Genetic level investigations into the species diversity, biogeography and trophic traits of Antarctic Polychaetes

A thesis submitted for the degree of Doctor of Philosophy

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Declaration

This thesis is an account of my research undertaken within the Life Science Department, Natural History Museum, London, UK, and the School of Environmental Sciences, Liverpool, UK, between December 2013 and December 2017. Unless acknowledged the material presented in this thesis is, to the best of my knowledge, original and has not been previously submitted.

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Abstract

The diversity and biogeography of the Antarctic benthos has been shaped by its unique history through glacial cycles, the influence of circumpolar current regimes and seasonal food inputs. There is currently a large international research effort to define levels of species diversity, biogeography, functional traits and their sensitivity to changing environmental conditions. These data are vital in setting ecological baselines to monitor the effects of climate change and manage the impacts of human activities in the Southern Ocean.

The findings from genetic level analyses into species diversity, biogeography and the trophic traits of two groups of benthic Antarctic polychaetes, an abundant taxa within macrofaunal communities are presented here. The first group contained free-living polychaetes collected from the Scotia, Amundsen and Weddell Seas, whilst the second group consisted of symbiotic polynoids taken from coral host species in the South Orkney Islands Southern Shelf Marine Protected Area.

The application of DNA barcoding to a subset of 15 morphologically identified polychaete species (morphospecies) from the free-living polychaetes, uncovered 10 additional cryptic species (these individuals are morphologically identical but genetically distinct) and 10 previously overlooked morphospecies. These findings suggest that the levels of Antarctic benthic diversity may be largely underestimated. The difficulty in determining true 'species' from genetic analysis for which there are no genetic cut offs or rules is discussed, as well as the causes of misidentification of soft bodied species within large sample sets.

The distribution of cryptic species are often more restricted that that of their original morphospecies. This is potentially related to geographic or reproductive isolation of populations during the speciation process. In this study, the cryptic species previously considered to be circum-Antarctic remained widespread. This demonstrates the importance of considering dispersal mechanisms, including developmental mode and larval biology and subsequently transport via cicrum-Antarctic currents.

The determination of trophic traits using both bulk and compound specific stable isotope analysis, revealed high levels of variability within and between species with the same categorical trophic traits. These data suggest a high degree of omnivory coupled with variation at the base of the food web i.e. in $\delta^{15}N$ of phytoplankton/phytodetritus. The use of genetic and

biochemical analyses to describe the symbiotic relationship between polynoid symbionts and their host corals identified polymorphisms with significantly different trophic signatures.

The relevance and significance of the findings are discussed with regard to environmental change in the Southern Ocean and the future of Antarctic marine management and scientific research. Antarctica represents one of the most rapidly changing and vulnerable ecosystems on our planet. Any means to mitigate the effects of climate change or to sustainably manage Antarctic marine resources requires international and multidisciplinary research collaborations. Future research should focus on understanding the interacting and changing relationships between the biological, chemical, physical and geological environments.

I have lots of heroes: anyone and everyone who does whatever they can to leave the natural world better than they found it.

- Sylvia Earle

Acknowledgments

My love of marine ecosystems evolved from many happy days face first in a rock pool in Cornwall whilst visiting my grandparents. I hope they'd both be proud of this thesis and all the adventures my PhD brought with it.

My interest in Antarctic biology came apparent during my undergraduate studies at the University of Southampton. I would like to thank Professor Paul Tyler for introducing me to the wonders of deep-sea life and all Antarctic science had to offer. My passion for polychaetes was perhaps an unexpected one. Shortly after graduating I was welcomed into Dr Adrian Glover's lab at the Natural History Museum, London. I soon realised there was a lot of polychaete related fun to be had. I feel very lucky to have been a part of Adrian's research group and am grateful for all friendships and fieldwork opportunities that came with it.

My PhD maintained connections with the Natural History Museum but the University of Liverpool soon became my new home. Whilst in Liverpool, my supervisor Dr Rachel Jeffreys became not only a great source of support and guidance through this PhD but also my 'academic mum'. Thank you Rachel for your kindness through the rough times and the laughs along the way. Thanks are also due to Professor George Wolff for keeping me on the straight and narrow to get this thesis finished. I would also like to thank Lenka Neal and Dr's Helen Wiklund and Anu Petal for their incredible patience with me in the lab and help in the collection of taxonomic, genetic and isotope data, and, Drs Henry Ruhl and James Harle for their collaborative work in the publication of my second chapter.

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During my PhD I had the opportunity to work with British Antarctic Survey in the Southern Ocean on the State of the Antarctic Ecosystem project. Thanks are due to Dr's Huw Griffiths and Susie Grant for this opportunity and the 3 month internship in Cambridge following the expedition. Whilst there I was able to collaborate further with both Dr's Katrin Linse and Phil Trathan who were most helpful in the production of the SOAntEco report for CCAMLR.

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I dedicate this thesis to my overwhelmingly supportive family, they saw it all from start to finish; the excitements, the disappointments, the frustrations and the breakthroughs. So to my loving bungles; Julie, Alan, Jenna and Sam, here some words about worms. Thank you for always encouraging me to take on the world one Antarctic animal at a time.

Glossary and Acronyms

1M – 1 month

1W – 1 week

1Y – 1 year

6M – 6 months

3’ prime end – the end of a single-stranded nucleic acid molecule which terminates in a hydroxyl group.

5’ prime end - the end of a single-stranded nucleic acid molecule which terminates in a phosphate group.

$\delta^{15}N$ – denotes the isotopic ratio of ^{15}N:^{14}N

AA – Amino Acid

AIC – Akaike Information Criterion

ACC – Antarctic Circumpolar Current

Ala – Alanine (amino acid)

ANDEEP - ANtarctic DEEP-sea benthic biodiversity

Asp – Aspartic acid (amino acid)

BAS – British Antarctic Survey

BIC – Bayesian Information Criterion

BIOPEARL - BIOdiversity dynamics: Phylogeography, Evolution, And Radiation of Life

BLAST - Basic Local Alignment Search Tool

BOLD – Barcode of Life Database

bp – base pair

CAML – Census of Antarctic Marine Life

CCAMLR – Commission of the Conservation of Antarctic Marine Living Resources

COI – Cytochrome c oxidase subunit 1

CSIA – Compound specific stable isotope analysis

DNA Barcode – short sequence of DNA used to identify species

EA/IRMS – Elemental Analyser Isotope Ratio Mass Spectrometry

E – Ethanol

FOODBANCS - FOOD for Benthos on the ANtarctic Continental Shelf

F – Formalin

Fr – Frozen

GC – Gas Chromatography

GC/C/IRMS – Gas Chromatography Combustion Isotope Ratio Mass Spectrometry

GenBank – Online genetic database within the National Center for Biotechnology Information

Glu – Glutamic acid (amino acid)

Gly – Glycine (amino acid)

GTR + I + G – General time reversible + invariable sites + gamma

Iso – Isoleucine (amino acid)

ITS – Internal transcribed spacer

JR144 – BIOPEARL I expedition led by British Antarctic Survey on the *RRS James Clarke Ross* to sample the benthic communities of the Scotia Arc.

JR179 – BIOPERAL II expedition led by British Antarctic Survey on the *RRS James Clarke Ross* to sample the benthic communities of the Amundsen Sea.

JR275 – British Antarctic Survey expedition on the *RRS James Clarke Ross* to sample benthic communities of the eastern Weddell Sea.

JR15005 – SOAntEco expedition led by British Antarctic Survey on the *RRS James Clarke Ross* to sample the benthic communities of the South Orkney Island Southern Slope Marine Protected Area.

K2P – Kimura two-parameter

Leu – Leucine (amino acid)

MAFT – Multiple Alignment using Fast Fourier Transform

MPA – Marine Protected Area

MUSCLE – Multiple Sequence Comparison by Log-Expectation

Nor – Norleucine (amino acid)

PCR – Polymerase Chain Reaction

Phe – Phenylalanine (amino acid)

RAMS – Register of Antarctic Marine Species

SAA – Source amino acids

SCAR-MarBIN – Scientific Committee on Antarctic Research, Marine Biodiversity Information Network

Ser – Serine (amino acid)

SO-DIET – Southern Ocean Diet and Energetics Database

SIA – Stable Isotope Analysis

TAA – Trophic amino acids

TEF – Trophic Enrichment Factor also referred to as Trophic Discrimination Factor (TDF).

Thr – Theorine (amino acid)

TL – Trophic Level also referred to as Trophic Position (TP).

TO – Time zero

Val – Valine (amino acid)

WoRMS – World Register of Marine Species

1 Introduction

Antarctic marine environments are biologically and financially important, harnessing high species diversity, providing commercial fisheries and attracting increasing levels of tourism (Aronson et al., 2011, Xavier et al., 2016). The direct and cascading effects of climate change are likely to lead to local species extinctions, invasions of warm water species, and shifts in species diversity and dominance, ultimately resulting in changes to both community and food web composition (Ingels et al., 2012). The monitoring of Antarctic ecosystems is essential in detecting these changes and in understanding the likely consequences for ecosystem functioning and for effective marine management practices (Griffiths et al., 2017). However, basic biological information including taxonomic and distributional data, a necessary baseline for all ecological studies, is still lacking from many regions (Grant et al., 2011, Kaiser et al., 2013, Xavier et al., 2016). Thus, research effort is required to improve our current understanding of the status of the Antarctic benthos which can then be used for comparison with future data to detect and interpret environmental change. This thesis applies genetic and isotopic analyses in an attempt to improve our understanding of the diversity, distribution and trophic traits of polychaetes from the Antarctic benthos. Polychaetes are abundant and diverse benthic macrofauna, but to date only species-specific genetic analyses have been conducted and species level trait data are lacking.

1.1 Evolution of the Antarctic benthos

The benthic communities of the Southern Ocean have been shaped by its unique evolutionary history. In a paleo-oceanographic context, Antarctica was originally a part of the super-continent Gondwanaland, which disintegrated at the end of the late Cretaceous, 60 to 80 million years ago, after which Antarctica migrated to its current position over the South Pole (Kennett, 1977). Land masses including Australia and South America continued to separate from Antarctica but temperatures around Antarctica remained subtropical until the late Eocene-Early Oligocene, ~40 million years ago (Kemp, 1972). Subsequently, temperatures fell to near-freezing around the coastline, triggering the onset of Antarctic Bottom Water production and the thermohaline circulation, ultimately leading to the formation of the large Antarctic ice sheets (Knox, 1994). The cooling caused a dramatic change in the biodiversity of the Southern Ocean, including the loss of major marine predators such as sharks and crabs and a decline in the number of bivalves, fish and decapod species (Clarke, 1983, Aronson and Blake, 2001,

Thatje et al., 2005a). In comparison to temperate and tropical regions, and in the absence of shell crushing predators, the benthic communities of Antarctica are dominated by echinoderms, pycnogonids and filter-feeding species (Clarke and Johnston, 2003, Aronson et al., 2007).

With the continuing separation of Antarctica from other continents, the Antarctic Circumpolar Current (ACC) was established around 30 million years ago (Kennett, 1977). The ACC lies south of the Polar Front, the northern most limit of the Southern Ocean, and flows eastward around Antarctica with its counter currents, East Wind Drift, running closer to the continent (Figure 1-1). Both currents have had a major role in controlling the distribution of marine life and connectivity within the Southern Ocean. Additional oceanic features include the Ross and Weddell Sea Gyres. In both regions, the near shore circulation is impacted significantly by the presence of large ice shelves and the permanent gyre circulations.

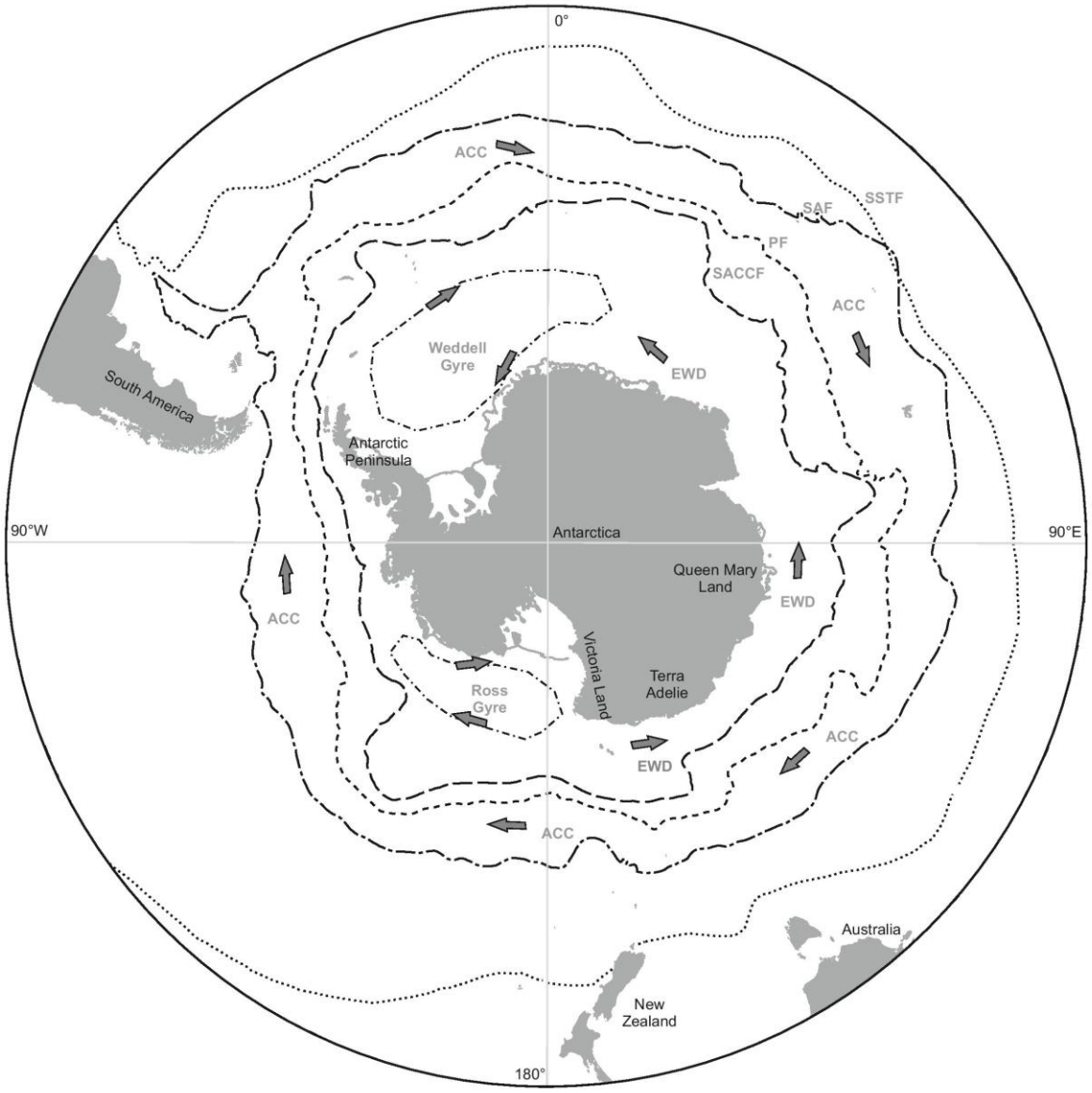

Figure 1-1 Position and direction of ocean currents including the Antarctic Circumpolar Current (ACC), East Wind Drift (counter current) (EWD) and the Weddell and Ross Sea Gyres. Positions of the Polar Front (PF), the Southern Antarctic Circumpolar Current Front (SACCF), Subantarctic Front (SAF) and the Southern Subtropical Front (SSTF) are also marked. Figure adapted from Brasier et al. (2017).

