal, and potentially impact both native communities and intertidal Pacific Oyster cultivation, the most valuable aquaculture in Scotland (Scottish Government 2011).

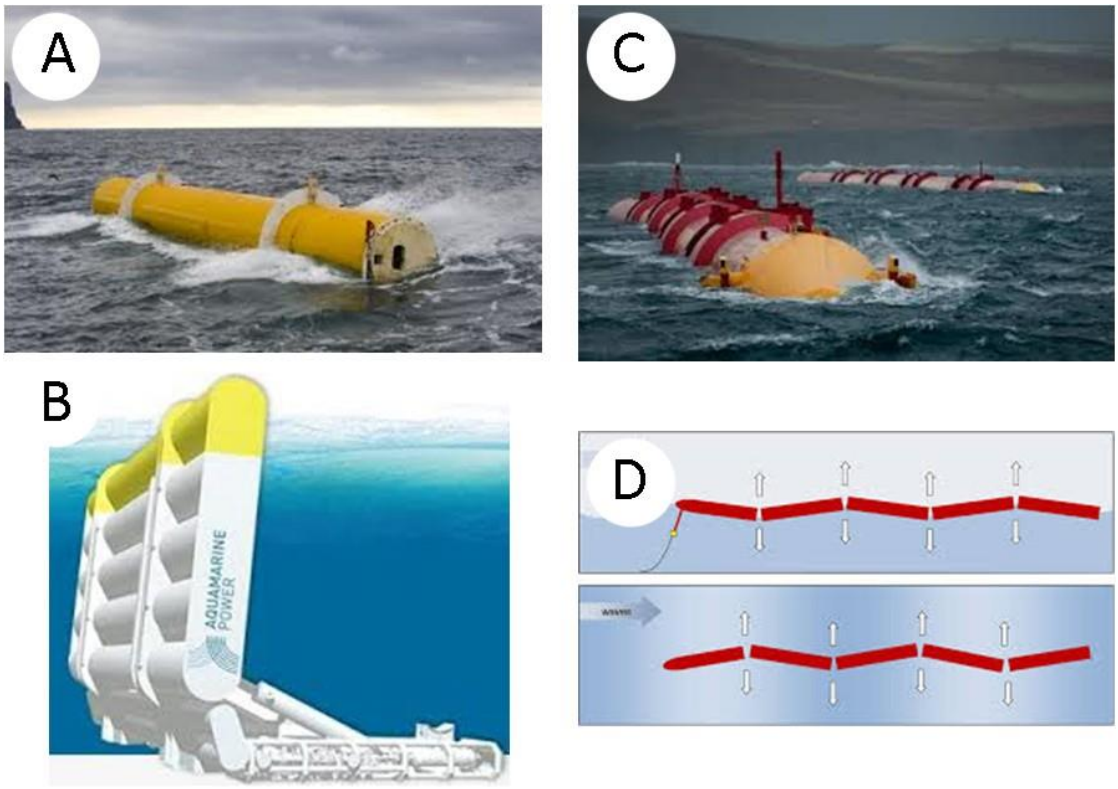

Figure 7.16: Leading technologies in wave power in Orkney. A-B) Aquamarine Power's Oyster 800 device (Aquamarine Power 2014) *A)in Billia Croo, West Orkney and B) diagram of design; C-D) Pelamis Wave Power's P2-002 device* (Pelamis Wave Power 2014) *A) In Billia Croo, West Orkney and B) diagram of P2-002 design.*

Through this route this non-native species could cause a threat to native biodiversity in the intertidal zone and progress from being considered a relatively benign non-native species to an invasive species (International Union for the Conservation of Nature 2000). Further research into *S. japonica* larval swimming distances, combined with ocean energy modelling would help elucidate the potential for larvae reaching the intertidal and successfully metamorphosing. Controlled competition studies between native intertidal communities and *S. japonica* would help us understand the extent of any ecological impact and potential loss in biodiversity resulting from its arrival.

7.7 Summary and Conclusions

Examination of the distribution of the non-native bryozoan, *Schizoporella japonica*, shows it to be established in eleven British marinas with a preference for cold full-salinity water. In Scotland it has been found to foul marina pontoons, pleasure-craft, small commercial vessels and marine renewable energy devices. It is suspected that small vessel hull fouling transported *S. japonica* from N. America to Scotland and is the primary vector for its movement around the UK.

S. japonica features more aragonite and less $MgCO_3$ in calcite than the native species, *S. unicornis* and has a highly complex and variable skeletal mineralogy and morphology. Neither *S. japonica* nor *S. unicornis* show evidence of the expected relationships of increasing aragonite and Mg-calcite with seasonal changes in seawater temperature. *S. japonica* shows a statistically significant negative correlation between $MgCO_3$ in calcite and temperature and, for this stenothermal cold-water species, the physiological preference for cold appears to over-ride any influence of seawater temperature on mineralogy. *S. japonica* is morphologically more variable than *S. unicornis* and features multi-ovicells, a trait unique to the *Schizoporella* genus, and possibly the species.

S. japonica shows differential deposition of $MgCO_3$ and aragonite in skeletal features for mechanical advantage and variable morphology is shown to be related to $MgCO_3$ in calcite.

A combination of morphological and mineralogical adaptability, prolific breeding and cold-adapted stenothermal preferences make *S. japonica* an effective competitor in fouling communities and give it the potential to cause both ecological and commercial impacts in Scotland. Commercially it may impact the aquaculture, shipping and marine renewable energy industries. Ecologically, if it is able to colonise the intertidal, it could increase its rate of spread around the UK, cause community and biotope changes and potentially threaten native biodiversity resulting in its reclassification as an invasive species.

Further research is recommended to 1) model the ecological niche of *S. japonica* and its potential future movements; 2) investigate the viability and occurrence of multi-ovicells; 3) examine larval swimming distance and potential of distribution into the intertidal and 4) test competition between *S .japonica* and native intertidal communities.

Chapter 8: General Discussion

Globally, our oceans are getting warmer and more acidic (Kerr 2013). For calcifying organisms this change in conditions has the potential to impact skeleton growth rates (e.g. Findlay et al. 2008; Anlauf et al. 2011), dissolution rates (e.g. Widdicombe & Spicer 2008) and physiology (e.g. Somero 2012). It is becoming increasingly important to understand the responses of marine calcifiers to environmental conditions as this knowledge may help us to predict which species will survive and how the composition of assemblages will change in the future. It is anticipated that marine animals which are able to vary their skeleton chemistry in response to environmental change may also be useful as an indicator of past, present and future ocean conditions (Fabry et al. 2009).

Between 1885 and 2012, the mineralogical analysis of ~2500 skeletal samples established that bryozoans exhibit a highly variable geochemistry within their skeletons (Smith et al. 2006; Lombardi et al. 2008). A wide spectrum of calcite and/or aragonite composition and differing levels of $MgCO_s$ incorporation within calcite have been observed (Smith et al. 2006; Taylor et al. 2009), with variation occurring both within and among species. Theories have been proposed attributing this variability to environmental and/or biological controls, however, within the bryozoan literature this debate is ongoing and, prior to this study, there was little conclusive evidence. Past mineralogical studies on bryozoans have been heavily constrained by the limited origin and availability of samples and associated metadata on environmental conditions. The mineralogy of less than 10% of extant bryozoan species have been examined to date and the majority of studies have involved single or low numbers of replicate analyses. No studies have been conducted investigating phylogenetic, seasonal, ecological or morphological patterns of bryozoan mineralogy.

This study aims to explore the skeletal mineralogy of Temperate and Polar bryozoans, investigating any variability within and between species in relation to: specimen preservation and cleaning; taxonomic and phylogenetic relationships; spatial considerations including, latitude, depth, region and distance between sites; environmental conditions and seasonality; ecological adaptations and morphology at the ultra, micro and macro-scale.

8.1 Empirical findings

8.1.1 Specimen preservation and cleaning and its impact on bryozoan mineralogy

For centuries bryozoan collections have been subjected to curational procedures involving a wide variety of preservation and cleaning techniques. To date, however, little consideration has been given to how cleaning and preservation may affect fragile or dissolution susceptible calcium carbonate within the skeleton, and consequently how this may influence mineralogical measurements. Studies have documented the impact of curational procedures on carbonate chemistry on a range of taxa (e.g. Tasch & Shaffer 1961; Milton & Chenery 1998; Edwards et al. 2002; Barker et al. 2003; Hedges et al. 2004). The aim of Chapter 2 was to investigate the impact of cleaning procedures and preservation on the skeletons of marine bryozoans. This represents the first study of cleaning or preservation impacts on mineralogy in bryozoans and one of the most in depth examinations conducted in any taxa. Findings of the cleaning experiment showed that:

i) More mass and $MgCO_3$ in calcite was lost during cleaning with higher bleach concentrations, over longer durations and with exposure to ultrasonic. This concurs with published observations of skeleton damage in bryozoans caused by ultrasonic (Sandberg 1971) and bleaching (Taylor & Weedon 2000).

ii) The loss of organic mass from the skeleton resulted in increased $MgCO_3$ loss; a pattern also observed in Crustacea (Inoue et al. 2008) and Mollusca (Zuschin et al. 2003), and attributed to the important role the organic matrix plays in holding the carbonate component together.

iii) Specimens with higher initial Mg-calcite were found to be especially susceptible to $MgCO_3$ loss during cleaning and although this has not been investigated or observed in previous studies, it is in accordance with what would be expected from solution equilibrium chemistry (Oomori et al. 1987) and reaction kinetics (Moore 2012).

Each cleaning treatment was conducted on triplicate specimens, and the complimentary use of XRD, ICP-AES, staining and mass measurements in the cleaning experiment provide a high degree of confidence in the results presented for *Flustra foliacea*. The study was, however, limited to a single species with a lightly calcified skeleton. It is recommended that similar studies should be conducted on additional bryozoan species with the aim to produce

a calibration curve for the back-calculation of initial mineralogy for cleaned museum samples.

Findings of the preservation experiment showed that aragonitic and HMC bryozoan species showed no impact of preservation on skeleton chemistry over the two year study period. LMC and IMC species, however, both showed an increase in wt% $MgCO_3$ in calcite over time with preservation. The results are in accordance with observations from fish otolith (Milton & Chenery 1998; Hedges et al. 2004) and Foraminifera (Ganssen 1981) studies, which also document an increase in $MgCO_3$ caused by preservation. The complex interaction between specimens and preservation solutions, is attributed to solution chemistry and reaction kinetics and has been previously observed in curation publications (Steedman 1976; Cato 1990; Moore 1999; Edwards et al. 2002; Marte et al. 2003; Waller & Simmons 2003; Kotrba & Golbig 2009; Garrigos et al. 2013). Strengths of this preservation study include the use of multiple species, regular timepoints and replicates however the experimental design could have been improved by more controlled conditions, increased preservation fluid analysis and a longer overall period of preservation. As such it is recommended that this preservation experiment be treated as a pilot study. The results provided by this pilot study, highlight a potential source of error in mineralogical analyses and future long-term studies into the impact of preservation on bryozoan mineralogy are, therefore, recommended.

Examination of published bryozoan mineralogy analyses showed that in 44% authors either didn't document or provided an incomplete description of cleaning techniques applied prior to sample analysis and 65% provided no information on preservation procedures. As a result of the findings of this study, which shows evidence of mineralogical impacts of cleaning and preservation, the results from some previous studies should be treated with caution. Publications documenting broad mineralogical trends derived from the comparison of multiple literary sources of bryozoan mineralogy (e.g. Smith et al. 2006; Taylor et al. 2009), are based on the assumption of accurate mineralogical reporting in previous publications, and should, therefore, also be treated with caution.

World-wide there is an abundance of historic samples stored in museum collections, which could potentially be used for mineralogical studies of palaeo-climate. In addition new specimens are being added to collections daily which may be used for future investigations into climate change effects. It is anticipated that this study will provide vital

recommendations to curators in order to preserve the geochemical usefulness of calcified specimens and increase the reliability of between-study comparability for mineralogical analyses in the future, both for bryozoans and other phyla. New material, cleaned and preserved with consideration for mineralogy, would help to ascertain correct mineralogy patterns in the future.

8.1.2 Taxonomic and phylogenetic patterns of bryozoan mineralogy

Chapter 3 represents the first phylomineralogy study in bryozoans and the results indicate that there is a strong relationship between the phylogenetic relatedness of species and the mineralogical composition of their skeletons. Specifically, cyclostomes were found to feature less aragonite and lower $MgCO_3$ in calcite than cheilostomes. This difference is attributed to the smaller genetic distance between cyclostomes and the shared (calcitic) evolutionary ancestor. The findings are in keeping with previous studies relating mineralogy to taxonomic patterns (Smith et al. 2006) and first appearance date (FAD) of families (Taylor et al. 2009). Mineralogical similarities between species from the same phylogenetic clades were further observed in Chapters 4, 5, 6 and 7 and these generally reflected taxonomic patterns reported by Smith et al (2006). Investigations into taxonomic patterns of mineralogy revealed that the majority of bryozoans considered to be highly plastic bio-mineralisers in the literature are actually a result of taxonomic instability of the species, or cryptic speciation (e.g. *Schizoporella*, *Bugula* and *Watersipora*). It is therefore recommended that phylogenetic data is used where possible, in the future, to predict and to confirm mineralogical patterns related to taxonomy. Phylogenetic analysis of mineralogical patterns in this study was limited by the number of species (n=39) for which both mineralogical and phylogeny data were available. Compared to recent phylo-mineralogy studies on coralline red algae (28 species) (Smith et al. 2012) and serpulids (18 genera) (Smith et al. 2013), however, this seems an acceptable quantity for a reliable interpretation of patterns in the first instance.

An implication of the findings regarding the taxonomic patterns observed in bryozoan mineralogy is that this information could be used to develop further hypotheses to test the potential for detecting cryptic speciation or specimen misidentification. An example of this was seen in Chapter 4 where unusual mineralogical measurements led to the detection of probable different species. A further implication of the reported phylogenetic patterns in bryozoan mineralogy is the relative impact of climate change and ocean acidification on the

different clades. Cyclostomes, with their generally calcitic skeletons and lower incorporation of $MgCO_3$ could potentially be expected to be less soluble under ocean acidification conditions than cheilostomes and may become more dominant in the future. On the other hand, cheilostomes feature a more adaptable skeleton and therefore may be able to accommodate warming and ocean acidification through range extension. Currently the majority of reported non-native species are cheilostomes, and this may increase further with climate change.

8.1.3 Consideration of spatial influences on mineralogical variability, including: latitude; depth; region and distance between sites

Investigations into the influence of latitude on the mineralogy of Scottish bryozoans identified trends of decreasing wt% aragonite and wt% $MgCO_3$ in calcite with increasing latitude and modelling showed latitude to explain the majority of mineralogical variability. This concurs with previous publications looking at latitudinal influences on mineralogy at a regional or global scale (Smith et al. 2006; Kuklinski & Taylor 2009). It is important to note, however, that although there is a correlation of Mg-calcite and aragonite with latitude it is highly unlikely that this is the cause of the variability. Although the study design was in line with previous investigations it is a very simplistic model and neglects to take into account other factors which vary with latitude including differing species, community structures, temperature, nutrient availability, seasonality, growth rates and seawater chemistry all of which have been shown to influence mineralogy. As such the value of observing high-level latitudinal correlations with mineralogy is questionable and misleadingly simplistic. It is recommended that future studies should move away from this methodology and towards more structured studies relating mineralogy to combinations of measurable environmental and biological factors.

Across all spatial studies no statistically significant relationship was found between depth and bryozoan skeletal mineralogy. This represents the first controlled study to investigate the effects of depth variation on bryozoan mineralogy however it was limited to the infralittoral/circalittoral zone. Environmental measurements taken at different depths within this zone show a well mixed water body and it is proposed that any future investigations into the effects of depth look at mineralogical variation over a greater range of depth. The challenge of finding individual species which reside over a great range of depths may, however, impact the feasibility of this approach.

At the global (temperate vs Polar), regional (within Scotland) local (<10km, Antarctica and Scotland) and close proximity (<10m, Antarctica only) scales, sites were shown to have statistically significant differences in $MgCO_3$ in calcite. The global scale results are in accordance with those presented by Kuklinski & Taylor (2009), which compared bryozoan mineralogy between the Arctic, Antarctic, New Zealand and Mediterranean and found Polar regions to be mineralogically distinct to temperate zones. This is the first study to investigate the potential impact of relative site proximity on mineralogy at the regional or finer scale. Mineralogical differences at the global and regional scale are most likely attributable to evolutionary adaptation to temperature and seawater chemistry occurring over long times-scales. This explanation is in accordance with that articulated by Taylor et al. (2009), Smith et al. (2006) and Kuklinski & Taylor (2008). At the < 10km scale mineralogical differences between sites are attributed primarily to oceanographic links effecting population connectivity sites. Particularly isolated populations may be experiencing enhanced genetic drift caused by the founder effect, enhancing the phenotypic differentiation between populations. Although the effect of spatial distribution and the impact on population connectivity and phenotypic variation has been investigated in bryozoans in both empirical (Hoare et al. 2001) and theoretical (Cheetham et al. 1994) publications, this study presents the first documented measurements of bryozoan mineralogy on a 'local' spatial scale. The results discussed here are derived from the analysis of ~ 1500 specimens, a vast dataset considering that the combined analyses of all previous bryozoan mineralogy publications amount to less than 2500. As such the results can be considered statistically robust.

An important implication of the observed findings regarding the measured mineralogical differences between sites pertains to the field of palaeoclimatography. Palaeoclimatography is currently a popular field of research as it is being used order to draw comparisons between past and present ocean conditions and relate these to climate change. The results of this study however, suggest that unless studies are comparing specimens collected from exactly the same site then observed differences may not be an accurate reflection of differences in seawater temperature and chemistry. Palaeoclimatic signals may also be being masked or overridden by mineralogical effects arising from adaptation of populations to site specific conditions. As a result the use of bryozoan skeletal mineralogy for

palaeothermometry would be susceptible to a high degree of error and is therefore not recommended unless species specific calibration has been conducted.

The study has shown that mineralogical variability between sites is caused by biological responses to localized environmental conditions; however, at present the mechanism of this is unknown.

8.1.4 Impact of environmental conditions and seasonality on bryozoan mineralogy

Across all components of this thesis no convincing relationships were found relating bryozoan skeletal mineralogy to the environmental factors of salinity, pH or carbonate chemistry, despite the larger number of replicates used than in previous studies. $MgCO_3$ in calcite is shown to be influenced by seasonal changes in temperature and site specific temperature; however, there is no consistency in the relationship with temperature between species. Key results from the thesis:

- In the species *Electra pilosa*, *Membraniporella nitida*, *Tubulipora* sp. and *Hippadenella inerma*, no statistically significant correlation was found between $MgCO_3$ in calcite and temperature. This is in accordance with published data by Crowley & Taylor (2000) which presented a further six New Zealand species which showed no correlation between skeletal mineralogy and temperature.
- The species, *Callopora craticula*, *Microporella ciliata*, *Celleporella hyalina* and *Fenestrulina malusii*, were shown to have a statistically significant positive correlation of increasing $MgCO_3$ in calcite with temperature. This finding is comparable to published results for *Pentapora foliacea* by Lombardi et al (2008) and a general trend described by Kuklinski & Taylor (2009). This positive relationship is thermodynamically favoured and is the basis for the use of $MgCO_3$ in calcite as a proxy for seawater temperature (Chave 1954).
- The species, *Escharella immersa*, *Schizoporella japonica*, *Fenestrulina rugula* and *Inversiula nutrix*, show negative relationships of increasing $MgCO_3$ in calcite with increasing temperature. This is in accordance with results for a single New Zealand species from Crowley & Taylor (2000) but is contrary to the widely expected thermodynamically controlled pattern.

Although temperature might not necessarily be the sole cause of the observed mineralogical variation, it is likely to be triggering mineralogical differences through its effect on growth

rate, food availability, breeding cycles and metabolism. It is suggested that the differential mineralogical responses to temperature between species may be directly linked to the ecological preferences of the species. Of the four species exhibiting negative correlations between temperature and mineralogy at least three are winter breeding, cold-water specialists and could be considered stenothermal cold-adapted species. In contrast, species which exhibited a positive relationship between temperature and mineralogy are all either documented summer breeders or have a predominantly Lusitanean/Boreal distributional range; as such they could be considered eurythermal. It seems possible, therefore, that the ecological preference of the species could be the determining factor to explain a mineralogical response to changes in temperature.

Although the study species from Chapter 5 show patterns derived from very small temperature ranges and individually may be considered weak, when these are combined with the longer term seasonality studies from Chapter 4 and 7 they construct a more robust dataset and provide useful evidence that suggests a differential relationship between temperature and $MgCO_3$ among bryozoan species.

The primary implication of this finding is that it raises doubt about the validity of using bryozoans as palaeoclimatic indicators, as they have been applied in the past. It is, therefore, recommended that the relationship between Mg-calcite and temperature in the Bryozoa should not be assumed but should be proven and calibrated for each species before it can be reliably used as a palaeoclimatic proxy in future studies.

8.1.5 Mineralogical variation as a result of ecological adaptation in Bryozoa

Evidence for the utilization of mineralogy as an ecological adaptation among species is provided from the measurements reported in Chapter 3. Some species are shown to utilize energetically expensive or complex skeletal chemistry which, through its mechanical properties, allows them to occupy specific ecological niches. Examples have been presented across HMC, bi-mineralic and aragonitic species where aragonite and HMC bring enhanced strength and reduced brittleness compared to calcite. This data adds to published evidence of ecological advantages provided by aragonite deposition in bryozoans (Smith & Girvan 2010) although further examples would need to be provided for the evidence to be considered conclusive.

Evidence is provided in Section 8.1.4 for the influence of a species ecological preference on the patterns of change in skeletal mineralogy as a response to changing temperature. This is a research area which has not been investigated previously in bryozoans. Published research on corals, however, shows that colonies only deposit their optimum skeleton when they are metabolically "comfortable" (Stanley & Hardie 1998). During times of metabolic stress, such as that caused by a stenothermal species approaching a thermal limit, energy is diverted from calcification to more vital physiological processes (Portner et al. 2000). It is possible that this is the mechanism in stenothermal cold-adapted bryozoan species which is overriding thermodynamics and resulting in decreased deposition of energetically expensive $MgCO_3$ during warmer months. Similarly we may expect an inverse response to lower seawater temperatures in stenothermal warm-water species. The flexibility to divert energy away from skeletal deposition and towards vital physiological processes such as feeding and breeding during times of stress would help a species conserve energy and survive sub-optimal ecological conditions. It may be speculated that this metabolic/mineralogical flexibility has aided the cold-adapted species, *Schizoporella japonica*, not just to survive, but actually to thrive across the ecologically wide range of non-native habitats in which it becomes established.