Over the last 3.5 million years there have been intervals of distinct climatic oscillations in the form of the glacial-interglacial cycles (Hays, 1969). These pronounced climatic fluctuations and at times extremely cold ocean temperatures would have had a significant effect on the evolution of the fauna in the Southern Ocean. During glacial maxima, grounded ice extended onto the continental shelf, physically removing or destroying shelf fauna. An early hypothesis suggested that surviving fauna were deposited off the shelf and down slope (Brey et al., 1996). Physiologically, organisms could survive at these greater depths. The isothermic water column and deep continental shelf around Antarctica which they had previously inhabited meant that the physical conditions within their new habitat were relatively similar. The Antarctic continental shelf averages 450 m depth, which is between 2 and 4 times deeper than other oceanic regions (Knox, 1994). This resulted from continued ice scouring during interglacial periods and depression by continental ice during glacial maxima. The continental shelf that surrounds Antarctica can extend to 1000 m depth (Clarke, 1996, Clarke and Johnston, 2003).

More recently, geophysical studies by Thatje et al. (2005b) suggest that grounded ice on the continental shelf and glaciogenic debris redeposited down slope would have made these regions unfavourable to benthic fauna during glacial maxima. Community survival may have only been possible in the deep sea off shelf, or at ice free regions on the continental shelf. During glacial retreat, previously ice-covered areas were recolonised by the surviving fauna from these habitats (Fraser et al., 2012). One way to identify the ancestry of current shelf populations and provide evidence for the different hypotheses regarding de-population/population of the benthic communities during glacial/inter-glacial cycles is the use of phylogenetic analyses (e.g. Hunter and Halanych, 2008, Thornhill et al., 2008, Wilson et al., 2009, Hoffman et al., 2010, Hunter and Halanych, 2010, Raupach et al., 2010, González-Wevar et al., 2011, Hoffman et al., 2011, Janosik et al., 2011, Hemery et al., 2012, Strugnell et al., 2012). Populations originating from ice-free refugia should theoretically have reduced genetic diversity resulting from a reduced population size and genetic mixing by physical isolation from other populations. In contrast, populations that recolonised shelf regions from the deep sea should have regional genetic structuring, as the retreat into and out of the deep sea could have occurred over larger geographic scales. In some cases the physical and reproductive isolation of refugia populations may also have led to the evolution of cryptic species (those that are morphologically identical but genetically distinct; (Thiel et al., 1996, Held, 2003). The dispersal of species around Antarctica following glacial periods could have been aided both by the circumpolar current system and the collapse of the West Antarctic ice shelf creating a ‘short

cut' between the Ross and Weddell Seas (Pollard and DeConto, 2009). Therefore, species with both pelagic larvae and direct development may have been able to disperse substantial distances around the continent establishing their current distributions.

The evolution of life at cold temperatures may have resulted in several life history and physiological traits, such as longevity and an increase in the number of species lacking larval dispersal phases (Thorson, 1936, 1950, Mileikovsky, 1971, Clarke, 1992, Arntz and Gili, 2001, Thatje, 2012). Some of these traits are associated with lower physiological and metabolic rates in comparison to warm water species (Clarke and Johnston, 1999, Peck and Conway, 2000, Peck, 2002). However, the limited and seasonal food supply in the Southern Ocean may have also played a role. The breakup of winter sea ice and increased hours of daylight in the austral summer triggers an intense and short lived phytoplankton bloom in the coastal waters of Antarctica (Clarke, 1988). The level of primary production varies between years and is influenced by longer climatic cycles such as El Nino Southern Oscillation and the Southern Annual Mode (Turner, 2004, Arrigo et al., 2008).

Primary production is variable both spatially and temporally in the open Southern Ocean away from the continent and sea ice zones, with occasional intense phytoplankton blooms (Arrigo et al., 1998). Net primary production is low despite an abundance of macronutrients; globally the Southern Ocean is the largest high nutrient-low chlorophyll zone (Martin, 1990, Moore and Abbott, 2000). These low production rates result from low sun angles, deep mixing of the upper water column, and trace metal limitation (Martin, 1990, Holm-Hansen and Mitchell, 1991, Boyd et al., 2000, Korb et al., 2005). Exceptions to this low productivity include areas around the Polar Front and regions surrounding islands such as South Georgia, Crozet and Kerguelen, where the divergence of surface waters, shallowing topography and trace metal enrichment generates a flux of nutrient rich water to the surface fueling enhanced phytoplankton growth (Moore and Abbott, 2000, Korb and Whitehouse, 2004). With the exception of chemosynthetic habitats such as those on the East Scotia Ridge (Rogers et al., 2012), the seasonal and variable food supply from the exported primary production is considered to be the greatest constraint to life in the Antarctic benthos (Clarke, 1988, Peck et al., 2006). Some species have evolved seasonal reproductive cycles (Grange et al., 2004, Grange et al., 2007, Galley et al., 2008) and increased omnivory with changing food availability (Knox, 1994).

1.2 Benthic research in Antarctica

The first benthic research in the deep Southern Ocean was conducted during the global oceanographic *Challenger* expedition between 1872 and 1876. Subsequent major expeditions within Antarctic waters included those of the RV *Belgica* (1897-1901), the RRS *Discovery* (1901-1904) and a series of voyages on the RV *Eltanin*. As well as discovering species and habitats, early expeditions sampled deep-sea sediments, documenting changes in dropstone abundance and grain size with distance from the continent and their chemical composition across the Polar Front (Brandt et al., 2007). The formation of SCAR (Scientific Committee on Antarctic Research) in 1958 promoted collaborative Antarctic research initiatives. In the 1990s, international research programmes such as EPOS (European Polarstern Studies) and EASIZ (Ecology of the Antarctic Sea Ice Zone) led to another surge in Antarctic expeditions which provided a wealth of taxonomic data from many previously unsampled areas (Arntz and Gutt, 1997, Arntz and Clarke, 2002, Arntz and Brey, 2003, Clarke, 2008).

Since the formation of SCAR, cooperative international efforts have continued, and the number of expeditions, their intensity and coverage is continually expanding. In the last decade, these expeditions have been supported by international initiatives including the International Polar Year (IPY, 2007-2008), the Census of Antarctic Marine Life (CAML) and the Evolution and Biodiversity in Antarctica (EBA). Certain areas of the Southern Ocean, including the Western Antarctic Peninsula (WAP) and the Weddell Sea, are now globally regarded as 'well-studied' marine habitats (Clarke, 2008). Major collaborative benthic sampling programmes have targeted the continental shelf for example, FOODBANCS (FOOD for Benthos on the ANtarctic Continental Shelf) which was designed to evaluate the seafloor deposition, and subsequent ecological and biogeochemical impacts, of the summer phytoplankton bloom at the West Antarctic Peninsula (Smith et al., 2008). Previously unexplored deeper regions of the Southern Ocean have also been sampled, one of the first comprehensive projects was ANDEEP (ANtarctic DEEP-sea benthic biodiversity: colonisation history and recent community patterns) which involved a two-leg expedition to the Weddell and Scotia seas in 2002, and a third expedition in 2005 to the Cape and Agulhas basins, the Weddell Sea and Bellingshausen Sea and Drake Passage (Brandt et al., 2007b). The ANDEEP results show a prevalence of "new" biodiversity (an abundance of undescribed species), and demonstrated that the number of species endemic to Antarctica varied among taxa (Brandt et al., 2007b, Brandt et al., 2007c). These archives have since been used for many taxonomic works including DNA analyses

(Brandt et al., 2007a, Schüller, 2008, Cedhagen et al., 2009, Riehl and Brandt, 2010, Schüller, 2011, Larsen et al., 2013).

Since ANDEEP, the BIOPEARL (BIOdiversity dynamics: Phylogeography, Evolution, And Radiation of Life) expeditions recovered one of the largest single collections of benthic fauna in Antarctica, covering the Scotia Sea (BIOPEARL I) and the Amundsen Sea (BIOPEARL II) in the austral summers of 2006 and 2008, respectively. The collections from the Amundsen Sea and Pine Island Bay area were the first benthic samples to be collected in this region (Linse et al., 2013). These programmes have provided a wealth of taxonomic data as well as evolutionary and biogeographic insight into several taxonomic groups including, for example, Mollusca (Strugnell et al., 2008a, Strugnell et al., 2008b, Moreau et al., 2013) Polychaeta (Brasier et al., 2016, Neal et al., 2017), Echinodermata (O'Loughlin and Vanden Spiegel, 2010, O'Loughlin et al., 2014) and Crustacea (Lörz et al., 2012, Kaiser, 2015).

As a result of the challenges arising from Antarctica's isolation and extreme physical environment, the application of more modern sampling methods has been limited compared to the world's other major oceans (Kaiser et al., 2013). Although traditional trawling methods are still used to collect physical specimens for taxonomy, there has been an increased use of remotely operated vehicles (ROVs), autonomous underwater vehicles (AUVs) and photographic surveying equipment. Such methods are benefical when accessing environments not suitable for trawling, and result in less destructive sampling (Clarke, 1996, Bowden et al., 2011, Gutt et al., 2011). Furthermore, the sampling expeditions discussed also collected physical environmental data, providing an opportunity to monitor the physical conditions and their potential relationship and influence on biological communities. Such data can also feed into modelling studies that can assist in the prediction of future biological change under future climatic conditions.

1.3 Advances in benthic taxonomic, biogeographic and trait analyses within the Southern Ocean

1.3.1 Molecular taxonomy

Taxonomy forms the basis of any ecological study, and is used to understand, define and compare different habitats and communities. The identification and number of species are used to estimate diversity, describe species distributions, and consider the health or vulnerability of

an ecosystem or any further biological analysis. In the early days of Antarctic research, the taxonomic work available to help identify marine species was mostly from northern regions, including Europe. This inherently led to the documentation of similar species in the Southern Ocean being described by comparison to species from northern localities (Schüller and Ebbe, 2014). With increased sampling, developments in microscopy and the use of DNA to identify species, our understanding of species diversity and their evolution has greatly increased. Until relatively recently, genetic data for Antarctic species was rare, with DNA barcodes available for only 2.6% of 3520 marine species listed in RAMS (Grant and Linse, 2009). Between 2009 and 2011 the number of Antarctic morphospecies with DNA barcodes increased from 90 to 2330 (Grant et al., 2011).

The use of DNA barcoding has enabled the identification of cryptic species, those that are morphologically identical but genetically distinct, in many taxa within and between populations outside of the Southern Ocean. Cryptic species have been found in most major taxonomic groups, including polychaetes, crustaceans, echinoderms and molluscs. The drivers of cryptic diversity have generally been associated with reproductive isolation during glacial maxima, when there would have been increased genetic divergence between small populations of the original species. For example, genetic analyses of *Pareledone turqueti* (Cephalopoda) populations identified a continental and sub-Antarctic lineage (Strugnell et al., 2012). The timing of *P. turqueti* diversification was estimated based on genetic mutation rates by Strugnell et al. (2012) and was predicted to have occurred during the mid-Pliocene. It is likely that during periods of glaciation, populations were using refugia at Shag Rocks and South Georgia as well as around the Antarctic continent in the Ross Sea, Weddell Sea and off Adélie Land. Over time, the lack of gene flow between, and physical separation of, *P. turqueti* populations may have led to the evolution of the lineages found today. The abundance of cryptic species and divergent lineages suggests that diversity of the Antarctic benthos may be greatly underestimated.

In Chapters 2 and 6 of this thesis, DNA barcoding is used to confirm the number of polychaete species identified by species morphology. By applying a phylogenetic species concept, phylogenetic and distance analyses are used to assess the abundance of cryptic species and the benefits of using molecular taxonomy for the identification of polychaete species are discussed.

1.3.2 Biogeography

Several biogeographical characteristics have been associated with the Antarctic benthos, including endemism, cicrumpolarity and eurybathy. The number of species endemic to the Southern Ocean is related to its isolation by the frontal systems at the northern limit of the Southern Ocean (Figure 1-1). Fronts can pose a physical barrier to the migration of many species, preventing their movement or dispersal into and out of Antarctic waters (Dell, 1972, Arntz et al., 1997, Clarke and Johnston, 2003). The circumpolar current system that encircles the Antarctic continent may have promoted broad distribution ranges of species around the continent (Arntz et al., 1997, Clarke and Johnston, 2003). Given the relatively uniform physical conditions across the continental shelf, individual settlement and survival of species are not restricted by their physiology e.g. temperature tolerance (Arntz et al. 1994). The abundance of eurybathic species, those exhibiting broad depth ranges, is likely a response to the deeper than average continental shelf and migration during glacial cycles (Dell, 1972, Knox and Lowry, 1977, Brey et al., 1996). Population establishment at different depths was possible due to similar physical conditions (e.g. temperature) on the shelf, slope and deep-sea floor, thus reducing the need for specific adaptations to survive in these environments (Clarke et al., 2009, Clarke and Crame, 2010).

Increased use of data portals such as SCAR Marine Biodiversity Information Network (SCARMarBIN) and the Register of Antarctic Marine Species (RAMS) to document the presence of species at different Antarctic locations has promoted large scale analyses of species diversity, species distribution patterns and the identification of biodiversity hotspots (Griffiths et al., 2009, De Broyer et al., 2014, Terauds and Lee, 2016). Additionally, the use of molecular taxonomy and the deposition of genetic data in databases such as GenBank (https://www.ncbi.nlm.nih.gov/genbank/), has improved our understanding of species biogeography by assessing the level of genetic connectivity between populations and their relationship to populations outside the Southern Ocean (Thornhill et al., 2008, Wilson et al., 2009, Janosik and Halanych, 2010, Riesgo et al., 2015, Galaska et al., 2017). When compared with oceanographic data, such studies can help elucidate connectivity pathways, the roles of current systems in larval dispersal, and identify possible stepping-stone populations (Young et al., 2015, Brasier et al., 2017).

The identification of cryptic species has also led to questions regarding truly circum-Antarctic and eurybathic species (e.g. Brandão et al., 2010, Schüller, 2011). When the distributions of

cryptic clades are compared to those of their original morphospecies, the presumed widespread species are found to include genetically distinct and geographically isolated cryptic species. In these cases, the distribution of species may be more restricted than originally believed (Raupach et al., 2007, Wilson et al., 2007, Arango et al., 2011, Havermans et al., 2011, Janosik et al., 2011); these factors are important factors when monitoring and managing Antarctic fauna.

Following the identification of polychaete species using DNA barcoding, Chapter 3 considers the distribution of the species identified compared to their former morphological species. Patterns such as species restrictions by location or depth as well as widespread distributions are discussed with regard to glacial history, Antarctic oceanography and species reproductive traits that could have influenced their evolution or dispersal.

1.3.3 Trait analysis

Across biological disciplines there has been an increasing amount of research into biological trait analyses. Traits are measurable properties of an organism such as body size, habitat, mobility, and trophic group. Species traits affect their ability to establish populations and the role they have within an ecosystem may be referred to as their 'functional' trait (Hooper et al., 2005). By defining the biological or functional traits of an individual species or group of organisms, they can then be classified according to their functional importance and ecological roles within an ecosystem (McGill et al., 2006). Such data allow investigations into the relationship between species and functional diversity and how species contribute to ecosystem functioning, which in turn have valuable applications in assessing the potential impacts of environmental change on ecosystem properties (Reynolds et al., 2002, McGill et al., 2006, Chown, 2012).