The evidence for ecologically driven mineralogical adaptation in the 12 species examined in this thesis is robust, however, this dataset represents only a handful of species from the overall 6000 number in existence. There is also only limited data available on the distributional range and breeding times of bryozoan species and it is therefore difficult to accurately categorize species as having cold or warm-water preferences. Given the uncertainties in ecological preferences amongst the Bryozoa, further research is recommended in order to further elucidate patterns of mineralogical specialization based on ecological adaptation. The proven relationship of ecological preference driving mineralogical adaptation in some bryozoan species does, however, provide evidence for the existence of a "vital effect" in bryozoans. This "vital effect" enables some species to deposit skeletal carbonate out of equilibrium with seawater temperature and is a further reason why some bryozoan species have skeletons that are unlikely to be useful as palaeoclimatic indicators of seawater temperature.

8.1.6 Morphology at the ultra, micro and macro scale

This thesis provides a preliminary examination of the relationship between bryozoan skeletal mineralogy and morphology at the ultra, micro and macro scale. Data presented in Chapter 6 documents clear evidence of a statistically significant relationship between $MgCO_3$ concentration in calcite and ultrastructure crystal size. Although the mechanism of this remains unclear, the dense packing enabled by these small crystals could be contributing to the increased hardness and decreased brittleness of HMC and aragonite compared to calcite. Across all 17 examined species evidence was found of mineral localization in skeletal features; strong evidence for the biological control of mineralogy for mechanical advantage. $MgCO_3$ was found to be localized in: avicularia and orifice openings, providing reinforcement in these "high-wear" areas; in the base of spines and internode junctions, where its low brittleness would decrease the risk of snapping; and in ovicells, where strength is important. A previous study by Loxton et al. (2012) also documented localization of $MgCO_3$ in the base of Polar bryozoan colonies, suggesting that this may be mechanically advantageous. Aragonite, with its documented hardness and strength, was found to be localized in frontal shields, as previously documented by Smith & Girvan (2010), and for the attachment of ovicells. Although the study was limited in the number of species examined, the fact that the patterns were found in all 17 species, across both cyclostomes and cheilostomes and across the full range of $MgCO_3$ incorporation in calcite (LMC, IMC and HMC) offers compelling evidence that this is likely to be a widespread feature among bryozoans. Although this is a relatively new area of research for bryozoans, localisation of minerals for ecological and mechanical advantage has been documented in studies on other calcifying taxa (e.g. Mann 2001; Loste 2003; Borzęcka-Prokop et al. 2007; Yurong & Limin 2010; Feng 2011). Sea urchins, for example, have been shown to have localisation of very high levels of $MgCO_3$ in teeth, where the hardness and strength of the material aids grazing (e.g. Yurong & Limin 2010; Feng 2011; Chen et al. 2012).

One implication of this observation is that $MgCO_3$ is predominantly localized in transient/ephemeral skeletal features and this may be adding to the seasonal variability of mineralogy. Evidence is provided for this by the study of *S. japonica* which shows that it is the number of ovicells present on specimens that best explains fluctuations in $MgCO_3$ in calcite between seasons. A second implication is that exclusively "passive" mineralization, in equilibrium with environmental drivers, is not common in bryozoans and may not even

occur at all; this is further evidence that caution and calibration is required if bryozoans are to be used as palaeoclimatic indicators. The final implication of differential localization of minerals within bryozoan skeletons is the potential for differential dissolution of the HMC and aragonite skeletal features of skeletons under future ocean acidification conditions. The complex skeletal features where minerals are predominantly localized are important for reproduction and defense and it is possible that species showing a high degree of mineral localization may be selected against under ocean acidification conditions. Further investigations into the dissolution patterns of (live) complex bryozoan skeletons would help to elucidate the risk of this occurring.

8.2 Core concept – environmental vs biological control

Prior to this study, the two main controls, which were believed to be exerted on bryozoan mineralogy within species, were predominantly summarised as biological and environmental control (Smith & Key 2004; Steger & Smith 2005; Smith et al. 2006; Lombardi et al. 2008; Kuklinski & Taylor 2009; Taylor et al. 2009), also known as "active" and "passive" control in some publications (Bader & Schäfer 2005; Schäfer & Bader 2008). Biological or "active" control indicates that calcium carbonate is deposited with a composition that is out of equilibrium with the seawater chemistry or temperature of the animal's habitat. Environmental control, or "passive" control, indicates that skeletal mineralogy is driven by the seawater within which the bryozoan lives with little or no physiological involvement from the animal itself.

All species examined in this thesis provided evidence of a "vital effect" involved in mineralization, indicating biological or "active" control. Biological involvement in the control of mineralogy was found at the phylogenetic, physiological and physical level. At the deeper phylogenetic level the data shows a high degree of conservation of mineralogical preferences within families and phylogenetic clades. Physiologically, the ecological adaptation of a bryozoan species was shown to play a role in determining the scale and direction of its mineralogical response to temperature. At the physical level all species examined showed localization of minerals within specific skeletal features for mechanical advantage. Together this evidence provides a compelling case for biological/ "active" control of skeletal mineralogy in bryozoans. In contrast no evidence was found of "passive" environmental control of $MgCO_3$ in the calcite of bryozoans. Some patterns were detected showing a correlation between environmental conditions and skeletal mineralogy although all of these can be considered "active", biological responses to environmental triggers as they resulted in skeletal deposition out of equilibrium with seawater temperature or seawater chemistry.

With regards to the debate about "active"/ biological versus "passive" /environmental control of bryozoan mineralogy, all evidence from this thesis indicates that the bryozoans studied here, predominantly, and possibly exclusively, exhibit "active" biological control over their skeletal mineralogy. This is contrary to previous publications which have suggested that both biological and environmental controls influence bryozoan aragonite

and Mg-calcite content within species (e.g. Kuklinski & Taylor 2008; Lombardi et al. 2008; Schafer & Bader 2008; Taylor et al. 2009). The arguments for both environmental and biological control in these publications are based predominantly on inorganic studies (e.g. Lowenstam 1954; Davis et al. 2000) or experiments involving other marine taxa (e.g. Chave 1954; Cohen & Branch 1992). None of the publications debating biological vs environmental control in bryozoan mineralogy provide any evidence of "passive" environmental control of mineralogy in extant bryozoans. Some of the reason for the ongoing debate about environmental and biological control of mineralogy in the bryozoan literature may be due to different interpretations of the terms "environmental" and "biological". The interpretation of the term "environmental control" seems to vary between publications with some defining it as any change in bryozoan mineralogy triggered by a changing environmental factor, regardless of whether skeletal deposition is deposited in equilibrium with the seawater temperature or chemistry within which the animal resides (e.g. Taylor et al. 2009; Kuklinski & Taylor 2009). This study challenges this definition on the basis that it makes it impossible to differentiate between environmental and biological control. The majority of biological mechanisms of mineralogy control (e.g. astogeny, breeding, growth rate) are influenced by environmental factors and conversely changes in a single environmental factor, such as temperature, may result in changes in multiple physiological processes which influence mineralogy. It is suggested that in the future, for clarity, the terms "active" and "passive" are always included in publications when debating environmental or biological controls of mineralogy.

There are two main implications of a predominantly "active" biological method of control in bryozoan mineralogy. The first implication is that, as previously discussed, the use of bryozoan skeletal mineralogy for palaeoclimatic interpretation is problematic, as physiological processes may be overriding or masking any environmentally driven pattern. The second implication is that it becomes more difficult to predict effects of climate change or ocean acidification on bryozoans. It is likely that ocean warming will affect different species in different ways, depending on the strength of their species specific "vital effect". More investigations will be required before generalizations on the effects of climate change or ocean acidification are possible across taxonomic or ecological groups of bryozoan species.

8.3 Recommendations for future research

- At present mineralogical studies have only been conducted on ~12% of extant bryozoan species. It is recommended that surveys of bryozoan species continue, particularly focusing on understudied regions and, where possible, including multiple analyses for individual species.
- It is recommended that future research into the relationship between temperature and bryozoan mineralogy is conducted at fixed sites over multiple seasons. It would be interesting to focus on a mixture of species which are at their northern or southern-most distributional limit and are known to be winter or summer breeders; this would help elucidate the impact of ecological preference on mineralogical variation with temperature. To compliment this it is recommended that a targeted study is undertaken investigating the mineralogical response of ecologically adapted species under "stress". A multi-disciplinary *in vitro* study on thermally "stressed" and "non-stressed" stenothermal bryozoan species recording respiration, proteomic stress response (e.g. expression of heat-shock proteins) and calcification rate alongside skeletal mineralogy is recommended.
- Further research is recommended into the localization of minerals within bryozoan skeletons and the associated mechanical properties of multi-phase and bimineralic skeletal structures. As well as enhancing our knowledge of the mechanisms of bryozoan skeletal function this area of research has potential commercial applications for bio-inspired design of products such as marine renewable energy devices.
- Future research into the mechanisms of skeletal deposition in bryozoans would greatly enhance the investigative techniques available to mineralogists. A fully sequenced and annotated bryozoan genome, and subsequent identification of genes and proteins involved in the calcification process, would enable monitoring of protein expression under different environmental conditions. This would facilitate a step-change in our understanding of potential impacts of climate change and ocean acidification.

8.4 Conclusion

This study set out to explore the skeletal mineralogy of temperate and Polar bryozoans, investigating variability within and between species in relation to methodological, biological and environmental factors. Oceans are becoming warmer and more acidic and it is becoming increasingly important to increase our knowledge about the responses of marine calcifiers to environmental conditions. Bryozoans are important components of the benthic community globally, however prior to this study relatively little was known about their skeleton composition. The skeletons of less than 10% of known bryozoan species had been determined from less than 2500 mineralogical analyses. Although a wide range of mineralogical variation was reported within Bryozoa, no dedicated studies had investigated the causes of mineralogical variability within or between different species.

This study has contributed over 1700 new mineralogical analyses to the field and provided new skeletal profiles for 115 bryozoan species. The study represents by far the most comprehensive regional profile of bryozoan mineralogy to date with 79% of Scottish species analysed. Targeted experiments have resulted in the documentation of impacts of curational procedures on skeletal mineralogy, resulting in recommendations which are pertinent across taxa in the wider fields of both curation and palaeoclimatography. Evidence is presented of mineral localization in specific skeletal features in bryozoan skeletons which adds to the growing body of data showing $MgCO_3$ localization for mechanical and ecological advantage in marine invertebrates.

Prior to this study it was proposed that both “active” biological and “passive” environmental controls influence bryozoan skeletal mineralogy. Through the examination of 156 Polar and temperate species, results from this study showed evidence of biological control of bryozoan mineralogy, while no evidence of passive environmental control was found. This finding precludes the use of bryozoan mineralogy for palaeoclimatic interpretation, for the species included in this study, and it is recommended that future species are chosen carefully and thoroughly calibrated prior to their use as palaeothermometers. Further investigation into the effects of ecological specification on the temperature/mineralogy response may, however, prove an area for fruitful research, enabling prediction of climate change effects on the bryozoan skeletons of ecologically specialised species and providing insights into changes in community composition.