Functional trait analysis has been conducted on well-studied shallow-water temperate marine communities (e.g. Bremner et al., 2003, 2006b, 2006a, Bremner, 2008). Following the success of these studies, trait based classification systems have been constructed for some of the major marine groups including polychaetes, fish and copepods (Froese and Pauly 2017, Barnett et al., 2007, Faulwetter et al., 2014). Comprehensive multiple species trait analyses of benthic communities in the Antarctic are rare but have been used to investigate iceberg disturbance, which has a major role in structuring shallow water habitats on the continental shelf (Gutt and Starmans, 2001, Teixidó et al., 2004, Smale, 2007). The recolonization of benthic habitats

following disturbance in Antarctica is much slower than at lower latitudes, and reflects the slower reproduction and growth rates of many Antarctic fauna (Barnes and Conlan, 2007). When the biological traits of species were examined along a disturbance gradient, less mobile species were more abundant at less disturbed sites, whilst species with higher dispersal capabilities and secondary consumers were more abundant at the disturbed sites (Smale, 2008). However, the effects of iceberg scouring on megabenthic assemblages differ with scale, species diversity being reduced at local scales (0 - 100 m) and increased at regional scales (1 - 100 km) (Gutt and Piepenburg, 2003). Additionally the presence and abundance of keystone and structural species (e.g. ascidians, sponges and corals) during the course of succession reflected their life history traits including growths rates and dispersal capabilities (Teixidó et al., 2004).

Functional trait data for a particular species may be missing or unknown, but can be defined using data from similar or related species, which may be from different locations (Tyler et al., 2012). For this reason, resolution of trait data is often defined at the family level. Defining and interpreting the functional traits of Antarctic species would provide further insight into the relationship between functional and species diversity. This would also identify groups that may be vulnerable to environmental change and the potential impact of community change on ecosystem function (Bremner, 2008). Such investigations require large amounts of data, and to date several trait databases exist including the World Register of Marine Species (WoRMs), Biological Trait Information Catalogue (BIOTIC, Marshall et al. (2006)) and taxa-specific databases such as FISHBASE (Froese and Pauly) and Polytraits (Faulwetter et al., 2014). Databases for the Southern Ocean include RAMS and the SCAR data base Southern Ocean Diet and Energetics Database (SO-DIET http://www.scar.org/data-products/southern-ocean-diet-energetics/). SO-DIET includes information related to diet and energy flow collected from biological and ecosystem investigations in the Antarctic.

The trophic traits of a species can be described either by feeding guild or trophic level. For polychaetes, as well as other marine taxa, gut content analyses and jaw morphology have been used to describe the categorical traits of many marine taxa which cannot be observed *in situ*. In Chapter 4, the lugworm, *Arenicola marina*, is used to develop bulk and compound specific stable isotope methods to define trophic position. These methods are then applied to free living polychaetes (Chapter 5) and symbiotic polychaetes (Chapter 6) from the Antarctic. The data provide insight into the trophic flexibility and regional variation in the trophic biology of Antarctic benthic species.

1.4 Antarctic Marine living resources

The exploitation of Antarctic marine living resources dates back to the late eighteenth century, and has targeted four major groups; seals, whales, finfish and krill (Miller, 1991, Brooks, 2013). The intense and sporadic cycles of exploitation have resulted in severe depletion of target stocks (Gulland, 1983, Miller, 1991). Seals and whales were the first groups to be targeted. Their unregulated exploitation led to near extinction of many fur and southern elephant seal populations, as well as the severe depletion of every Antarctic whale population, with the exception of Antarctic minke whales (Knox, 1984, Sage, 1985, Laws, 1989, Brooks, 2013). This was followed by full scale commercial fishing of finfish which started in the early 1960s on the major shelf areas of the Southern Ocean (Kock et al., 1985). Since then, there has been a notable collapse in the marbled rock cod (*Notothenia rossii*) in the early 1970s and, more recently, some stocks of Patagonian toothfish (*Dissostichus eleginoides*), which are still being exploited (Constable et al., 2000).

The exploitation of Antarctic marine resources is now regulated through international conventions including: the 1946 International Convention for the regulation of Whaling, the 1972 Convention for the conservation of Antarctic Seals, and the 1980 Convention for the Conservation of Antarctic Marine Living Resources (CCAMLR) as a part of the Antarctic Treaty System. Given the impacts of unregulated exploitation in Antarctica, the CCAMLR convention was negotiated in response to the declining krill (*Euphausia superba*) stocks. Krill is a key prey species in the Southern Ocean and is essential in the recovery of exploited mammals (Edwards and Heap, 1981, Constable, 2001). The primary objective of CCAMLR is to conserve Antarctic marine living resources; this differs from most marine management practices which primarily manage fisheries (Brooks, 2013). To achieve its principles, CCAMLR adopts a precautionary ecosystem-based management approach (Stokke, 1996). This includes the precautionary catch limits, which are designed to manage current areas of exploitation, and are determined by various ecosystem indicators and monitoring schemes. CCAMLR is also responsible for the designation of closed areas within Antarctica; these areas serve both scientific study and conservation purposes. In the last decade, there has been a global increase in the designation of Marine Protected Areas (MPAs), where human activity is limited or prohibited to ensure the long term health and sustainable use of marine habitats (Lester et al., 2009, Brooks, 2013).

The first CAMMLR MPA workshop was convened in 2005 to develop advice on the designation of MPAs and their potential to achieve the CCAMLR conservation objectives. In 2009, the South Orkney Island Southern Slope MPA (SOISS MPA) became the first MPA in the Southern Ocean, with an area of 94,000 km^2 designated as "no-take" to commercial fishing (CCAMLR, 2009). At present CCAMLR continues to work towards the designation of a network of MPAs across nine different planning domains in the Southern Ocean. Reflecting the scale and location of current research efforts, these domains provide a mechanism to plan and report on the development of the MPA network (CCAMLR, 2017). The largest MPA to date in the Southern Ocean is in domain 8 (Figure 1-2), the Ross Sea MPA, which was designated in 2016 and covers 1.55 million km^2 (CCAMLR, 2016). Proposals for future MPAs are being considered, including within the Weddell Sea and the Antarctic Peninsula (Teschke et al., 2013).

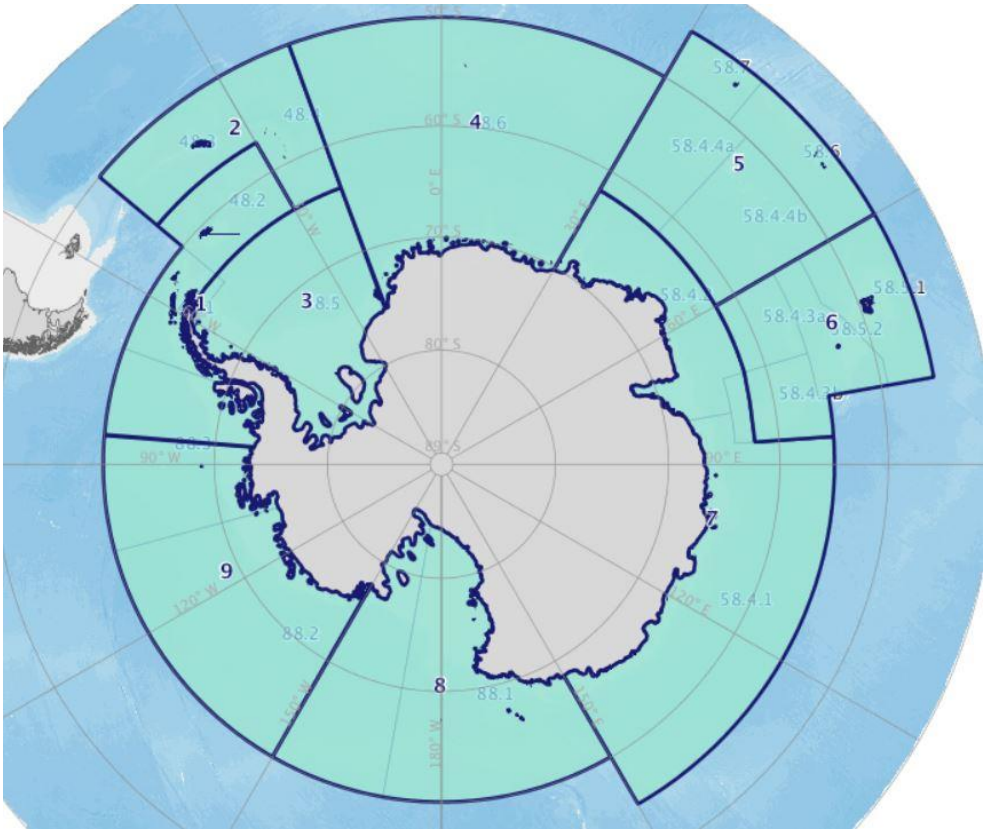

Figure 1-2 CCAMLR's nine MPA planning domains defined during the 2011 CCAMLR workshop on MPAs. Domain 1 = Western Peninsula – South Scotia Arc, domain 2 = North Scotia Arc, domain 3 = Weddell Sea, domain 4 = Bouvet Maud, domain 5 = Crozet – del Cano, domain 6 = Kerguelen Plateau, domain 7 = Eastern Antarctica, domain 8 = Ross Sea and domain 9 = Amundsen – Bellingshausen. Source = https://www.ccamlr.org/en/science/marine-protected-areas-mpas

1.5 Impact of climate change on the Antarctic marine benthos

In addition to the fishing and exploitation pressures on marine ecosystems, global climate change is altering the presence, distribution and behaviour of marine species (Aronson et al., 2007, Clarke et al., 2007). Using 50 years of climate data, the Antarctic Peninsula has been classified as an area of rapid regional warming, having experienced some of the fastest rates of warming anywhere on Earth (Vaughan et al., 2003, Turner et al., 2005). Within the next 100 years, sea surface temperatures in the Southern Ocean are predicted to increase by 0.5-1.0°C in summer, with a 0.25°C increase in waters deeper than 4000 m, and a significantly greater warming expected over the continental shelf (Turner et al., 2009). These changes may seem small, but the fauna of the Southern Ocean will not only experience a change in temperature. Other physico-chemical changes are expected that will ultimately impact the benthos including pH, salinity, oxygen levels and ice density/scouring (Figure 1-3). Such alterations to environmental conditions will have direct impacts on the benthic biota and communities, as well as triggering cascade effects that could influence food quantity and quality. Ecosystem responses to these changes are likely to be complex, due to the interacting and synergistic relationships between taxa (Ingels et al., 2012).

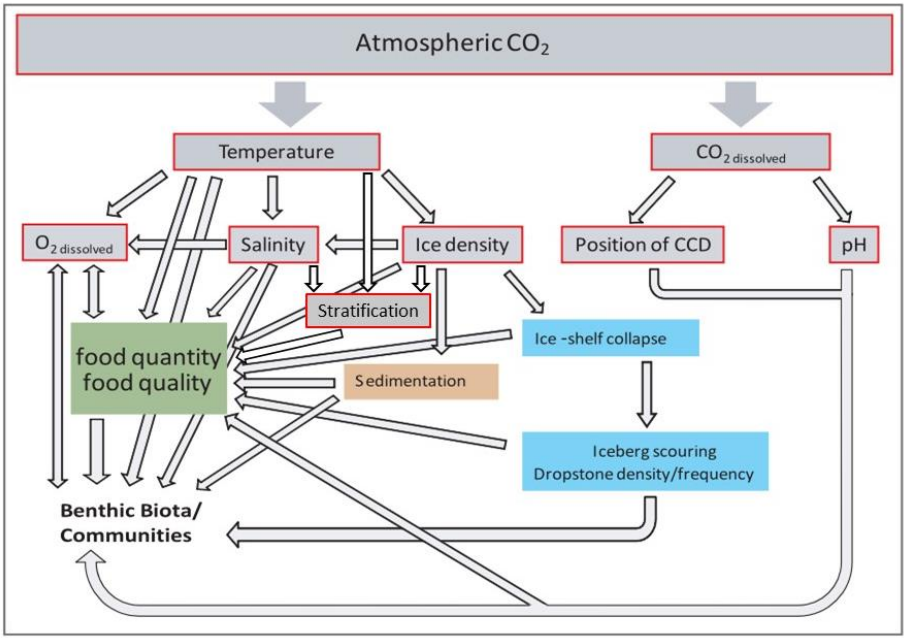

Figure 1-3 Flow chart showing the main effects of climate change and how these will ultimately impact the benthic marine environment. Red-framed boxes indicate interacting physio-chemical variables; blue, brown and green boxes are factors that are affected by the physio-chemical variables which may interact with each other and cause a type of disturbance to the benthic biota/communities. CCD = Calcite compensation depth, the depth which the rate of supply of calcite (a form of calcium carbonate) is lower than the rate of calcite dissolution, such that no calcite is preserved. Figure adapted from Ingels et al. (2012).

1.5.1 Increasing temperatures

The elevated air and sea temperatures are resulting in the collapse of ice shelves, the retreat of glaciers and the exposure of new terrestrial habitats on the continent (Clarke et al., 2007). Major alterations in the timing and extent of winter sea ice cover have been recorded in the Bellingshausen and Amundsen seas, and along the Western Antarctic Peninsula (Vaughan and Doake, 1996, Zwally et al., 2002, Vaughan, 2006). The most recent iceberg to break free was over 6,000 km^2, representing 10% of the Larsen C ice shelf (BAS, 2017). The break-up of ice shelves such as this can cause increased scouring in shallower regions (< 500 m), and also an increase in dropstone density (Gutt et al., 1996, 2011). As well as physical disturbance, the loss of ice will have direct impacts on the ice-dependant life stages of different species, including krill (Smetacek and Nicol, 2005). Other impacts are associated with the exposure of 'new habitats' influencing the timing and intensity of the phytoplankton bloom, the quantity and quality of phytodetritus and so impacting higher trophic levels through ecological and food-web interactions.

Rising air temperatures will also increase deglaciation on land, resulting in increased glacial discharge and sedimentation rates, that will have a localised physical disturbance on benthic communities (Grange and Smith, 2013, Sahade et al., 2015). Over longer time scales, increased melting and freshwater input to the coastal waters of Antarctica will reduce salinity (Meredith and King, 2005). This could affect the physical properties of the water column, such as stratification, and subsequently the timing, composition, magnitude and frequency of the phytoplankton bloom. Reduced salinity will also influence animal physiology. Furthermore, a reduction in the solubility of oxygen will also occur with increased temperatures. With enhanced stratification reducing the flow of dense oxygen rich waters to the deep sea, as well as other physiological stressors, a reduction in oxygen levels of benthic habitats may promote the development of hypoxic zones (Matear et al., 2000, Hofmann and Schellnhuber, 2009).

Several laboratory studies of shallow water benthic fauna have observed and determined the physiological limits of several Antarctic species. In general, Antarctic species are considered to have poorer physiological capacities to cope with changing conditions than species elsewhere (Peck, 2005). This is due to their evolution in relatively low and stable temperatures resulting in mostly cold-stenothermal species with slow growth rates and extended generation times (Everson, 1977, Arntz et al., 1994, Peck et al., 2000). When *in vivo* temperatures were raised by 2-3°C above the annual mean, many species lost the ability to perform essential

functions e.g. swimming in scallops or burrowing in infaunal bivalves. When temperatures were raised further to 5-10°C above the annual mean, many species reached their physiological limits and died (Peck, 2005). Sensitivity to elevated temperatures varies between species. For example, the ophuroid *Ophionotus victoriae* has been identified as one of the most vulnerable of those tested, unable to acclimate to an increase in temperature from 0°C to 2-3°C (Peck et al., 2009a). Multispecies analyses have also shown that size and activity may affect acclimation abilities; the greatest survival at elevated temperatures was recorded in smaller and more mobile species, which were deemed less vulnerable to warming events (Peck et al., 2009b). The impact of ocean warming on marine fauna will depend on both the rate and level of change that is experienced and the impact of any synergistic factors such as changes in food supply via primary production, salinity, oxygen levels and ocean acidification (Chown et al., 2015).