Appendix A: Details of previous analyses of Scottish species

		all previous analyses			analyses from Scottish waters			
genus+species	publications	N	wt. % $MgCO_3$	wt. % calcite	Locality	N	wt. % $MgCO_3$	wt. % calcite
Amphiblestrum flemingii	Taylor 2009,	1	4.70	100				
Bugula neritina	Clarke & Wheeler 1922, Borisenko & Gontar 1991, Smith et al. 2006	3	7.30	100				
Caberea ellisii	Borisenko & Gontar 1991	1	1.50	100				
Callopora craticula	Taylor 2009,	2	0.30	100				
Callopora lineata	Taylor 2009,	3	5.60	100	Shetland (2)	2	5.85	100
Carbasea carbasea	Taylor 2009,	3	8.23	100	Leith(1)	1	7.40	100
Cauloramphus spiniferum	Taylor 2009,	2	7.10	100				
Cellaria salicornioides	Poluzzi & Sartori 1974	1	6.00	100				
Cellepora pumicosa	Taylor 2009, Poluzzi & Sartori 1974	3	7.10	100				
Celleporella hyalina	Taylor 2009,	1	0.13	100	Shetland(3)	3	0.30	100
Celleporina caliciformis	Poluzzi & Sartori 1974	1	9.00	100				

Chartella papyracea	Taylor 2009,	1	10.60	100
Chorizopora brongniartii	Poluzzi & Sartori 1974	1	7.00	100
Conopeum seurati	Poluzzi & Sartori 1974	1	7.00	100
Cribrilina annulata	Taylor 2009,	3	6.70	100
Cribrilina cryptooecium	Taylor 2009,	7	4.10	100
Crisia eburnea	Borisenko & Gontar 1991	1	1.30	100
Cryptosula pallasiana	Taylor 2009, Poluzzi & Sartori 1974, Schopf & Allan 1970, Smith et al 2006	14	5.30	93
Dendrobeania murrayana	Taylor 2009,	2	4.30	100
Electra pilosa	Taylor 2009,	2	10.10	100
Escharella immersa	Taylor 2009,	2	5.15	88
Escharella variolosa	Poluzzi & Sartori 1974	1	9.00	77
Escharoides coccinea	Taylor 2009, Poluzzi & Sartori 1974	4	6.70	71
Eucratea loricata	Taylor 2009, Borisenko & Gontar 1991	9	5.50	100
Fenestrulina malusii	Poluzzi & Sartori 1974	1	9.00	100

Flustra foliacea	Taylor 2009, Schopf & Allan 1970, Borisenko & Gontar 1991	5	4.30	100				
Haplopoma impressum	Poluzzi & Sartori 1974	1	?	100				
Hippoporina pertusa	Poluzzi & Sartori 1974, Smith et al 2006	3	6.20	43				
Idmidronea atlantica	Poluzzi & Sartori 1974, Smith et al 2006	4	5.10	100				
Membranipora membranacea	Taylor 2009, Clarke & Wheeler 1922	5	0.00	37				
Schizoporella unicornis	Taylor 2009, Poluzzi & Sartori 1974, Schopf & Allan 1970, Clarke & Wheeler 1922, Smith + 2006	29	5.77	45				
Scrupocellaria reptans	Taylor 2009,	2	5.10	100	Shetland(1)	1	3.50	100
Scrupocellaria scrupea	Poluzzi & Sartori 1974	1	8.00	100				
Securiflustra securifrons	Taylor 2009, Poluzzi & Sartori 1974	3	7.65	100	Scotland(1)	1	8.80	100
Setosella vulnerata	Poluzzi & Sartori 1974	1		0				
Stomachetosella cruenta	Taylor 2009,	3	5.17	98				
Tegella unicornis	Taylor 2009,	3	2.07	100				
Tervia irregularis	Poluzzi & Sartori 1974	1	2.00	100				
Tessaradoma boreale	Poluzzi & Sartori 1974	1	7.00	100				

Tricellaria ternata	Taylor 2009, Borisenko & Gontar 1991	5	2.56	100
Tubulipora phalangea	Poluzzi & Sartori 1974	1	3.00	100

__ indicates analysis from Scottish waters

Appendix B: Full details of all specimen sources

genus	species	family	wt. % $MgCO_3$	wt. % calcite	Locality	Specimen ID
Aetea	anguina*	Aeteidae	7.86	100	St Abbs	22316
Aetea	sica*	Aeteidae		100	Flotta, Orkney	22574
Aetea	truncata*	Aeteidae		100	Lamlash Bay, Arran	26804
Alderina	imbellis	Calloporidae	6.06	100	Bell Rock	NMSZ 2000.280.383
Amphiblestrum	auritum	Calloporidae	5.59	100	St Abbs	22296
Amphiblestrum	flemingii	Calloporidae	3.64	100	St Abbs	22274
Amphiblestrum	solidum	Calloporidae	7.16	100	Shetland	NMSZ 1999.239.006
Anarthropora	monodon	Exechonellidae		0	Shetland/Faroe Channel	NMSZ 1999.239.139
Annectocyma	major*	Annectocymidae	6.40	100	Shetland	1911.10.1.31
Beania	mirabilis	Beaniidae	7.18	100	St Abbs	22342
Anarthropora	monodon	Exechonellidae		0	Shetland/Faroe Channel	NMSZ 1999.239.139
Anarthropora	monodon	Exechonellidae		0	Shetland/Faroe Channel	NMSZ 1999.239.139
Bicellariella	ciliata	Bugulidae	5.03	82	Bird Rock, Barra	NMSZ 1993.065.1456
Bicellarina	alderi	Bugulidae	2.81	57		11.10.1.298
Bicrisia	abyssicola	Crisiidae	2.44	100	Stromness, Orkney	22379
Bugula	avicularia	Bugulidae	4.80	100		11.10.1.257
Bugula	flabellata	Bugulidae	3.85	58	Holy Isle, Arran	22269
Bugula	fulva	Bugulidae	6.59	100	Kirkwall marina, Orkney	22374
Bugula	neritina	Bugulidae	5.14	100	unspecified - J Porter coll.	22503
Bugula	plumosa	Bugulidae	7.39	100	St Kilda	NMSZ 1998.55.115
Bugula	purpurotincta	Bugulidae	3.89	89	Loch Crinan	NMSZ 1993.065.1455
Bugula	simplex	Bugulidae	3.85	49	Largs marina, Ayrshire	22428
Bugula	turbinata	Bugulidae	2.16	100	Largs marina, Ayrshire	22430
Buskea	dichotoma	Celleporidae	4.49	100	Scotland, exact location unknown	22437
Buskea	nitida	Celleporidae	4.56	100	Holy Isle, Arran	26914

Caberea	ellisii	Candidae	4.88	100	Scotland, exact location unknown	22480
Callopora	craticula	Calloporidae	4.26	100	W Sgeir Ulibhe (Loch Hourn)	22305
Callopora	dumerilii	Calloporidae	7.52	100	Kyle of Lochalsh	NMSZ 2009.026.225
Callopora	lineata	Calloporidae	5.24	83	St Abbs	22300
Callopora	rylandi	Calloporidae	6.85	100	St Abbs	22279
Carbasea	carbasea	Flustridae	6.29	100	Leith (1792)	NHM specimen
Cauloramphus	spiniferum	Calloporidae	3.78	85	St Abbs	22332
Cellaria	fistulosa	Cellariidae	4.78	100	Lamlash Bay, Arran	22271
Cellaria	fistulosa	Cellariidae	5.75	100	Lamlash Bay, Arran	22271
Cellaria	fistulosa	Cellariidae	4.84	100	Lamlash Bay, Arran	22271
Cellaria	fistulosa	Cellariidae	5.73	100	Lamlash Bay, Arran	22271
Cellaria	fistulosa	Cellariidae	5.17	100	Lamlash Bay, Arran	22271
Cellaria	fistulosa	Cellariidae	7.05	100	Lamlash Bay, Arran	22271
Cellaria	fistulosa	Cellariidae	6.44	100	Lamlash Bay, Arran	22271
Cellaria	fistulosa	Cellariidae	3.45	100	Lamlash Bay, Arran	22271
Cellaria	fistulosa	Cellariidae	4.30	100	Lamlash Bay, Arran	22271
Cellaria	fistulosa	Cellariidae	3.94	100	Lamlash Bay, Arran	22271
Cellaria	fistulosa	Cellariidae	5.03	100	Lamlash Bay, Arran	22271
Cellaria	fistulosa	Cellariidae	6.15	100	Lamlash Bay, Arran	22271
Cellaria	fistulosa	Cellariidae	5.30	100	Lamlash Bay, Arran	22271
Cellaria	fistulosa	Cellariidae	4.60	100	Lamlash Bay, Arran	22271
Cellaria	fistulosa	Cellariidae	4.96	100	Lamlash Bay, Arran	22271
Cellaria	fistulosa	Cellariidae	4.00	100	Lamlash Bay, Arran	22271
Cellaria	salicornioides	Cellariidae	3.68	100		99.5.1.30
Cellaria	sinuosa	Cellariidae	3.94	92		19.11.10.1.766
Cellepora	pumicosa	Celleporidae	4.69	89	St Abbs	22315
Celleporella	hyalina	hippothoidae	1.73	100	Clashfarland, Isle of Cumbrae	223125
Celleporina	caliciformis	Celleporidae	4.90	100	Deer Sound, Orkney	22371
Celleporina	pygmaea	Celleporidae	7.20	100	Shetland/Faroe Channel	NMSZ 1999.239.082

Chartella	barleei	Flustridae	9.58	28	Shetland	NMSZ 1999.239.006
Chartella	papyracea	Flustridae	9.03	100	Lyme Bay	NMSZ unregistered
Chorizopora	brongniartii	Chorizoporidae	5.49	100	St Abbs	22367
Conopeum	reticulum	Membraniporidae	5.04	100	Kinneil survey station 02/1	NMSZ 2000.295.219
Conopeum	seurati	Membraniporidae	9.10	95		1998.11.11.1
Coronopora	truncata	Lichenoporidae	7.78	100	Rockall (Dr Jim Drewery, MS)	26912
Cribrilina	annulata	cribrilinidae	4.95	89	St Abbs	22366
Cribrilina	cryptooecium	cribrilinidae	2.87	100	St Abbs	22276
Cribrilina	punctata	cribrilinidae	4.99	100	Houton Bay, Orkney	25751
Crisia	aculeata	Crisiidae	4.84	90	Stromness, Orkney	22383
Crisia	denticulata	Crisiidae	3.31	100	Clashfarland, Isle of Cumbrae	223122
Crisia	eburnea	Crisiidae	3.35	100	Saulmore Point, Oban	22372
Crisia	eburnea	Crisiidae	3.33	100	Saulmore Point, Oban	22372
Crisia	ramosa	Crisiidae	2.32	100	St Kilda	NMSZ 1998.55.104
Crisidia	cornuta	Crisiidae	5.04	100	Stromness, Orkney	22398
Cryptosula	pallasiana	Cryptosulidae	4.57	59	Hoy, Orkney	22524
Dendrobeania	murrayana	Bugulidae	4.73	100	Scotland, exact location unknown	12.12.21.886
Diplosolen	obelia*	Diastoporidae	5.75	100	Shetland	11.10.1.148
Disporella	hispida	Lichenoporidae	5.17	100	Eyemouth	22304
Doryporellina	reticulata*	Doryporellidae	7.15		Rockall	1911.10.1.1152
Einhornia	crustulenta	Electridae	4.11	100	Loch of Stenness, Orkney	22350
Electra	monostachys	Electridae	7.34	91	Solway Firth	NMSZ 2005.049.0067
Electra	pilosa	Electridae	8.14	78	Clashfarland, Isle of Cumbrae	22440
Electra	pilosa	Electridae	9.76	84	Clashfarland, Isle of Cumbrae	22442
Electra	pilosa	Electridae	9.89	53	Clashfarland, Isle of Cumbrae	22438
Electra	pilosa	Electridae	9.71	80	Clashfarland, Isle of Cumbrae	22438
Electra	pilosa	Electridae	8.77	84	Clashfarland, Isle of Cumbrae	22441
Electra	pilosa	Electridae	9.22	87	Clashfarland, Isle of Cumbrae	22441
Electra	pilosa	Electridae	8.04	69	Clashfarland, Isle of Cumbrae	22440

Electra	pilosa	Electridae	6.79	82	Clashfarland, Isle of Cumbrae	22440
Electra	pilosa	Electridae	7.90	64	Clashfarland, Isle of Cumbrae	22440
Electra	pilosa	Electridae	8.26	86	Clashfarland, Isle of Cumbrae	22442
Electra	pilosa	Electridae	8.61	81	Clashfarland, Isle of Cumbrae	22442
Electra	pilosa	Electridae	8.46	88	Clashfarland, Isle of Cumbrae	22442
Electra	pilosa	Electridae	8.35	94	Clashfarland, Isle of Cumbrae	22438
Electra	pilosa	Electridae	8.28	100	Clashfarland, Isle of Cumbrae	22438
Entalophoroecia	deflexa	Diaperoeciidae	4.50	100	St Kilda	NMSZ 2001.035.215
Escharella	abyssicola	Romancheinidae	7.49	94	Shetland/Faroe Channel	NMSZ 1999.239.253
Escharella	immersa	Romancheinidae	5.64	86	Loch Sween	22354
Escharella	labiosa*	Romancheinidae	5.80	100	Mull Sound	1997.8.4.14
Escharella	laqueata*	Romancheinidae	6.00	100	Skye	11.10.1.1010
Escharella	octodentata	Romancheinidae	7.32	100	Shetland/Faroe Channel	NMSZ 1999.239.002
Escharella	variolosa	Romancheinidae	6.55	88	St Abbs	22275
Escharella	ventricosa	Romancheinidae	5.60	100	Flotta, Orkney	25586
Escharina	alderi	Escharinidae	7.00	100	Shetland/Faroe Channel	NMS 2003.06.1045
Escharina	dutertrei haywardi	Escharinidae	7.39	100	Shetland/Faroe Channel	NMSZ 2003.106.1059
Escharina	johnstoni*	Escharinidae	7.20	100	The Minch	11.10.1.1208
Escharoides	coccinea	Exochellidae	3.30	64	Stromness, Orkney	22381
Escharoides	mamillata	Exochellidae	6.96	100	Houton Bay, Orkney	223139
Eucratea	loricata	Eucrateidae	7.74	85	Scotland, exact location unknown	86.1.26.3
Eurystrotos	compacta	Oncousoeciidae	6.26	100	Fair Isle	26919
Fenestrulina	malusii	Microporellidae	2.84	88	St Abbs	22294
Filicrisia	geniculata	Crisiidae	3.86	86	St Kilda	NMS 1998.55.86
Flustra	foliacea	Flustridae	6.18	100	Scotland, exact location unknown	22471
Flustra	foliacea	Flustridae	6.99	100	Scotland, exact location unknown	22471
Flustra	foliacea	Flustridae	7.83	100	Scotland, exact location unknown	22471
Flustra	foliacea	Flustridae	7.85	100	Scotland, exact location unknown	22471

Flustra	foliacea	Flustridae	7.96	100	Scotland, exact location unknown	22471
Flustra	foliacea	Flustridae	7.99	100	Scotland, exact location unknown	22471
Flustra	foliacea	Flustridae	8.01	100	Saulmore Point, Oban	22449
Flustra	foliacea	Flustridae	8.04	100	Scotland, exact location unknown	22471
Flustra	foliacea	Flustridae	8.33	100	Scotland, exact location unknown	22471
Flustra	foliacea	Flustridae	8.84	100	Scotland, exact location unknown	22471
Flustra	foliacea	Flustridae	9.01	100	Saulmore Point, Oban	22449
Flustra	foliacea	Flustridae	9.06	100	Scotland, exact location unknown	22471
Flustra	foliacea	Flustridae	9.29	100	Scotland, exact location unknown	22471
Flustra	foliacea	Flustridae	9.29	100	Scotland, exact location unknown	22471
Flustra	foliacea	Flustridae	9.30	100	Scotland, exact location unknown	22471
Flustra	foliacea	Flustridae	9.72	100	Saulmore Point, Oban	22449
Flustra	foliacea	Flustridae	10.25	100	Scotland, exact location unknown	22471
Flustra	foliacea	Flustridae	10.32	100	Scotland, exact location unknown	22471
Flustra	foliacea	Flustridae	10.55	100	Scotland, exact location unknown	22471
Haplopoma	graniferum	Haplopomidae	3.06	75	Stromness, Orkney	26806
Haplopoma	impressum	Haplopomidae	1.81	100	Orkney	22397
Haplopoma	planum	Haplopomidae	6.14	100	Shetland	NMSZ 1999.239.022
Haplopoma	sciaphilum*	Haplopomidae	7.49		St Kilda	1985.1.10.29
Hemicyclopora	polita	Romancheinidae	7.26	100	Shetland	NMSZ 1999.239.022
Herentia	hyndmanni*	Escharinidae	6.21	100	Orkney	1899.7.1.2468
Hippoporina	pertusa*	Bitectiporidae	6.28	100		11.10.1.1181
Hippothoa	divaricata*	Hippothoidae		0	Mull of Cantyre	88.4.21.6
Hornera	lichenoides	Horneridae	5.55	94	Shetland/Faroe Channel	NMSZ 2003.106.1032
Idmidronea	atlantica	Tubuliporidae	3.77	100	W.S. Bruce Collection (Arctic 77.55N)	NMSZ EA 2/21/16
Lagenipora	lepralioides*	Celleporidae	5.17	100	Shetland	1899.7.1.3565
Larnacicus	corniger	Chaperiidae	7.66	94	Shetland/Faroe Channel	NMSZ 2003.106.1038
Lepraliella	hippopus	Lepraliellidae	7.69	96	Elmwood	NMS 1921.145.508

Marguetta	lorea	Bryocryptellidae	8.49	100		1911.10.1.1593
Megapora	ringens	Calloporidae	6.50	44	Shetland/Faroe Channel	NMSZ 1999.239.139
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256

Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membranipora	membranacea	Membraniporidae		0	Bridge over the Atlantic, ClachanSeil	22256
Membraniporella	nitida	Cribrilinidae	2.22	100	St Abbs	223123
Microporella	ciliata	Microporellidae	4.50	61	Eday Sound, Orkney	22412
Notoplites	jeffreysii	Candidae	5.87	13	Shetland	NMSZ 1999.239.006
Omalosecosa	ramulosa	Celleporidae	5.39	100	Lamlash Bay, Arran	22443
Omalosecosa	ramulosa	Celleporidae	4.78	100	Lamlash Bay, Arran	22443
Omalosecosa	ramulosa	Celleporidae	6.50	100	Lamlash Bay, Arran	22443
Omalosecosa	ramulosa	Celleporidae	6.25	95	Lamlash Bay, Arran	22443
Omalosecosa	ramulosa	Celleporidae	7.68	100	Lamlash Bay, Arran	22443
Omalosecosa	ramulosa	Celleporidae	10.36	100	Lamlash Bay, Arran	22443
Omalosecosa	ramulosa	Celleporidae	11.75	100	Lamlash Bay, Arran	22443
Omalosecosa	ramulosa	Celleporidae	10.67	91	Lamlash Bay, Arran	22443
Omalosecosa	ramulosa	Celleporidae	12.24	100	Lamlash Bay, Arran	22443
Omalosecosa	ramulosa	Celleporidae	12.48	92	Lamlash Bay, Arran	22443
Omalosecosa	ramulosa	Celleporidae	11.37	100	Lamlash Bay, Arran	22443
Omalosecosa	ramulosa	Celleporidae	12.00	100	Lamlash Bay, Arran	22443
Omalosecosa	ramulosa	Celleporidae	11.62	100	Lamlash Bay, Arran	22443
Omalosecosa	ramulosa	Celleporidae	13.55	100	Lamlash Bay, Arran	22443

Omalosecosa	ramulosa	Celleporidae		95	Lamlash Bay, Arran	22443
Oncousoecia	diastoporides	Oncousoeciidae	6.87	87	Fair Isle	26918
Oncousoecia	dilatans	Oncousoeciidae	7.81	100	Shetland	NMSZ 1999.239.010
Oshurkovia	littoralis	Umbonulidae	6.02	48	Millport, Isle of Cumbrae	26920.00
Palmicellaria	elegans*	Celleporidae	7.41	100		65.7.5.16
Palmiskenea	skenei	Bryocryptellidae	5.90	100		1959.3.6.1
Parasmittina	trispinosa	Smittinidae	7.73	80	Eyemouth	22303
Pentapora	fascialis	Bitectiporidae	8.35	54	St Kilda	NMS 1998.55.133
Phaeostachys	spinifera	Escharinidae	5.66	63	Saulmore Point, Oban	22373
Phylactella	eximia*	Smittinidae	5.00	100		11.10.1.1.1549a
Plagioecia	patina	Plagioeciidae	6.24	100	Eyemouth	223126
Porella	alba	Bryocryptellidae	3.99	94	Stromness, Orkney	22375
Porella	compressa	Bryocryptellidae	8.21	100	Scotland, exact location unknown	22435
Porella	concinna	Bryocryptellidae	8.18	99	Kyle of Lochalsh	NMSZ 2009.026.254
Porella	concinna	Bryocryptellidae	8.11	100	Shetland	NMSZ 1999.239.292
Porella	laevis	Bryocryptellidae	10.17	100		11.10.1.1383
Porella	struma*	Bryocryptellidae	5.93	100	Shetland	11.10.1.1379
Porella	tubulosa*	Bryocryptellidae	6.96	100	Shetland	11.10.1.1527A
Pseudoflustra	virgula	Smittinidae	5.60	100	Shetland/Faroe Channel	NMSZ 2004.128.0006
Puellina	innominata	Cribrilinidae	8.75	100	Lamlash Bay, Arran	26786
Pyripora	catenularia	Electridae	8.68	90	Flotta, Orkney	25585
Ragionula	rosacea*	Romancheinidae	7.22	100	Loch Fyne	11.10.1.1167
Ramphonotus	minax	Calloporidae	6.85	100	Shetland	NMSZ 1999.239.136
Reteporella	beaniana	Phidoloporidae	8.54	100		1911.10.1.1361
Reteporella	incognita	Phidoloporidae	8.36	100	Rockall (Dr Jim Drewery, MS)	26910
Reteporella	watersi	Phidoloporidae	5.75	87		11.10.1.799
Rosseliana	rosselii*	Antroporidae	6.48	100	Shetland	11.10.1.636
Schizomavella	auriculata	Bitectiporidae	5.24	27	Houton Bay, Orkney	25641
Schizomavella	linearis	Bitectiporidae	6.37	1	Flotta, Orkney	22562