1.5.2 Ocean acidification

The carbonate saturation state in the Southern Ocean is close to being undersaturated in surfaces waters, because of the inverse relationship between calcium carbonate ($CaCO_3$) solubility and temperature (Revelle and Fairbridge, 1957, Guinotte and Fabry, 2008). This makes the deposition of $CaCO_3$ (in the form of calcite or aragonite) for calcifying organisms, e.g. for shell and skeletal structures, an energetically costly processes (Arnaud, 1974, Vermeij, 1978). Ocean acidification, i.e. the ongoing decrease in ocean pH resulting from the uptake of atmospheric carbon dioxide (CO_2), will therefore pose a serious threat to any calcifying animals. For example, planktonic pteropods are already close to the limit of their ability to secrete $CaCO_3$ shells (Aronson et al., 2011) and are likely to be impacted.

Around 40% of the total oceanic uptake of anthropogenic CO_2 occurs south of 40°S (Sabine et al., 2004, Khatiwala et al., 2009). Models predict that under "business as usual" CO_2 emission scenarios, the surface waters of the Southern Ocean will become under-saturated in $CaCO_3$ in the next 100-150 years (Orr et al., 2005, Fabry et al., 2009, Steinacher et al., 2009). Calcifying marine organisms, including pteropods, foraminifera, cold-water corals, echinoderms and molluscs, which comprise a significant component of the rich communities at high latitudes, are thought to be at risk from increasing ocean acidification (Orr et al., 2005, Gutt et al., 2015). The increased absorption of CO_2 and decrease in ocean pH may further cause the dissolution of external skeletal elements (McClintock et al., 2009). Indeed, experimental studies have shown shell and structural deterioration in acidified pH levels (7.4) compared to ambient (8.2), in bivalve, gastropod and coralline algae species.

The effects of decreasing pH have also been considered with regard to early life stages (Clark et al., 2009). Early life history stages may be the bottleneck for species persistence and ecological success under changing pH, as these are often more sensitive than adult stages (Dupont and Thorndyke, 2009, Gibson et al., 2011). For benthic organisms with dispersive larval stages, this could impact their abundance and their ability to disperse between populations, affecting genetic connectivity. Among the most studied Antarctic larvae are echinoderms; these generally omnivorous keystone species have a structuring role in the Antarctic benthos (Clarke and Johnston, 2003, Dupont et al., 2010). Under lower pH conditions, the Antarctic echinoderm *Sterechinus neumayeri* showed reduced growth rates and smaller body size associated with reduced feeding efficiency (Clark et al., 2009). Ocean acidification is predicted to affect the entire Southern Ocean (Gutt et al., 2015). Further studies are needed to understand the potential species-specific effects, and how they could vary with life stage and other environmental stressors (Constable et al., 2014).

1.5.3 Range shift

When a species, population or community experiences environmental change beyond its physiological limits, they must either evolve and adapt to their new environment or migrate to a habitat in which conditions are more favourable in order to survive. Failure to adapt or migrate will result in population decline and possible extinction (Barnes et al., 2009, Ingels et al., 2012, Griffiths et al., 2017). The distributional range of benthic species has been documented and investigated to highlight the areas where the distributional limits of Antarctic and sub-Antarctic species coincide (Barnes et al., 2009). Monitoring regions, such as South Georgia and the Kerguelen Plateau, may provide insight into the onset of species shifts and biological change. The combined analysis of distributional and temperature data along with future climate scenarios predicts a decline in suitable temperature habitats for 79% of the ca. 1000 shelf species investigated (Griffiths et al., 2017).

Range shifts have already been recorded in some species, including shell crushing predators, which were regarded as absent from the shallow water Antarctic benthos. The lack of shell crushing predators has previously been explained by the inverse relationship between calcium carbonate solubility and temperature (Aronson et al., 2007). However, for reptant decapods, their exclusion from cold waters is associated with their inability to regulate magnesium ions in their hemolymph at cold temperatures (Frederich et al., 2002). Since the early 2000s, there has been an increasing number of recorded ‘invasions’ of lithodoids, or king crabs, onto the

continental slope and shelf around the Antarctic Peninsula (Thatje and Arntz, 2004, Thatje and Lörz, 2005, Thatje et al., 2008, Smith et al., 2012, Aronson et al., 2015b). This expansion of king crab populations is thought to have been facilitated by increased temperatures, removing the physiological barrier to their dispersal. If populations continue to migrate, this could alter the species living on the shelf regions and the distinct characteristics of Antarctica (i.e. an abundance of echinoderms and bivalve molluscs), and benthic communities may become more similar to those in temperate and tropical regions (Aronson et al., 2015a).

1.6 Aims and hypotheses

In order to monitor and predict the impacts of climate change and resource exploitation on the Antarctic benthos we must first:

- Understand the current levels of species diversity to provide accurate baseline data. Such data may also identify diversity "hotspots", once found these can be monitored and managed more effectively, ensuring the protection of the most valuable and/or vulnerable regions.
- Assess the current distribution patterns of Antarctic species and understand the environmental forcing and biological controls behind different species ranges. This again may highlight vulnerable species or regions of the Antarctic that should be incorporated into monitoring or management regimes.
- Explore the role and importance of species diversity in ecosystem function, for example are certain traits more abundant or more resilient than others? What would the consequences of species loss be?

This thesis uses a large sample set of free-living polychaete worms collected during the British Antarctic Survey BIOPEARL sampling programme to being to address these questions. More specifically it investigates the genetic diversity, biogeography and trophic traits of different species. DNA and stable isotope analyses (SIA) are used to:

- Compare, using a subsample from a large Antarctic sample set, the number of polychaetes species obtained from morphological and genetic analyses
- Analyse the distribution of genetically distinct populations and cryptic species of polychaetes

- Determine the effects of different preservation methods on the $\delta^{15}N$ bulk and amino acid signatures of polychaetes using *Arenicola marina*
- Define the trophic traits of polychaetes using stable isotope analysis and how these might vary with depth and location

The bulk SIA and compound specific stable isotope analysis (CSIA) method was developed using the lugworm *Arenicola marina* and applied to a limited Antarctic collection of polychaetes. Additionally, symbiotic polynoids (scale worms) were collected during the British Antarctic Survey South Orkneys – State of the Antarctic Ecosystem (SO-AntEco) expedition to the South Orkney Islands Southern Slope MPA and the same methods applied in attempt to define the relationship between the symbiotic polynoids and their host species (corals).

By completing the aims listed above the following overarching hypotheses are investigated:

1. As a result of cryptic species, estimates of Antarctic polychaete diversity are greater from combined morphological and genetic analysis than morphological analysis alone.
2. Genetically divergent or cryptic species are geographically isolated from one another with more restricted distributions than their original morphospecies.
3. The SIA and CSIA trophic traits of Antarctic polychaetes reflect their categorical feeding traits but vary with depth and location.
4. Genetic diversity is reflected in functional diversity, i.e. cryptic species have different isotopic signatures.

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2 Cryptic diversity within Antarctic Polychaetes

The data presented in this chapter were published in Brasier et al. (2016) 'DNA Barcoding uncovers cryptic diversity in 50% of deep-sea Antarctic polychaetes' in Royal Society Open Science.

2.1 Introduction

2.1.1 DNA barcoding and the identification of cryptic species

Traditionally marine species are identified by their morphological characteristics; it is now possible to identify them genetically by comparing DNA nucleotide sequences. As described in Hebert et al. (2003a), (2003b) DNA sequences from specific genes represent a natural 'barcode', which is embedded in every cell of the organism, unique to its species, hence the term 'DNA barcoding'. It was proposed that a 658-base pair (bp) region at the 5' end of the mitochondrial cytochrome *c* oxidase gene (COI or cox-1) should serve as the universal standard barcoding region (Hebert et al., 2003a). The COI gene is considered to have various attributes over other mitochondrial and nuclear genes that make it the best suited for this role; the most important perhaps is its fast-evolving nature. Evolution of the COI gene is nearly three times greater than rRNA genes and can be used to discriminate between very closely related species (Bucklin et al., 2011). Other associated advantages of using the COI gene include its ease of amplification across a range of taxa using universal primers (Folmer et al., 1994, Hebert et al., 2003a) and its lack of indels (the insertion or deletion of nucleotides) that can complicate sequence alignment and comparative analysis (Bucklin et al., 2011).

The application of DNA barcoding in marine diversity and ecological studies has shown to have various benefits over morphological identification uncovering previously overlooked or unforeseen results (Blaxter, 2004, Dasmahapatra and Mallet, 2006, Bucklin et al., 2011). Only a small fragment of material is required for DNA extraction and thus it is possible to obtain DNA barcodes and identify damaged or fragmented organisms. This can be advantageous when working with soft-bodied fragile marine organisms e.g. gelatinous species such as zooplankton as well as polychaetes that can easily be damaged during collection, reducing or removing discriminating morphological characteristics. DNA barcodes can be obtained from all life stages including larvae, juveniles and adult specimens. Given the difficulty of studying the entire life cycle of many marine organisms that can undergo many ontogenetic changes, previously unidentified larvae can be matched to their corresponding adult form. The

comparison of DNA barcodes obtained from several individuals within a species can also uncover cryptic species; species that are morphologically identical but genetically distinct. The ability to identify cryptic and non-cryptic species using DNA barcoding works on the basis that the genetic variation between species (interspecific) is greater than the genetic variation within species (intraspecific) (Hebert et al., 2003b). Thus, ideally, there should be a lack of overlap between intra- and interspecific sequence variation, commonly referred to as the 'barcoding gap' as it can be shown diagrammatically using frequency distributions (Meyer and Paulay, 2005).

In order to identify known species or detect novel sequences, DNA barcodes from sequenced specimens need to be compared to a reference library. There are currently several major genetic databases. The two that are most commonly used in marine diversity investigations are GenBank and Barcode of Life Data System (BOLD). GenBank contains roughly 260,000 publicly-available nucleotide sequences of formally-described species (Benson et al., 2013). DNA sequences can be compared to those within this database using the Basic Local Alignment Search Tool (BLAST), which provides a similarity score as well as a significance value for each match. The BOLD database was introduced as an informatics workbench for a single, high-volume DNA barcode facility (Ratnasingham and Hebert, 2007). Unlike GenBank, BOLD only accepts COI sequences and has a strict set of criteria that must be met for a sequence to obtain the formal barcode status. These include the species name, a voucher catalogue number, COI nucleotide sequence, polymerase chain reaction (PCR) primers used in replication, the collection record and the identifier of the specimen (Ratnasingham and Hebert, 2007). These data elements, when available, are also submitted with GenBank sequences together with any publications associated with the specific sequence. All DNA sequences on GenBank and those within open access databases on BOLD can be downloaded and used for phylogenetic analysis. This allows further analyses of barcodes such as Bayesian analysis and distance analysis to document species diversity and infer phylogenetic relationships amongst closely related species or within higher taxonomic groups e.g. at the family or phylum level.

Although COI is now widely recognised as the barcoding gene of choice there are other genes deemed suitable, based on the criteria of Hebert et al. (2003a), which for some taxa are more appropriate. Another mitochondrial gene frequently used is the 16S region. The 16S gene may be more conserved and slower evolving than COI, however, mutations within its variable regions can allow for species discrimination. An advantage of 16S over COI is that it is often

easier to obtain due to its more conserved primer binding regions (Vences et al., 2005b). Although 16S sequences will not be given formal barcode status on BOLD, these sequences are stored within other databases including GenBank. However the sequencing of COI alongside 16S is advised for newly barcoded species to avoid potential false negatives (Vences et al., 2005b). Many genetic diversity studies also include nuclear genes such as 18S and 28S, as well as the internal transcribe spacer region (ITS). In most taxa these genes are more conserved and most often used to elucidate phylogenetic relationships at higher taxonomic levels rather than at species level. Using a combination of mitochondrial and nuclear genetic markers is advised for the detection of cryptic species and species delineations (Ferguson, 2002). Nuclear genes can also provide useful information on gene flow, phylogeography and phyloecology, which may not be evident from single mitochondrial genes.

It should be noted that the use of DNA barcoding is generally considered to be an additional method of species identification and description rather than a replacement (DeSalle et al., 2005). Sequence data can direct taxonomic efforts towards unusual or unexpected phylogenetic groupings. In such cases, morphological characters may not adequately represent true phylogenies or levels of species diversity (Mincks Hardy et al., 2011). Morphological analyses provide insight into phenotypic variation and the functional ecology of a species, however they are also needed to identify true cryptic species (McManus and Katz, 2009). Additionally DNA barcoding is not always successful, in some taxa including Porifera, Ctenophora and Anthozoa, the evolutionary rate of the COI and other mitochondrial genes is too low to allow reliable discrimination between closely related species (Huang et al., 2008, Shearer et al., 2002). In such cases other genetic data such as microsatellites or single nucleotide polymorphisms may be investigated.

2.1.2 DNA barcoding Antarctica species

Antarctica is a fragile environment currently undergoing some of the fastest rates of climatic change on the planet (Vaughan et al., 2003). These changes are predicted to have a significant impact on its marine communities if species cannot adapt to their new conditions (Peck et al., 2004, Clarke et al., 2007). In order to detect and document any changes with environmental conditions, our knowledge of species diversity and biogeography needs to be improved. For these reasons there has been an increased effort to accurately document and assess our understanding of current species diversity globally and within Antarctic waters. In the last few decades several global initiatives have been set up to try and document marine species, quantify

marine biodiversity and make these data readily available through public databases. The Census of Marine Life (CoML; www.coml.org) project ran from 2000-2010; its goal was to create a global synthesis of the state of marine biodiversity. CoML comprised several subprojects including the Census of Antarctic Marine Life (CAML; http://www.caml.aq) that aimed to investigate the distribution and biodiversity of marine animals within the Southern Ocean and Antarctic Islands. As a part of this initiative, Grant and Linse (2009) collated and quantified the DNA barcode data available for Antarctic marine species in GenBank. This study revealed that for the 3,520 marine invertebrate species included, genetic data were available for 90 (2.6%) of them. However, these sequences only covered a limited range of taxa and areas, with the majority of sequences obtained from Mollusca and Crustacea groups. Furthermore, the sequenced specimens were mostly collected from the Weddell Sea and the Antarctic Peninsula, the areas that have received the greatest sampling effort. These results were revaluated by Grant et al. (2011). During this time the number of Antarctic barcoded sequences, either processed or within the pipeline, rose from 432 to 20,355. The increased barcoding effort was associated with both CAML and the 18 research voyages in 2008-2009 within the International Polar Year framework.