Schizoporella	japonica	Schizoporellidae	6.45	19	Portavadie marina	26921.00
Schizoporella	japonica	Schizoporellidae	5.77	38	Portavadie marina	26922.00
Schizoporella	japonica	Schizoporellidae	6.21	23	Portavadie marina	26923.00
Schizoporella	japonica	Schizoporellidae	3.91	62	Kirkwall marina, Orkney	22501
Schizoporella	japonica	Schizoporellidae	4.05	73	Stromness marina, Orkney	22341
Schizoporella	japonica	Schizoporellidae	4.20	60	Stromness marina, Orkney	22341
Schizoporella	japonica	Schizoporellidae	4.38	56	Stromness marina, Orkney	22502
Schizoporella	japonica	Schizoporellidae	4.62	62	Kirkwall marina, Orkney	22500
Schizoporella	japonica	Schizoporellidae	4.75	80	Stromness marina, Orkney	22341
Schizoporella	japonica	Schizoporellidae	4.85	46	Kirkwall marina, Orkney	22500
Schizoporella	japonica	Schizoporellidae	4.90	66	Kirkwall marina, Orkney	22501
Schizoporella	japonica	Schizoporellidae	5.17	62	Kirkwall marina, Orkney	22501
Schizoporella	japonica	Schizoporellidae	5.20	82	Stromness marina, Orkney	22341
Schizoporella	japonica	Schizoporellidae	5.42	56	Holyhead marina, Wales	22505
Schizoporella	japonica	Schizoporellidae	5.51	46	Holyhead marina, Wales	22505
Schizoporella	japonica	Schizoporellidae	5.88	86	Stromness marina, Orkney	22502
Schizoporella	japonica	Schizoporellidae	6.23	53	Holyhead marina, Wales	22505
Schizoporella	japonica	Schizoporellidae	6.68	62	Kirkwall marina, Orkney	22500
Schizoporella	japonica	Schizoporellidae	7.35	50	Stromness marina, Orkney	22502
Schizoporella	patula	Schizoporellidae	8.14	13	Shetland/Faroe Channel	NMS 2001.035.221
Schizoporella	unicornis	Schizoporellidae	6.04	59	Stromness, Orkney	22368
Schizoporella	patula	Schizoporellidae	7.63	1	Shetland	NMSZ 1999.239.082
Scruparia	ambigua	Scruparidae	8.05	100	St Kilda	NMS - 98.55.107
Scruparia	chelata	Scruparidae	7.91	100	Clashfarland, Isle of Cumbrae	22359
Scrupocellaria	reptans	Candidae	3.83	65	Saulmore Point, Oban	22456
Scrupocellaria	reptans	Candidae	6.76	94	Scotland, exact location unknown	1994.8.25.3
Scrupocellaria	reptans	Candidae	4.61	88	Scotland, exact location unknown	11.10.1.353
Scrupocellaria	scrupea	Candidae	1.42	100	Kirkwall marina, Orkney	22402
Scrupocellaria	scruposa	Candidae	6.30	57	Cava, Orkney	22264

Scrupocellaria	scruposa	Candidae	3.32	100	Saulmore Point, Oban	22369
Scrupocellaria	scruposa	Candidae	3.56	100	Cava, Orkney	22265
Scrupocellaria	scruposa	Candidae	4.31	100	Cava, Orkney	22265
Scrupocellaria	scruposa	Candidae	5.40	100	Cava, Orkney	22264
Securiflustra	securifrons	Flustridae	9.84	91	Scotland, exact location unknown	1993.6.28.2
Setosella	vulnerata	Setosellidae		0	Shetland	NMSZ 1999.239.165
Smittina	bella*	Smittinidae		0	Aberdeen	1911.10.1.1402
Smittina	crystallina	Smittinidae	8.11	67	Hoxa Head, Orkney	26917
Smittoidea	marmorea*	Smittinidae		0		11.10.1.1582
Smittoidea	reticulata	Smittinidae	6.28	16	Flotta, Orkney	22560
Stigmatoechos	violacea	Stigmatoechidae	7.36	100	Rockall (Dr Jim Drewery, MS)	26911
Stomachetosella	cruenta*	Stomachetosellidae	8.96		Shetland	1911.10.1.1093
Stomachetosella	sinuosa*	Stomachetosellidae	6.46		Summer Isles	1960.4.6.22
Stomatopora	gingrina	Stomatoporidae	7.15	93	Rockall (Dr Jim Drewery, MS)	26835
Tegella	unicornis	Calloporidae	3.56	100	Scapa Flow, Orkney	22260
Temachia	microstoma*	Romancheinidae	6.25	100	Shetland	1899.7.1.3629
Tervia	irregularis	Terviidae	7.95	100	Rockall (Dr Jim Drewery, MS)	26909
Tessaradoma	boreale	Tessaradomidae	6.37	100	St Kilda	NMSZ 2001.035.219
Tricellaria	inopinata	Candidae	2.40	100	Houton Bay, Orkney	223130
Tricellaria	ternata	Candidae	5.00	100	Bass Rock, North Berwick	NMSZ unregistered.
Tubulipora	liliacea	Tubuliporidae	2.65	97	Eyemouth	22302
Tubulipora	liliacea	Tubuliporidae	2.56	100	Eyemouth	22302
Tubulipora	penicillata	Tubuliporidae	4.66	100	Loch Laxford	NMSZ 2006.026.0625
Tubulipora	phalangea	Tubuliporidae	1.92	100	Eday Sound, Orkney	22417
Tubulipora	plumosa	Tubuliporidae	1.03	100	Scapa Flow, Orkney	22388
Turbicellepora	avicularis	Celleporidae	8.98	100	St Abbs Head	NMS 1997.130.129
Turbicellepora	boreale	Celleporidae	6.96	100	Rockall (Dr Jim Drewery, MS)	26913

__ indicates first analysis of genera or family * indicates analysis conducted using micro-XRD.

Appendix C: Environmental Data for Panel Settlement Sites

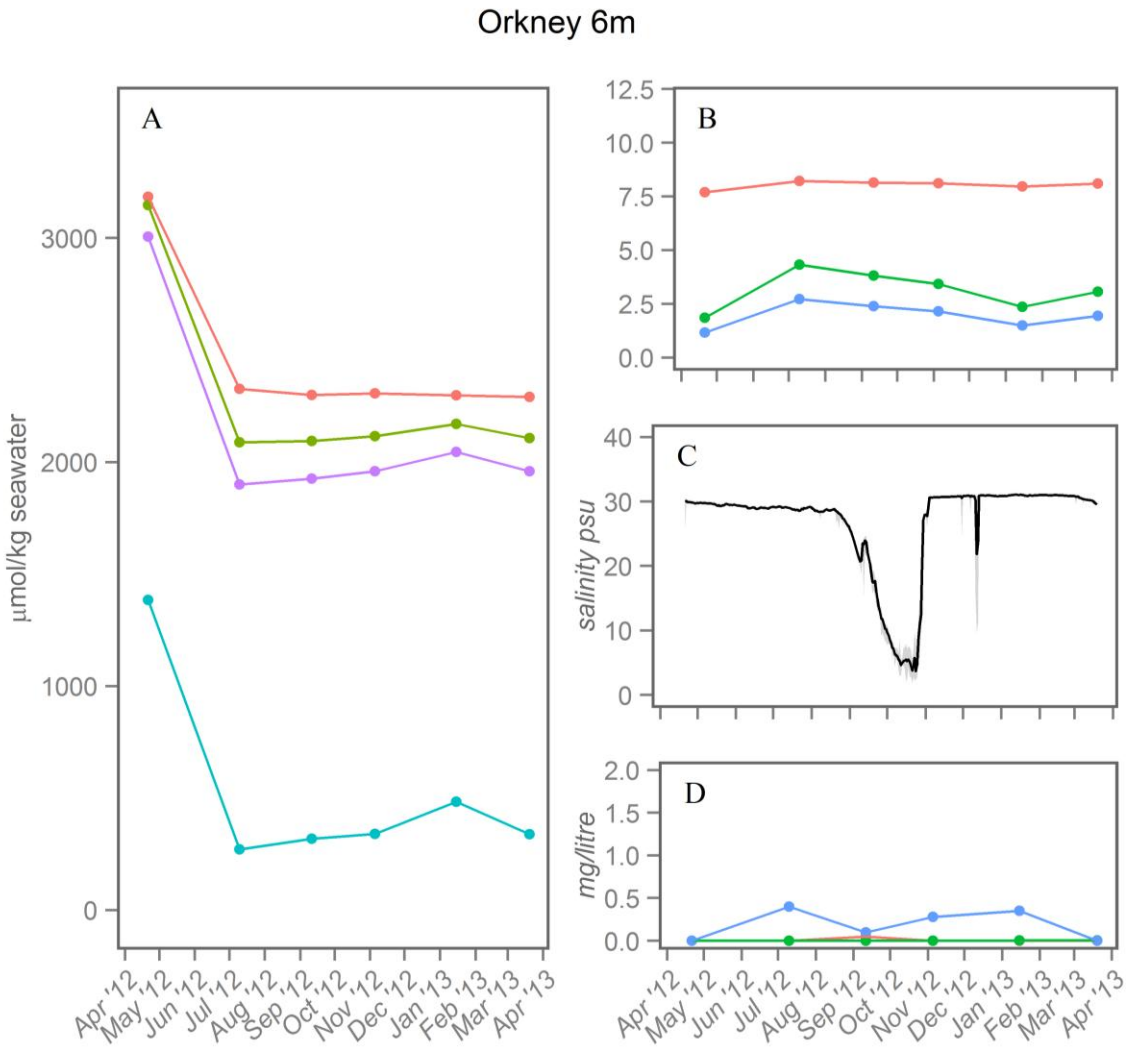

Figure 1: Environmental variables between April 2012 and April 2013in Orkney at 6m depth. A: red = Total alkalinity, TA (µmol/kg seawater), green = Dissolved Inorganic Carbon, DIC (µmol/kg seawater), purple = HCO_3 (µmol/kg seawater), blue = pCO_2 (µatm); B: red = pH, green = Ω calcite, blue = Ω aragonite; C: black line = daily mean salinity (psu), grey = daily salinity range; D: green = nitrate, red = phosphate, blue = silicate (mg/l).