As discussed in Grant et al. (2011) cryptic species appear to be a common feature within Antarctic fauna. Some areas within the Southern Ocean, such as the Scotia Arc, are considered potential hotspots of cryptic diversity (Linse et al., 2007). This is perhaps not surprising given the isolated nature and glacial history of Antarctic waters, which could have promoted cryptic speciation. It has been proposed that the ecological impacts of repeated glacial and interglacial cycles could act as an Antarctic diversity pump (Clarke and Crame, 1989, 1992). Ice advances during glacial maxima physically remove most of the marine benthos inhabiting the continental shelf depositing it on to the continental slope. Thus for species to persist through these glacial periods they would have had to survive within the deep sea or in shelf refugia within areas of no sea ice (Thatje et al., 2005, Thatje et al., 2008). During this time gene flow between populations would have been non-existent leading to increased genetic variation between populations. Under extreme environmental conditions there may be increased selection pressures on behaviour and physiological character rather than functionality, thus reducing or eliminating morphological changes that can accompany speciation (Bickford et al., 2007). So if the functionality of the isolated populations remained constant, it is likely that their morphology would have gone unchanged, thus potentially resulting in high levels of cryptic species. To date evidence of cryptic species has been documented in several Antarctic marine

phyla including crustaceans (Held, 2003, Havermans et al., 2011), molluscs (Linse et al., 2007, Allcock et al., 2011), polychaetes (Schüller, 2011, Neal et al., 2014), echinoderms (Wilson et al., 2007, Janosik and Halanych, 2010) and nematodes (Thornhill et al., 2008).

2.1.3 Cryptic diversity within marine polychaetes

Polychaetes represent one of the most dominant taxa in benthic marine communities, including Antarctica waters, where they are can account for more than 70% of macrofaunal individuals (Gambi et al., 1997, Glover et al., 2008). To date, the number of genetic investigations into the prevalence of cryptic species amongst polychaetes is somewhat limited. However, as discussed in a recent review (Nygren, 2014), there is evidence to suggest that cryptic species may be common amongst all polychaetes making up a significant portion of their biodiversity. The abundance of cryptic species within polychaetes could be assigned to both biological and methodological factors. Several polychaete families lack distinguishing characteristics and can be hard to identify beyond genus level. Furthermore, given the soft-bodied nature of polychaetes, they can be easily damaged during collection and sorting, or once preserved, they can lose species-specific characteristics including their colouration and appendages. For these reasons the use of molecular methods to identify polychaete specimens is essential for accurate measures of species diversity.

Within the last decade the majority of published studies on cryptic polychaete species use discrete mitochondrial and/or nuclear sequence data. Prior to this alternative methods such as fragment based analyses e.g. RAPD and protein electrophoresis were used (Nygren, 2014). These methods, whilst useful for identifying cryptic species and understanding evolutionary processes, are more costly and the data harder to collect and interpret. The first major comprehensive DNA barcoding project of polychaetes was by Carr et al. (2011). These authors sequenced specimens from waters surrounding Alaska and the Canadian Arctic. Out of the 333 morphologically identified species, approximately 25% contained two or more distinct genetic lineages. Results such as this suggest that polychaete identification based on morphological characters alone may significantly underestimate species diversity. Whether or not cryptic species are more prevalent within certain polychaete families, functional groups or environments, is unknown. Cryptic species have been documented in many polychaete families with differing levels of morphological complexity, modes of larval development, distribution ranges and niche requirements. These findings reduce our ability to target certain species in order to document cryptic diversity and limit our abilities to estimate true levels of species

diversity. Another factor that should be considered in diversity evaluations is the presence of polymorphic species. Like cryptic species these are most easily identifiable from sequence analysis. Polymorphic species contain several different morphotypes, which are often thought to be separate species but are genetically identical. Polychaete examples include the described scale worms *Arctonoe fragilis* and *A. vittata* (Carr et al., 2011) and the known colour morphs of *Harmothoe imbricata* (Nygren et al., 2011).

2.1.4 Phylogenetic species concept

The presence of cryptic species not only prevents accurate estimates of species diversity, it also creates great taxonomic challenges including the identification and description of species (Knowlton, 2000, Bickford et al., 2007). The biological species concept used by most biologists arose from Mayr (1963) where a species includes populations of organisms that interbreed or have the potential to produce fertile offspring. The relationship between genetic variation and reproductive compatibility is highly complex even within taxa. For example, within marine polychaetes the amount of genetic difference required for two populations to be reproductively isolated from one another is variable. For example a 5% genetic difference within *Ophryotrocha* sp. was enough to suggest reproductive isolation and cryptic species across sympatric clades. However 5% genetic difference would not be sufficient in other genera, even between geographically isolated populations, as seen in *Streblospio* (Schulze et al., 2000, Wiklund et al., 2009). Such differences can be assigned to the presence or lack of 'barcoding gap' and relatively 'high' or 'low' variation within and between cryptic clades.

For the reasons discussed there is currently no universal 'cut off' at which a certain genetic distance indicates a separate species (Bickford et al., 2007, Nygren, 2014). This uncertainty can leave genetic taxonomists unable or unwilling to confirm the presence of cryptic species, with many authors maintaining a conserved approach. A minimum of 10 times the average intraspecific variation between clade differences was suggested by Hebert et al. (2004b) as a rule of thumb for identifying cryptic species. This was used to identify provisional species in a major polychaete barcoding project (Carr et al., 2011). Phylogenetics can also be used to determine the presence of cryptic species. Most geneticists will include their sequences within a phylogeny of closely related taxa using sequences from public databases. This allows the comparison of genetic distances between proposed cryptic species and known morphologically described species. If the genetic distance is greater than or comparable to the genetic distance

between known species the author may use this as evidence to confirm the presence of cryptic species.

2.1.5 Aims

The data presented in this chapter will be used to compare our ability to identify polychaetes using morphology versus DNA analysis, and assess the level of cryptic diversity present within Antarctic polychaetes. Any new species found will be designated using a phylogenetic approach. Given the lack of genetic 'rules' or 'thresholds' for determining species, the sequence data will be compared to other species and cryptic species within the same family and compared to previous taxonomic literature.

2.2 Methods

2.2.1 Sample collection

The macrobenthic samples were collected using both an epibenthic sled (EBS) and Agassiz trawl between 100 and 3500 m depth during the BIOPEARL I (JCR144) and II (JCR179) expeditions on the RRS James Clarke Ross. For specific sampling protocols and preservation procedures see Linse et al. (2008), Neal et al. (2014) for EBS, and Griffiths et al. (2008) for Agassiz trawl. Additional specimens were donated by Katrin Linse; these were collected during the JCR275 expeditions which sampled the Southern Weddell Sea. These specimens were collected using the same sampling and preservations procedures followed for the BIOPEARL macrobenthic collections. In total there were 16 EBS and 55 Agassiz trawl deployments in different areas of the Weddell Sea across 6 depth horizons between 400 and 2000 m depth during JCR275. The location of the sampled sites within the Scotia Arc (BIOPEARL I), the Amundsen Sea (BIOPEARL II) and Weddell Sea (JR275) are shown in Figure 2-1.

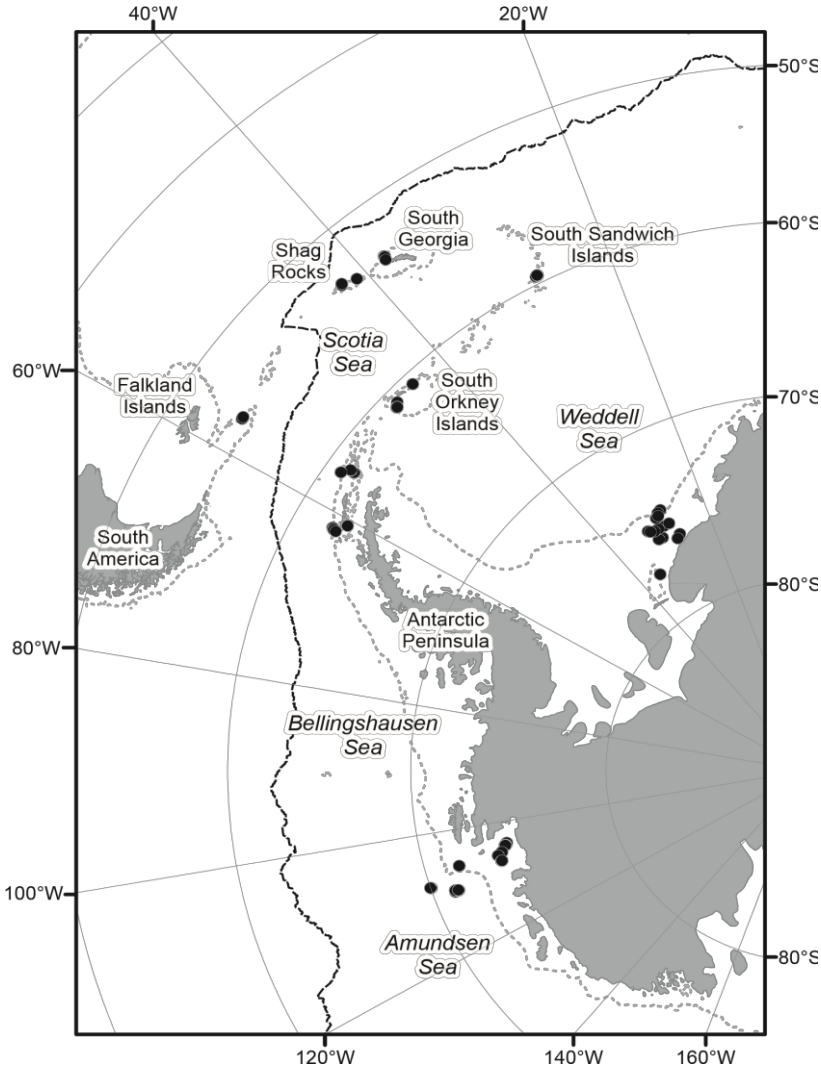

Figure 2-1 Location of all the EBS stations from the BIOPEARL I, II and JR275 cruises (Figure by Katrin Linse).

2.2.2 Morphological species identification

Around 20,000 individual polychaetes collected during BIOPEARL I and II were identified from morphological characters by Lenka Neal. Where possible, individuals were assigned to named species using published dichotomous keys. However, in many cases species lacked description and were assigned a morphological operational taxonomic unit at the highest identifiable taxonomic level. For example, some specimens could be resolved to a genus level e.g. *Flabelligena* sp. A and *Flabelligena* sp. B, whereas others were only identifiable to the family level e.g. Polynoidae sp. A. The additional samples from the Weddell Sea were sorted for targeted species whose morphological identification was also confirmed by Lenka Neal to ensure consistency.

2.2.3 Specimen selection for DNA barcoding

The selection of target species for DNA barcoding was a non-random process; it was based on an informed combination of methodological requirements and research considerations. As the second part of this thesis will investigate the trophic traits of Antarctic polychaetes, the target species chosen for DNA sequencing covered as broad a trait range as possible. The biological traits of polychaete families present in the BIOPEARL samples were compared using Adrian Glover's Polychaete trait database (Unpublished data). The categorical traits compared included body size, habitat, mobility, trophic, reproductive parameters. The database provided trait data at the genus level. When examined however, the majority of traits were consistent throughout the BIOPEARL families. Furthermore, the use of family level traits to assess trait coverage was also supported from the discussions at the EMODNet Traits Workshop (Paris, 2014). Discussions highlighted that most of our current knowledge of marine traits is at the family level. This led to the suggestion that at least one target species should be selected from each polychaete family within the BIOPEARL samples.

Choosing a target species from a range of families could also avoid taxonomic biase e.g. favouring species within a particular family, in which cryptic speciation is more common. However, our knowledge of the occurrence of cryptic species across polychaete families appears to be as limited as our knowledge of polychaete traits, as discussed in the recent review by Nygren (2014).

To allow for investigations into the variability of trophic traits with depth and location (Chapter 5), target species were also chosen on the basis of their distribution. The BIOPEARL sites consist of six locations in the Scotia Arc (BIOPEARL I) and five in the Amundsen Sea (BIOPEARL II), Figure 2-1. Within the Amundsen Sea, BIO3 and BIO6 are grouped as the ‘Outer Amundsen Sea’ and BIO4 and BIO5 sites as the ‘Inner Amundsen Sea’; unfortunately no specimens were suitable for sequencing from the abyssal BIO8 site. Those species with broader distributions were favoured over more restricted ones, provided they met the methodological requirements below. This approach also assured the inclusion of several currently considered ‘cosmopolitan’ and ‘circum-Antarctic’ distributed species.

The methodological constraints on species selection included: preservation, size and number of individuals. Half the BIOPEARL II specimens were preserved in formalin. Formalin denatures DNA and thus reduces the chance of usable DNA barcodes. Approximately 5 mg of tissue is required for compound specific stable isotope analyses (CSIA), so larger individuals

were favoured over smaller ones during specimen selection. Species with multiple individuals from the same site/depth were also favoured as they may provide insight into within species variation under the same environmental conditions. The families from which target species were successfully sequenced are shown in Table 2-2. To summarise, 15 polychaete morphospecies were selected from the ~400 available. The chosen species covered 12 out of the 28 families present in the sample set, Figure 2-2. All chosen specimens are listed in Appendix 1 with their NHM voucher and GenBank Accession numbers.

Figure 2-2 Photos of the 15 original target morphospecies selected for DNA barcoding, scale bar 1000 µm. In alphabetical order by family a) *Flabelligena* sp. A, b) *Flabelligena* sp. B (Acrocirridae), c) *Chaetozone* sp. A (Cirratulidae), d) *Euphrosinella cirratoformis* (Euphrosinidae), e) *Glycera capitata* (Glyceridae), f) Hesionidae sp. A (Hesionidae), g) *Lumbrineris kerguelensis-cingulata* (Lumbrineridae), h) *Maldane sarsi* (Maldanidae), i) *Aglaophamus trissophyllus* (Nephtyidae), j) *Aricidea simplex* (Paraonidae), k) *Harmothoe fuligineum*, l) *Macellicephala* sp. A, m) *Macellicephaloides* sp. B (Polynoidae), n) *Scalibregma inflatum* (Scalibregmatidae) and o) *Laonice weddellia* (Spionidae). From Brasier et al. (2016).

2.2.4 Molecular work

The selection of the exact section of each specimen to dissect for DNA extraction varied between families depending on their most useful taxonomic characteristics. This allowed for re-examination of taxonomic characters after DNA sequencing. For example, parapodia were taken from Polynoidae specimens, mid-body segments from Glyceridae and ventral tissue from Nephtyidae. DNA was extracted using Qiagen DNeasy Blood and Tissue kit. Of the 463 extractions, 131 were extracted using individual spin columns following the protocol provided by the manufacturer and the remaining 332 extracted by the NHM Sequencing Facility using a Hamilton Microlab STAR Robotic Workstation.

Part of the mitochondrial protein-coding COI (the so-called 'Folmer fragment', around 660-bp) gene was the primary gene targeted for this project. The COI gene was chosen because of its evolutionary rate, resulting in a greater degree of genetic distance between than within species (Hebert et al., 2003a). However, following variable PCR success with COI primers in this project, for many target species the non-coding mitochondrial 16S rRNA gene (around 500-bp) was also sequenced. This gene can be used in a similar way to COI for species discrimination (Vences et al., 2005a, 2005b). Furthermore it is often easier to obtain, and in the case of Antarctic invertebrates, most widely available (Grant and Linse, 2009).

DNA extractions were amplified using a PCR mix of 21 μL Red Taq DNA Polymerase 1.1X MasterMix (VWR), 1 μL of each primer (10 μM) and 2-5 μL of DNA extract. The PCR temperature profile consisted of an initial 5-minute denaturation stage at 95ºC, followed by 35 cycles of 95ºC denaturation for 1 minute, 55ºC annealing for 1 minute, 74ºC extension for 2 minutes with an additional 5 minutes extension phase after the last cycle. For primer sequences and references see Table 2-1. PCR products were purified using a Millipore Multiscreen 96-well PCR purification system and sequenced on a ABI 3730XL DNA Analyser (Applied Biosystems) at the Natural History Museum Sequencing Facility.

Primer name	Sequence (5-3')	Reference
LCO	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)
HCO	TAAACTTCAGGGTGACCAAAAA ATCA	Folmer et al. (1994)
CO1-E	TATACTTCTGGGTGTCCGAAGAATCA	Bely and Wray (2004)
polyLCO (F)	GAYTATWTTCAACAAATCATAAAGATATTGG	Carr et al. (2011)
polyHCO (R)	TAMACTTCWGGGTGACCAAARAATCA	Carr et al. (2011)
PolyshortCOIR (R)	CCNCCTCCNGCWGGRTCRAARAA	Carr et al. (2011)
Ann16Sr	TCCTAAGCCAACATCGAGGTGCCAA	Sjölin et al. (2005)
Ann16Sf	GCGGTATCCTGACCGTRCWAAGGTA	Sjölin et al. (2005)

Table 2-1 Primers used for PCR of polychaetes.

2.2.5 Sequence Analysis

Overlapping sequences (from forward and reverse primers) were assembled into a consensus sequences and aligned in Geneious 7.1.4 (Drummond et al., 2007). For phylogenetic analysis, additional sequences from the same, or when limited, closely related families were downloaded from GenBank (Benson et al., 2009, Sayers et al., 2009). For some species additional COI sequences were also included from private databases within BOLD (Ratnasingham and Hebert, 2007). COI sequences were aligned using MUSCLE (Edgar, 2004) and 16S using MAFTT (Katoh et al., 2002) both using the default settings and provided as plug-ins in Geneious. At least one outgoup was chosen for each alignment, this species or species' were selected from either a sister taxa or family within the same order. If available the choice of outgroups for some families was also inferred from previously published phylogenies.

Bayesian phylogenetic analyses were conducted for each species investigated using the separate 16S dataset and where possible the separate COI dataset. For each dataset the best nucelotide substitution model was chosen using the jModelTest Akaike and Bayesian information criterion (Posada, 2008). Either GTR+I+G or GTR+G models were chosen as the best-fit model for each alignment dependant on the jModelTest results. All analyses were run three times for 10,000,000 generations using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) with 2,500,000 generations discarded as burn-in. All phylogenetic trees were edited in FigTree 1.4 (Rambaut, 2007) and Adobe Illustrator CS5.1.

Kimura's two-parameter substitution model (K2P) (Kimura, 1980) was calculated in Mesquite for pairwise comparisons of sequence divergence within and between species, based on the number of nucleotide substitutions. Intra- and interclade K2P distances of the target specimens are listed in Appendix 2 by family, comparisons with non BIOPEARL species are presented separately, where discussed, in section 2.3.

Following phylogenetic analysis the specimens within any potential cryptic species were re-examined to rule out misidentification. Secondary examination allowed for the identification of previously overlooked or unrecognised morphological differences. With the exception of the Nepthyidae and Maldanidae specimens that were sent to taxonomic specialists Dr. Ascensão Ravara (University of Aveiro, Portugal) and Dr. Jon Anders Kongsrud (University Museum of Bergen, Norway) respectively, all specimens were re-examined by Lenka Neal. Specimens that were reassigned to other taxa are all named and discussed. Any potential cryptic species found were assigned an MB number after their species names e.g. Hesionidae sp. MB1 and MB2. Potential clades within species were assigned an additional letter e.g. Chaetozone sp. MB1a, MB1b, MB1c. If individuals did not match publically available sequences of their morphospecies, the species name was removed e.g. *Scalibregma inflatum* became *Scalibregma* sp. (MB#). If there was uncertainty about the species identification cf. was used e.g. *Eusphrosinella* cf. *cirratoformis*.

2.3 Results

The phylogenetic and distance analysis revealed evidence for cryptic diversity within at least six of the 12 families investigated. In some cases multiple cryptic species were identified from the one morphospecies targeted. Additional to the cryptic species identified, overlooked morphological differences were also found during secondary examinations in seven of the 12 families. A summary of these results is shown in Table 2-2. More detailed discussions of the barcoding results for each species are presented by family in the following sections. In some taxa the distinction between potential cryptic species and intraspecific variation was ambiguous. In such cases there was a general lack of structure to the phylogenetic groupings, overlapping inter and intra clade K2P distances and an absence of COI data for comparison to 16S. To decide whether these species were potential cryptic species or not the data were compared to previous work within the same family, expert taxonomic opinion and the data currently available. In cases of severe uncertainty a conserved approach was always taken i.e. cryptic species were considered to be absent.

Primary species identification	Number of DNA extractions	Success rate COI	Success rate 16S	Secondary identification	Number of COI sequences	Number of 16S sequences	Evidence of cryptic species
Glycera capitata	63	20.6%	52.4%	*Glycera* sp. (MB1)	4	17	Yes
				Glycera sp. (MB2)	9	15	
Scalibregma inflatum	23	43.5%	82.6%	*Scalibregma* sp. (MB1)	6	14	Yes
				Scalibregma sp. (MB2)	1	1	
				Scalibregma sp. (MB3)	3	4	
Chaetozone setosa	26	7.7%	69.2%	*Chaetozone* sp. (MB1a)	1	7	Species complex
				Chaetozone sp. (MB1b)	-	1	
				Chaetozone sp. (MB1c)	-	2	
				Chaetozone sp. (MB2)	-	3	No
				Chaetozone sp. (MB3)	-	1	No
				Chaetozone sp. (MB4)	1	2	No
				Chaetozone sp. (MB5)	-	2	No
Maldane sarsi	22	0.0%	56.6%	*Asychis amphiglyptus* (MB)	-	4	No
				Eupraxillela cf. *antarctica* (MB)	-	5	No
				Maldane sasrsi antarctica (MB)	-	2	Yes
				Maldanidae sp. (MB)	-	1	No
				Praxiella sp. (MB)	-	1	No
Laonice weddellia	36	25.0%	94.4%	*Laonice weddellia* (MB)	7	23	No
				Laonice anarctica (MB)	2	6	No
				Laonice vietezi (MB)	0	5	No
Aricidea simplex	24	0.0%	95.8%	*Aricidea simplex* (MB)	-	9	No
				Aricidea cf. *belgicae* (MB1)	-	10	Yes
				Aricidea cf. *belgicae* (MB2)	-	2	
				Aricidea cf. *belgicae* (MB3)	-	1	
				Aricidea cf. *pulchra* (MB)	-	1	No
Euphrosinella cirratoformis	20	0.0%	75.0%	*Euphrosinella* cf. *cirratoformis* (MB1)	-	10	Yes
				Euphrosinella cf. *cirratoformis* (MB2)	-	3	
				Euphrosinopsis cf. *antarctica* (MB)	-	2	No
Lumbrineris kerguelensis-cingulata	18	0.0%	88.8%	*Lumbrineris kerguelensis-cingulata* (MB1a)	-	5	Species complex
				Lumbrineris kerguelensis-cingulata (MB1b)	-	1	
				Lumbrineris kerguelensis-cingulata (MB1c)	-	5	

				Lumbrineris kerguelensis-cingulata (MB1d)	-	2	
				Lumbrineris kerguelensis-cingulata (MB1e)	-	1	
				Lumbrineris kerguelensis-cingulata (MB1f)	-	2	
				Aglaophamus trissophyllus (MB1a)	3		
				Aglaophamus cf. *trissophyllus* (MB1b)	2	8	
Aglaophamus trissophyllus				*Aglaophamus* cf. *trissophyllus* (M1c)	1		Yes
Aglaophamus digitatus	36	30.5%	86.1%	*Aglaophamus* sp. (MB2)	3	22	
Aglaophamus foliosus				*Aglaophamus* sp. (MB3)	2		
				Aglaophamus sp. (MB4)	-	1	No
				Harmothoe fuligineum (MB)	5	15	
Harmothoe fuligineum	21	28.6%	76.1%	*Harmothoe* cf. *fuligineum* (MB)	1	1	No
				Macellicephala sp. (MB1)	3	9	
Macellicephala sp. A	14	28.6%	85.7%	*Macellicephala* sp. (MB2)	1	3	Yes
				Macellicephaloides sp. (MB1a)	-	12	
Macellicephaloides sp. B	15	0.0%	80.0%	*Macellicephaloides* sp. (MB1b)	-	2	No
				Hesionidae sp. (MB1)	5		
Hesionidae sp. A	29	44.8%	82.8%	Hesionidae sp. (MB2)	8	24	Yes
Flabelligena sp. A	30	33.3%	66.6%	*Flabelligena* sp. A (MB)	8	12	No
Flabelligena sp. B				*Flabelligena* sp. B (MB)	2	8	No

Table 2-2 The number of DNA extractions for each target species and the barcoding percentage success rate ((number of sequences/number of extractions)*100) for both COI and 16S. Secondary identification/s each target species as labelled in the subsequent phylogenetic trees as decided from both morphological and phylogenetic analysis. The number of COI and 16S sequences obtained for each species, NB merged 16S cells indicate inability to distinguish between species using this gene, and whether there was evidence for cryptic species within each target species.

2.3.1 Glyercidae - *Glycera capitata* Orsted, 1843

Glycera capitata was described from European waters, type locality Norway, but has since been documented globally from littoral to abyssal depths and it is currently accepted that *G. capitata* is a eurybathic cosmopolitan. The occurrence of *Glycera* sp. in the Southern Ocean is well documented since the early 20th century (Hartman, 1964), however whether these individuals are truly *Glycera capitata* has been debated. Early morphological analyses suggested that Antarctic specimens had distinctly longer dorsal chaetal lobes than the described European species (Hartmann-Schröder, 1986) and were later assigned to the described *Glycera kerguelensis* (McIntosh, 1885). However, *G. kerguelensis* became a synonym for *G. capitata*, when its validity as a species was questioned based on morphological characteristics (Boggemann 2002). For this reason the BIOPEARL specimens were identified and named *G. capitata* and not *G. kerguelensis*.

After the morphological identification of the BIOPEARL material, the first phylogenetic investigation of Antarctic *Glycera* using specimens collected from the Weddell Sea was published by Schüller (2011). Prior to sequencing the author considered all sequenced individuals to belong to *G. kerguelensis* based on the morphological differences from *Glycera capitata* described in McIntosh (1885). Sequences were obtained from 38 specimens collected from three localities within the Weddell Sea covering a depth range of 2000 to 5300 m. Bayesian and K2P analysis of COI sequences revealed the presence of three distinct clades. Given the genetic variation within the sample area, Schüller questions whether any of these clades represent the originally described *G. kerguelensis* whose type locality is the shallow waters of Kerguelen Island (McIntosh, 1885).

Bayesian analyses of both 16S and COI sequences obtained from 32 (16S) and 13 (COI) BIOPEARL and JR275 individuals suggest the existence of at least two cryptic *Glycera* species. Comparison with publically available sequences indicate that neither of these potential species are *Glycera capitata*. However, one of these clades did match the *Glycera* sp. clade II described in Schüller (2011) and is labelled '*Glycera* sp. (MB2), Figure 2-3. No reference 16S sequences are available for Schuller's (2011) clades, thus corresponding 16s clades were determined using the BIOPEARL specimens from which both genes were sequenced and are labelled appropriately, Figure 2-4.

The K2P ranges were compared between and within *Glycera* clades. For both genes within clade variation was much lower than between clades, Table 2-3. Note the lower K2P range

(0.15-0.51) observed between *Glycera* sp. clade II and *Glycera* sp. (MB2) is additional confirmation that these BIOPEARL specimens are the same species as Schüller (2011)'s clade II. Where available for comparison, between clade variation was noticeably greater in COI.

***Glycera* clades**	***G. capitata***	***Glycera* sp. Clade I**	***Glycera* sp. clade II**	***Glycera* sp. clade III**	***Glycera* sp. (MB1)**	***Glycera* sp. (MB2)**
G. capitata	0.00 – 0.92				15.19 – 17.07	16.82 – 18.06
***Glycera* sp. Clade I**	18.29 – 18.98	0.00 – 0.00				
***Glycera* sp. Clade II**	24.48 – 25.21	19.07 - 19.30	0.34 - 0.51			
***Glycera* sp. Clade III**	25.52 – 26.54	25.86 - 26.11	25.78 – 26.30	0.17		
***Glycera* sp. (MB1)**	19.17 – 20.57	4.94 - 5.33	17.20 – 19.14	25.10 – 25.60	0.00 – 0.44 / 0.00 – 2.74	8.17 – 12.80
***Glycera* sp. (MB2)**	23.25 – 24.33	19.07 – 19.40	0.15 – 0.51	26.04 – 26.41	17.12 – 18.05	0.00 – 0.30 / 0.00 – 1.60

Table 2-3 Minimum and maximum K2P distances (%) within and between COI (white cells) and 16s (grey cells) sequences within and between *Glycera* clades. * no sequence data available for comparison or only one sequence available, bold indicates only one pairwise comparison.

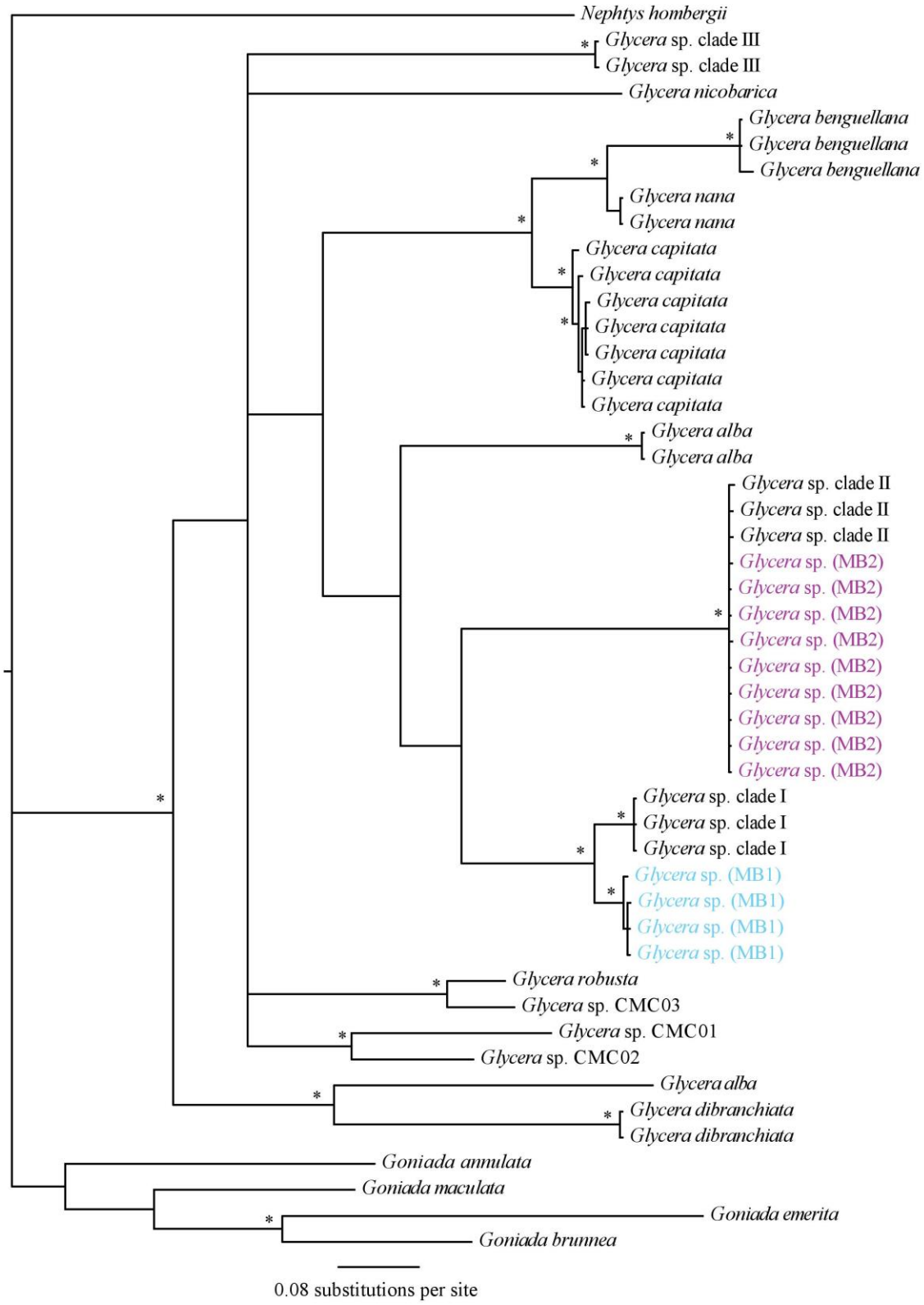

Figure 2-3 Phylogenetic tree of Glyceridae and Goniadidae from Bayesian analysis using COI (mtDNA) only. Including sequences from BIOPEARL and JR275 specimens labelled (MB#). Outgroup: *Nepthys hombergii* (Nepthyidae), * indicates significant node values (>95%) for Bayesian posterior probabilities.