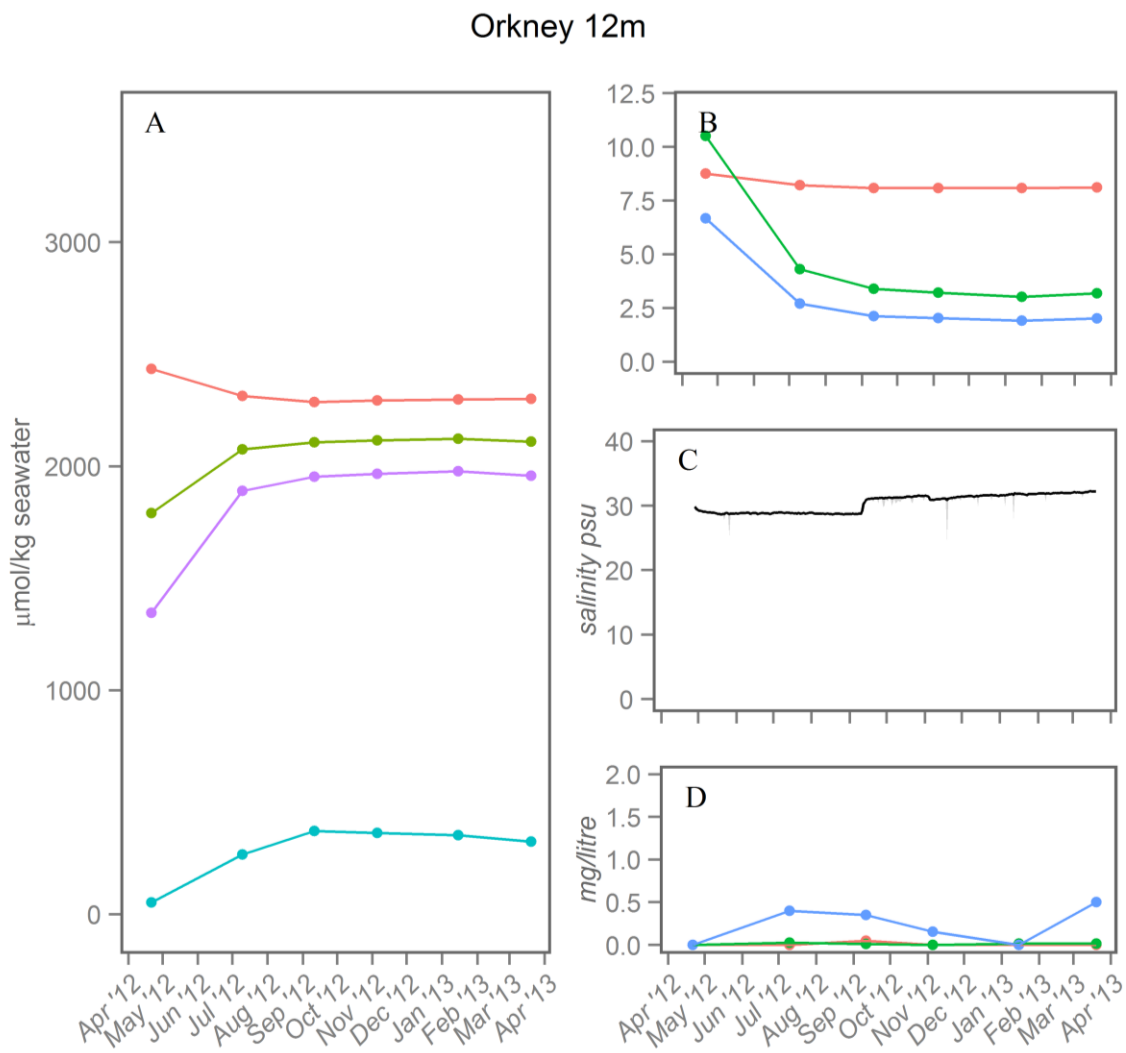

Figure 2: Environmental variables between April 2012 and April 2013in Orkney at 12m depth. A: red = Total alkalinity, TA (µmol/kg seawater), green = Dissolved Inorganic Carbon, DIC (µmol/kg seawater), purple = HCO_3 (µmol/kg seawater), blue = pCO_2 (µatm); B: red = pH, green = Ω calcite, blue = Ω aragonite; C: black line = daily mean salinity (psu), grey = daily salinity range; D: green = nitrate, red = phosphate, blue = silicate (mg/l).

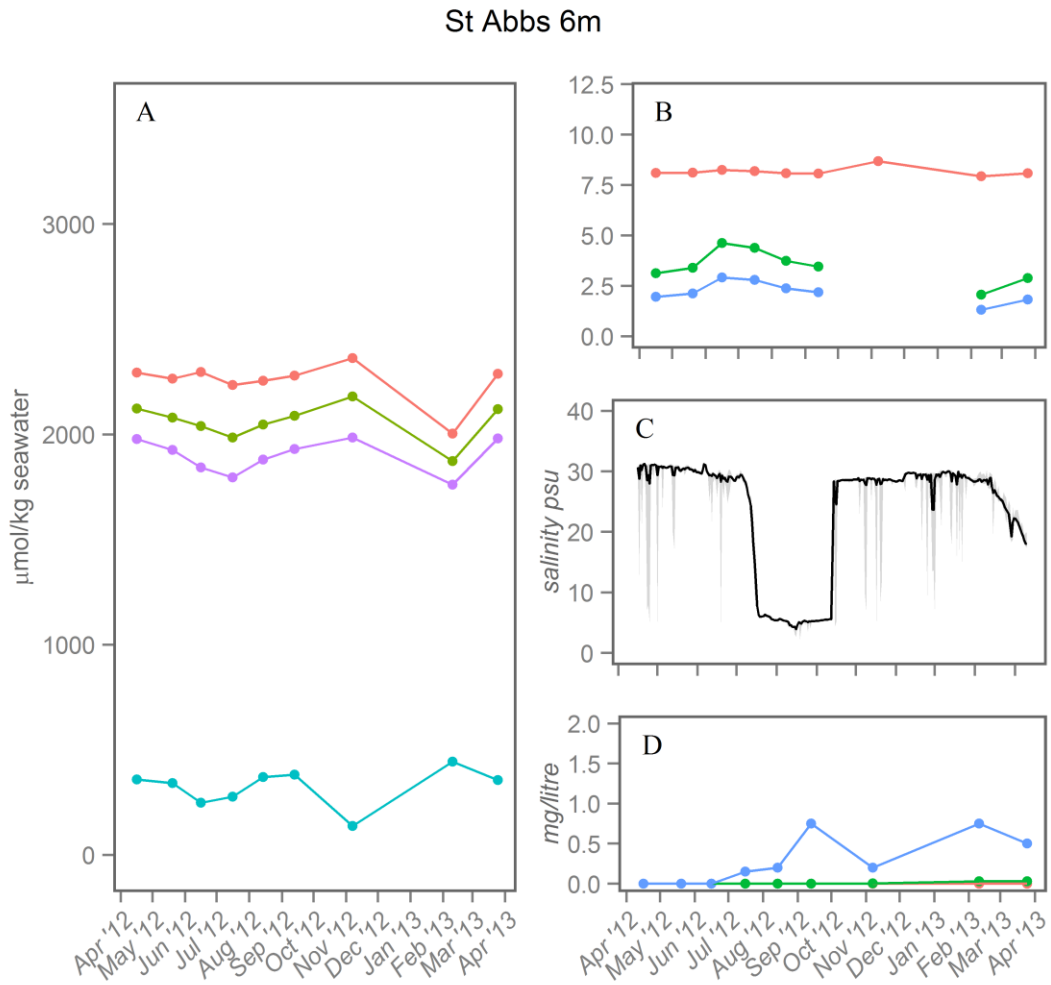

Figure 3: Environmental variables between April 2012 and April 2013in St Abbs at 6m depth. A: red = Total alkalinity, TA (µmol/kg seawater), green = Dissolved Inorganic Carbon, DIC (µmol/kg seawater), purple = HCO_3 (µmol/kg seawater), blue = pCO_2 (µatm); B: red = pH, green = Ω calcite, blue = Ω aragonite; C: black line = daily mean salinity (psu), grey = daily salinity range; D: green = nitrate, red = phosphate, blue = silicate (mg/l).

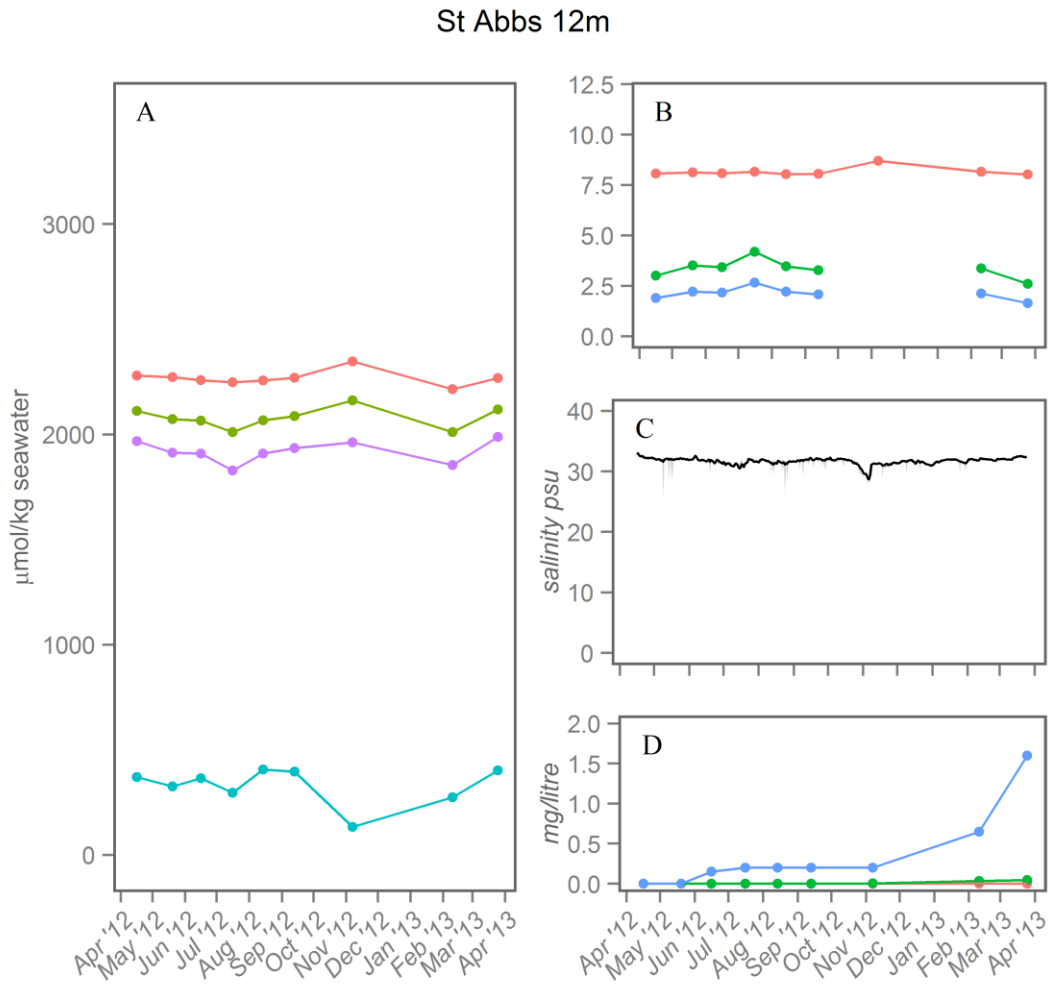

Figure 4: Environmental variables between April 2012 and April 2013in St Abbs at 12m depth. A: red = Total alkalinity, TA (µmol/kg seawater), green = Dissolved Inorganic Carbon, DIC (µmol/kg seawater), purple = HCO_3 (µmol/kg seawater), blue = pCO_2 (µatm); B: red = pH, green = Ω calcite, blue = Ω aragonite; C: black line = daily mean salinity (psu), grey = daily salinity range; D: green = nitrate, red = phosphate, blue = silicate (mg/l).