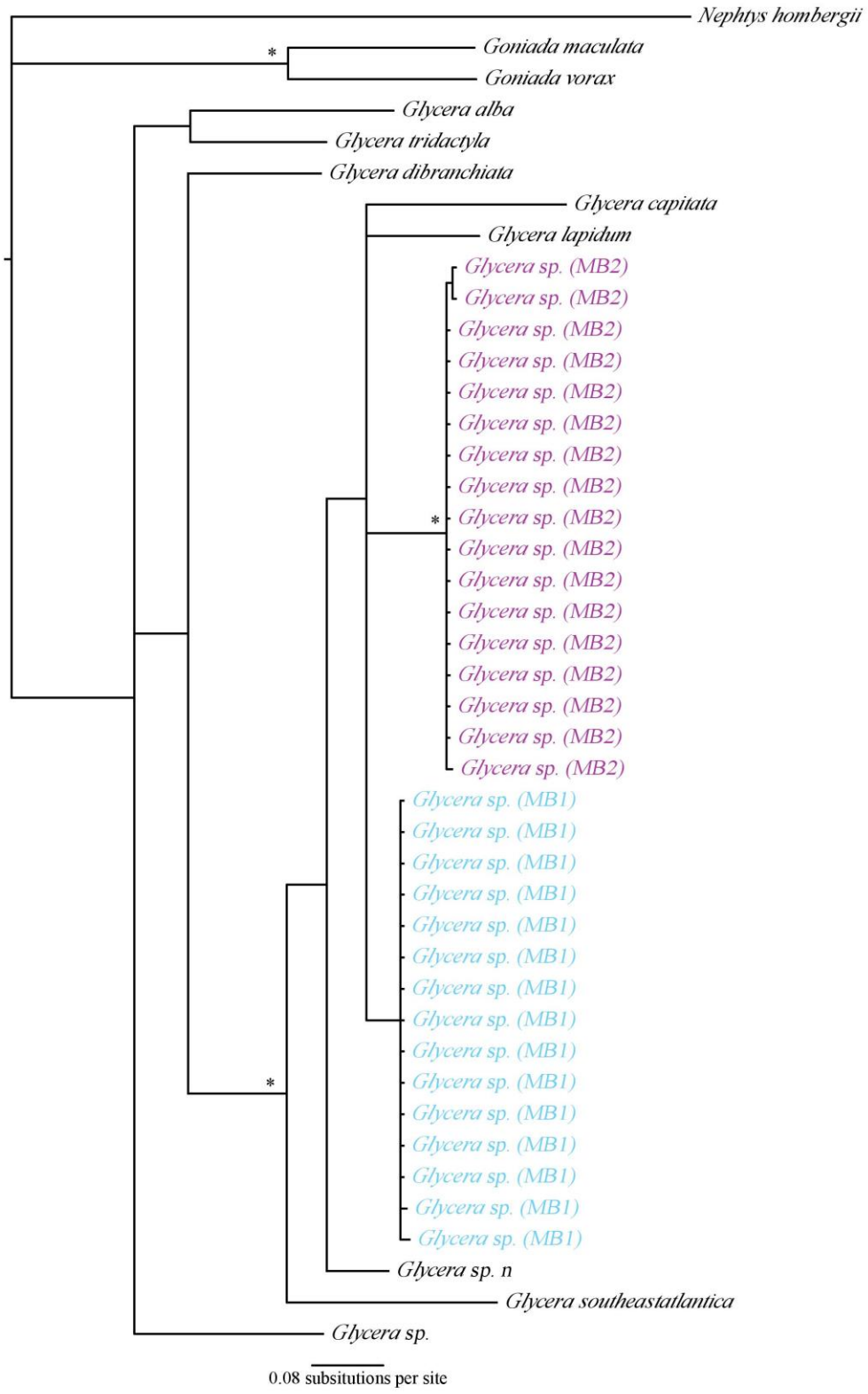

Figure 2-4 Phylogenetic tree of Glyceridae and Goniadidae from Bayesian analysis using 16S (rDNA) only. Including sequences from BIOPEARL and JR275 specimens labelled (MB#). Outgroup: *Nepthys hombergii* (Nepthyidae), * indicates significant node values (>95%) for Bayesian posterior probabilities.

2.3.2 Scalibregmatidae - *Scalibregma inflatum* Rathke, 1843

Scalibregma inflatum, whose type locality lies within Norwegian waters, is considered the most widespread Scalibregmatidae. The apparent lack of moprphological characters of *Scalibregma* has led to the classification of several synonyms of *S. inflatum,* with six listed on the WoRMs database at present. The cosmopolitan nature and diversity of the species has and is still being questioned by on-going morphological and molecular analyses. Mackie (1991) suggested that at least four species occur in European waters from detailed morphological analyses. During COI sequence analyses of Canadian specimens, Carr et al. (2011) revealed two genetically distinct populations of *S. inflatum* including Arctic and Pacific lineages. The relatively high abundance of *S. inflatum* within the BIOPEARL sample provided an opportunity to further investigate the level of diversity within the species and to determine through comparison with publically available sequences from its type locality, whether any of the BIOPEARL specimens are true *S. inflatum.*

Sequences were obtained from 20 individuals morphologically identified as *S. inflatum.* Bayesian analyses of both COI and 16S sequences indicated that at least three clades of *Scalibregma* exist within the Antarctic samples (Figure 2-5, Figure 2-6). Several reference sequences from morphologically identified *S. inflatum* specimens were available. For COI there were three Norwegian specimens (Ann150, TB530, TB549), a Spanish specimen (Tsatk13) and three Canadian species from Carr et al. (2011), (CMC01, CMCO2 and CMCO3). For 16S a German specimen (*Scalibregma inflatum*) (Bleidorn, 2005) and an American specimen (SIO BIC) (Law et al., 2014). The phylogenetic positions of the BIOPEARL clades (Figure 2-5), and the K2P distances between these specimens (Table 2-4) confirm that the BIOPEARL clades are additional cryptic species to those already identified. Given the type locality of *S. inflatum* the Norwegian sequences might be considered 'true' *S. inflatum* and thus the BIOPEARL and JR275 sequences are labelled *Scalibregma* sp. (MB#). As observed with the *Glycera* results, the COI K2P ranges between MB clades were greater than for 16S, however, within clade distances were slightly higher for 16S than COI.

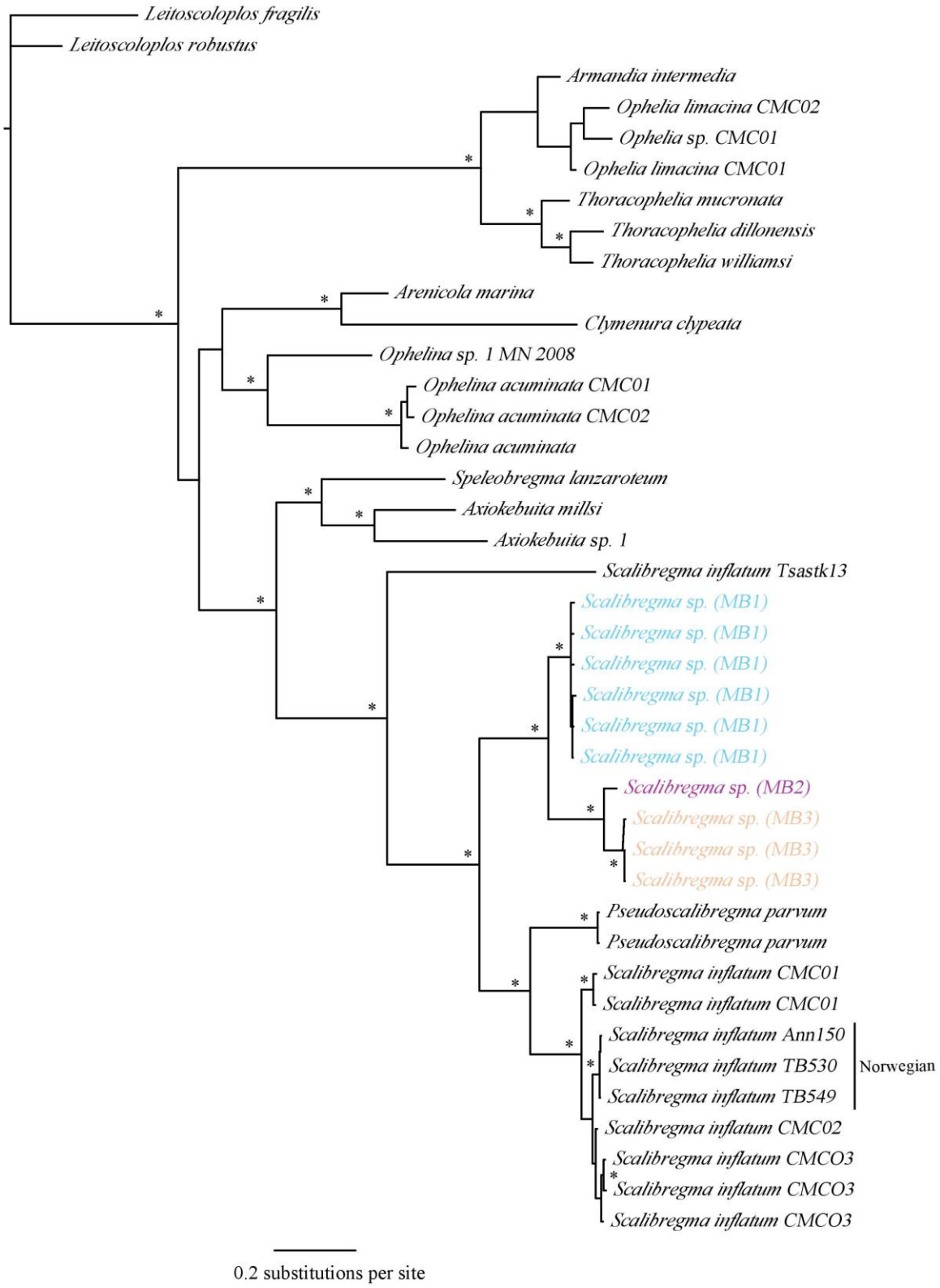

Figure 2-5 Phylogenetic tree of Scalibregmatidae from Bayesian analysis using COI (mtDNA) only. Including sequences from BIOPEARL and JR275 specimens labelled (MB#). Outgroup: *Leitoscoloplus fragilis* and *L. robustus* (Orbiniidae), * indicates significant node values (>95%) for Bayesian posterior probabilities.

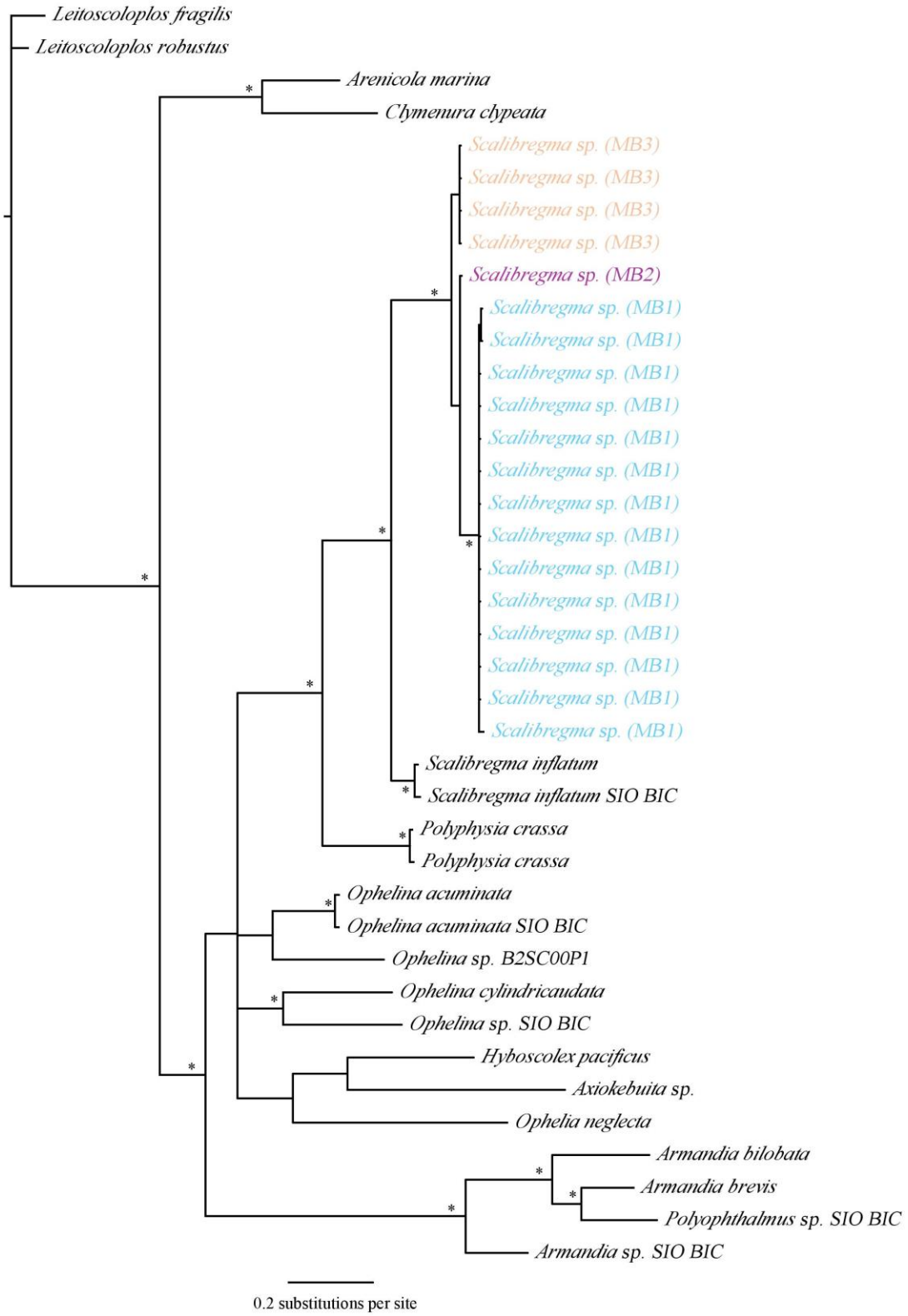

Figure 2-6 Phylogenetic tree of Scalibregmatidae from Bayesian analysis using 16S (rDNA) only. Including sequences from BIOPEARL and JR275 specimens labelled (MB#). Outgroup: *Leitoscoloplus fragilis* and *L.robustus* (Orbiniidae), * indicates significant node values (>95%) for Bayesian posterior probabilities.