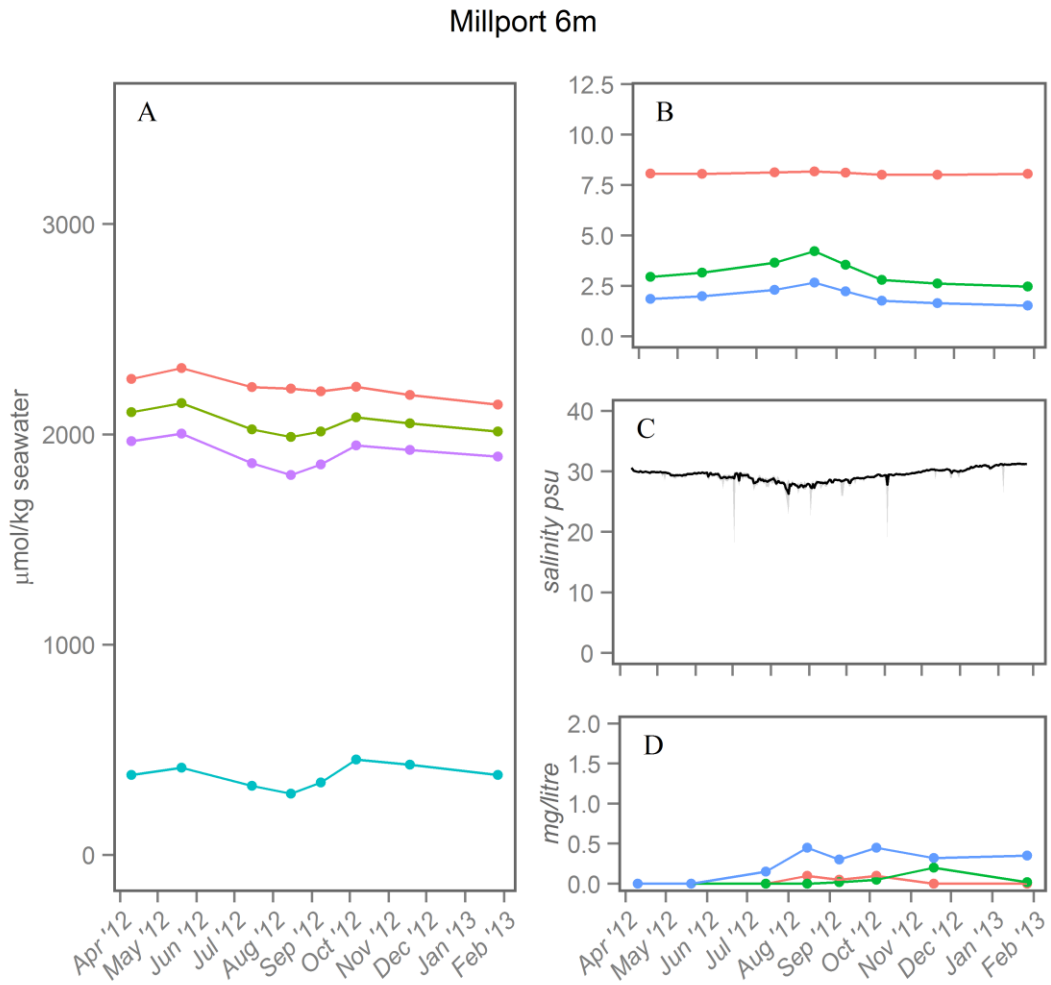

Figure 5: Environmental variables between April 2012 and April 2013in Millport at 6m depth. A: red = Total alkalinity, TA (µmol/kg seawater), green = Dissolved Inorganic Carbon, DIC (µmol/kg seawater), purple = HCO_3 (µmol/kg seawater), blue = pCO_2 (µatm); B: red = pH, green = Ω calcite, blue = Ω aragonite; C: black line = daily mean salinity (psu), grey = daily salinity range; D: green = nitrate, red = phosphate, blue = silicate (mg/l).

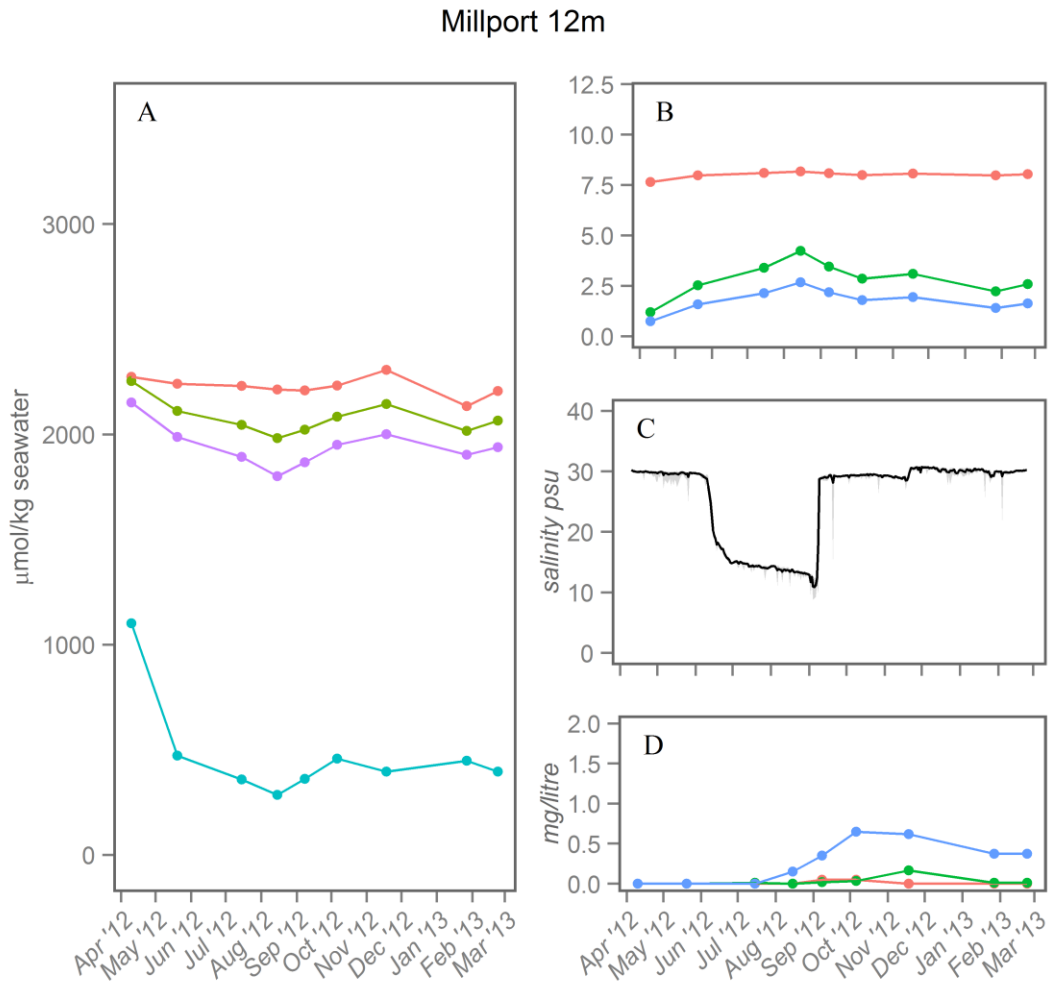

Figure 6: Environmental variables between April 2012 and April 2013in Millport at 12m depth. A: red = Total alkalinity, TA (µmol/kg seawater), green = Dissolved Inorganic Carbon, DIC (µmol/kg seawater), purple = HCO_3 (µmol/kg seawater), blue = pCO_2 (µatm); B: red = pH, green = Ω calcite, blue = Ω aragonite; C: black line = daily mean salinity (psu), grey = daily salinity range; D: green = nitrate, red = phosphate, blue = silicate (mg/l).

Appendix D: Environmental Data for Stromness Marina

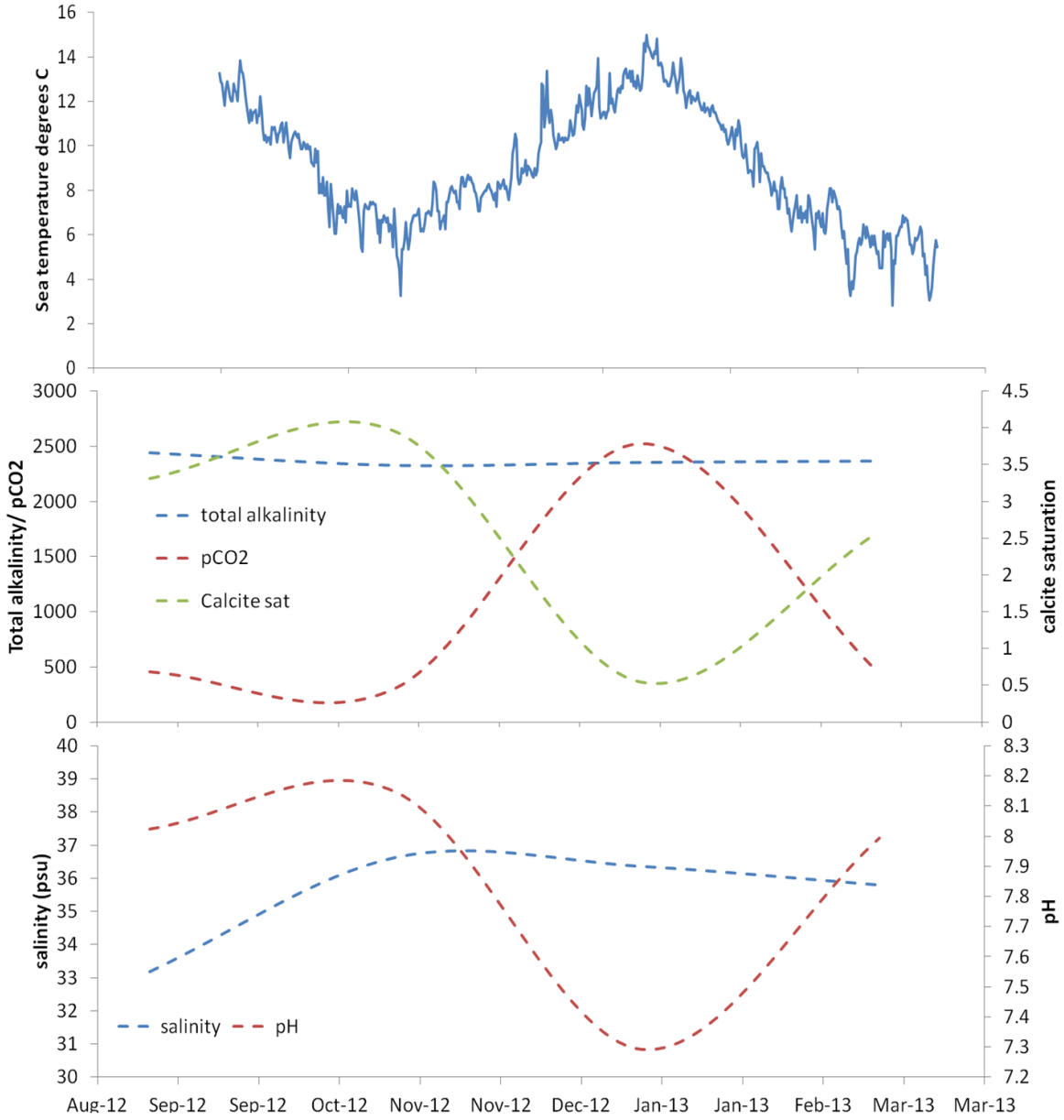

Figure 1: Collated graphs showing variation in environmental measures between August 2012 and March 2013 at Stromness marina. Top) Seawater temperature; Middle) blue dashed line = total alkalinity (left y-axis), red dashed line = pCO_2 (left y-axis), green dashed line = $\Omega_{calcite}$. (right y-axis); Bottom) red dashed line = pH (right y-axis), blue dashed line = salinity(left y-axis).

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