Scalibregma clades	*Scalibregma* sp. (MB1)		*Scalibregma* sp. (MB2)		*Scalibregma* sp. (MB3)		*S. inflatum* CMC01	*S. inflatum* CMC01	*S. inflatum* CMC03	*S. inflatum* Norwegian
***Scalibregma* sp. (MB1)**	0.14-1.48	0.00-2.86	4.92-6.66		6.66-9.82					
***Scalibregma* sp. (MB2)**	13.99-14.53		*	*	2.13-2.44					
***Scalibregma* sp. (MB3)**	14.34-15.33		6.51-6.77		0.00-1.46	0.00-1.56				
***S. inflatum* CMC01**	21.07-22.00		23.77-24.41		25.75-24.39		0.66			
***S. inflatum* CMC01**	22.00-22.95		23.45		25.36-25.37		4.55-4.79	*		
***S. inflatum* CMC03**	23.34-25.22		23.25-23.81		23.89-24.45		5.27-6.25	1.56-1.78	0.76-0.99	
***S. inflatum* Norwegian**	22.76-24.07		22.90-22.90		23.76-23.76		5.02-5.51	2.01-2.23	2.87-4.09	0.00-0.15

Table 2-4 Mean pairwise K2P distances (%) within and between COI (white cells) and 16s (grey cells) sequences of *Scalibregma inflatum* clades. * only one sequence available so no intraspecific comparison, NB 16S sequences only available for MB clades. Norwegian specimens include *Scalibregma inflatum* Ann150, TB530, TB549 from Figure 2-5.

2.3.3 Hesionidae - Hesionidae sp. A

The Hesionidae family consists of around 170 species within 28 genera (Ruta et al., 2007). Most species are described from shallow water, and more recently several species have been described from deep-sea habitats including hydrothermal vents, cold seeps and whale falls (e.g. Blake and Hilbig, 1990, Desbruyeres and Toulmond, 1998, Summers et al., 2015). In total, seven Hesionidae morphospecies were identified from the BIOPEARL material, none of which could be assigned to any known species or genus. The most abundant of these Hesionidae species, Hesionidae sp. A, was targeted in this study. Specimens were collected from the Scotia Arc and Amundsen Sea sites and were not found within the JR275 Weddell Sea samples. Bayesian analysis of the 13 COI and 24 16S sequences collected produced different results (Figure 2-7, Figure 2-8). For COI two distinct clades are present with relatively low within clade variation, this is also supported by K2P ranges at 0.14-0.51% and 0.0-1.01% variation within clades I and II respectively, and 10.59-12.41% between the two clades. For 16S all sequenced specimens, including all of the 13 individuals from which COI was obtained, fell within the same clade. Thus for Hesionidae sp. A 16S would not be suitable to differentiate between potential cryptic species.

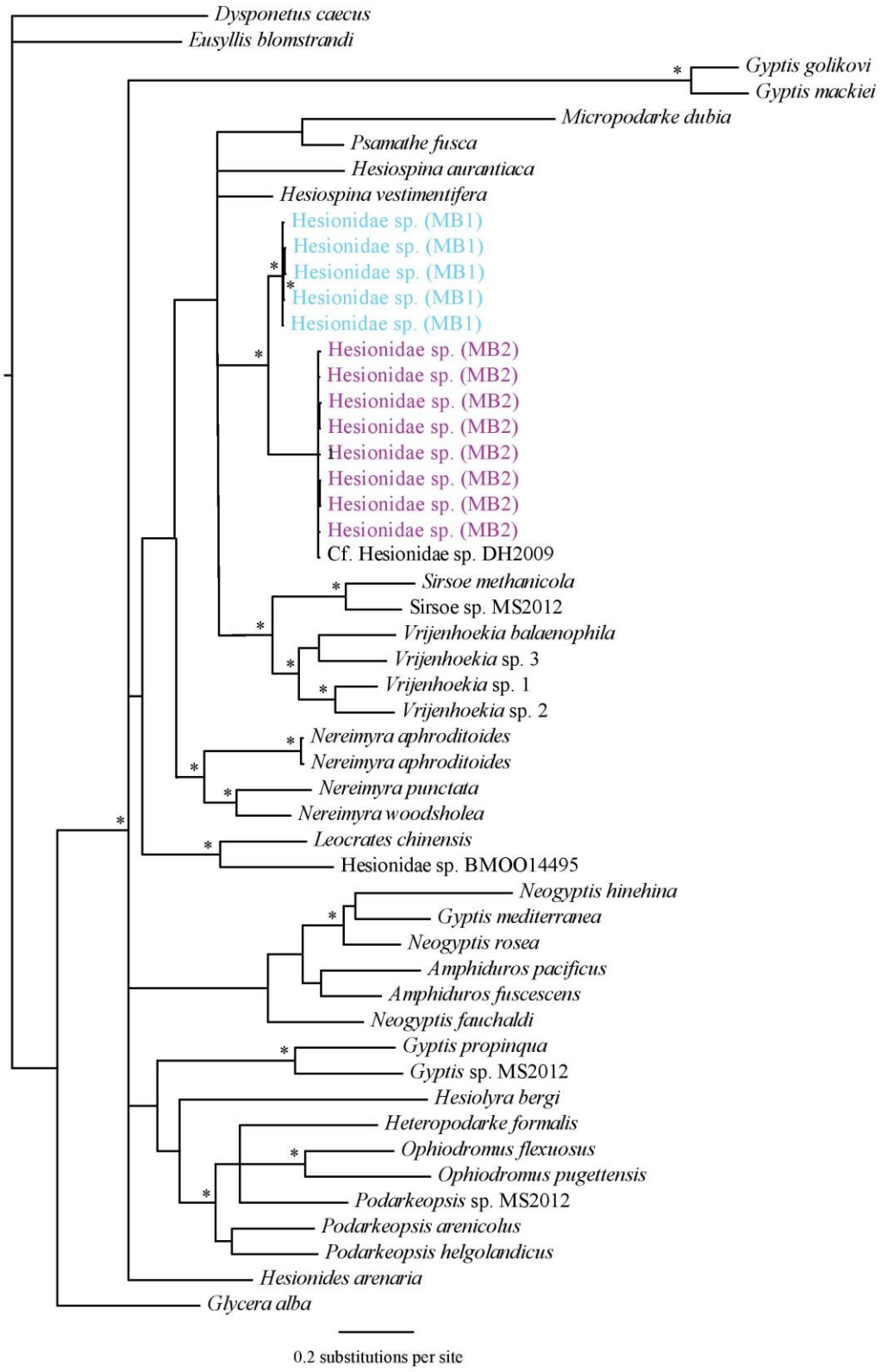

Figure 2-7 Phylogenetic tree of Hesionidae from Bayesian analysis using COI (mtDNA) only. Including sequences from BIOPEARL specimens and labelled (MB#). Outgroups: *Dysponetus caecus* (Chrysopetalidae) and *Eusyllis blomstrandi* (Syllidae), * indicates significant node values (>95%) for Bayesian posterior probabilities.

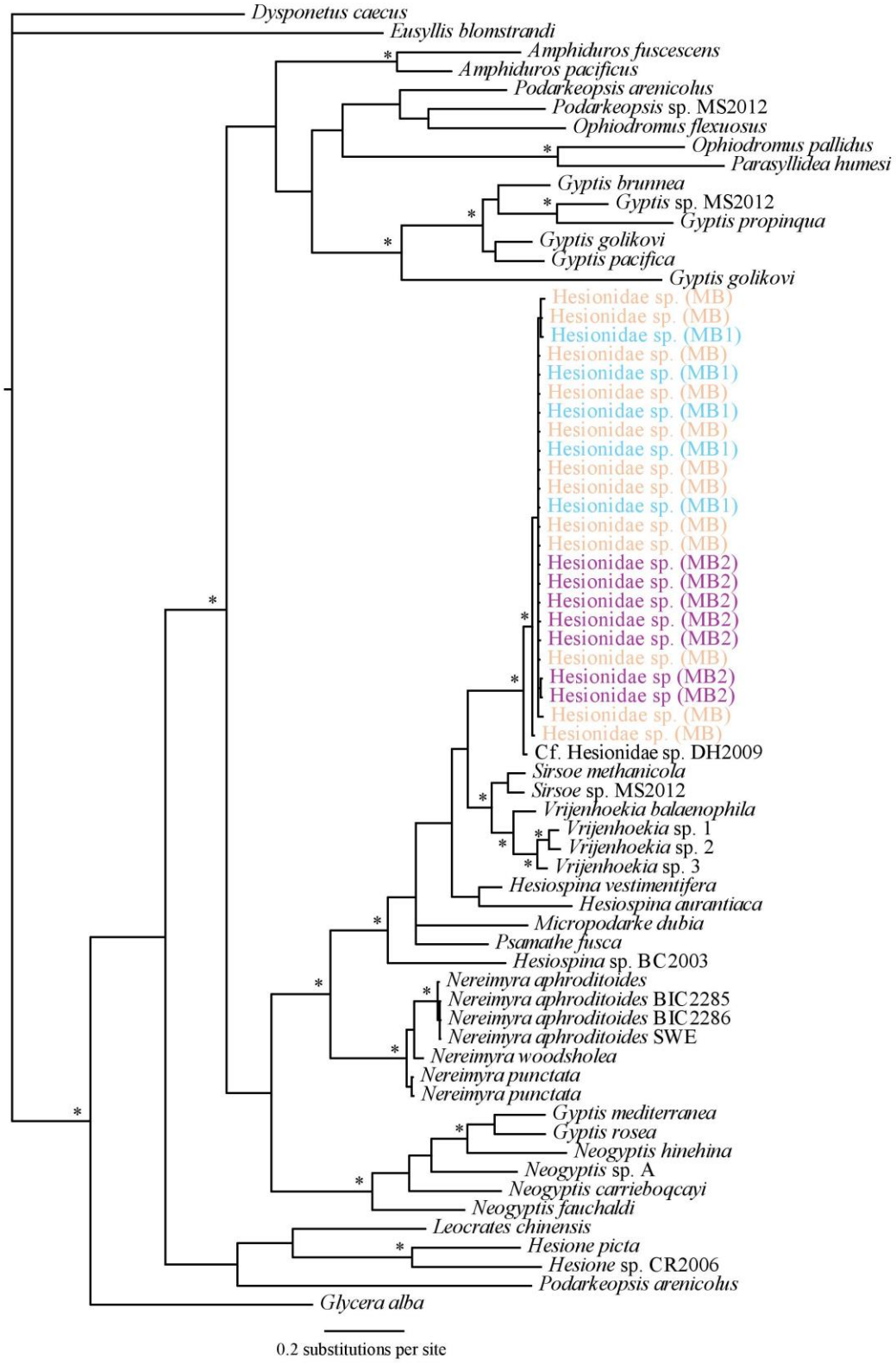

Figure 2-8 Phylogenetic tree of Hesionidae from Bayesian analysis using 16S (rDNA) only. Including sequences from BIOPEARL specimens and labelled (MB) or (MB#). Outgroups: *Dysponetus caecus* (Chrysopetalidae) and *Eusyllis blomstrandi* (Syllidae), * indicates significant node values (>95%) for Bayesian posterior probabilities.

Whether this is a universal or frequent trait amongst cryptic Hesionidae is unknown as the number of investigations into the cryptic diversity within this family is limited. There are several phylogenetic studies that include COI and 16S as well as nuclear genes (18S and/or 28S). However, these studies used combined analysis to understand phylogenies rather than delineate species (Ruta et al., 2007, Pleijel et al., 2008, Pleijel et al., 2012). Summers et al. (2015) described three cryptic species of *Vrijenhoekia* from whale falls, included in Figure 2-7 and Figure 2-8 as *Vrijenhoekia* sp. 1, 2 and 3. As observed in Hesionidae spp., the 16S between-clade distances for the *Vrijenhoekia* spp were up to 4 times lower than COI.

Hesionidae sp. (MB2), Figure 2-7, matched a larval sequence from the Ross Sea, Cf. Hesionidae sp. DH2009 (Heimeier et al., 2010). Given the morphological ontogenetic changes that occur in polychaetes, the larval forms of many polychaete species are unknown, thus DNA barcoding has allowed the pairing of this larval specimen to an adult form. During re-examination of the BIOPEARL specimens and comparison with photos in Brueggeman (1998), morphological similarities between Hesionidae spp. and *Psamathe fauveli* (Averincev, 1962) were noted. The original description of *P. fauveli* is not available for comparison but we know that its distribution ranges from the Antarctic Peninsula to the Ross Sea at depths up to 1040 m (Brueggeman, 1998). Given the visual similarities and overlapping distribution records it is not unreasonable to suggest that BIOPEARL Hesionidae could belong to the morphospecies *P. fauveli*. However genetic evidence may suggest otherwise. The BIOPERAL Hesionidae spp. sequences did fall within the Psamathini clade described in Pleijel et al. (2008) and Ruta et al. (2007), which contains the following Hesionidae genera; *Hesiospina, Micropodarke, Psamathe, Sirsoe, Vrijenhoekia, Syllidia, Bonuania* and *Nereimyra* (WoRMs, 2015). However, the only *Psamathe* avaliable, *Psamathe fusca,* did not cluster with the Hesionidae spp within either the COI or 16S tree (Figure 2-7, Figure 2-8). The unresolved cladistics and limited number of sequences does not assist in the genus identification of Hesionidae sp. A.

2.3.4 Euphrosinidae - *Euphrosinella cirratoformis* (Averincev, 1972)

The Euphrosinidae family consists of approximately 70 species mostly described from temperate and polar regions (Borda and Kudenov, 2014). The most abundant of the five Euphrosinidae species identified within the BIOPEARL material was *Euphrosinella cirratoformis*. Bayesian analysis of the 15 16S sequences resulted in three genetically distinct clades (Figure 2-9). Secondary morphological examination revealed one of these clades was misidentified and individuals were assigned to *Euphrosinopsis antarctica* (Hartmann-Schröder

& Rosenfeldt 1992). The other two clades did match the *Euphrosinella cirratoformis* description and are presented here as two potential cryptic species. The K2P pairwise distances also support this result ranging from 0-2.42% and 0.27-0.28% variation within species MB1 and MB2 respectively, and 5.50-7.53 between them. As no reference sequence is publically available for this species it is not possible to suggest either clade should be assigned as *Euphrosinella cirratoformis* and not the other, hence both are referred to as *Euphrosinella* cf. *cirratoformis* (MB#; Figure 2-9). The classification of five Antarctic Euphrosinidae species has recently been re-evaluated in Borda and Kudenov (2014). Within this review they highlight the need for new morphological characteristics within species descriptions. If new characters are included in future descriptions the cryptic diversity observed here might be explained by morphology. However based on the current species descriptions available, we can only suggest that this is a true case of cryptic diversity.

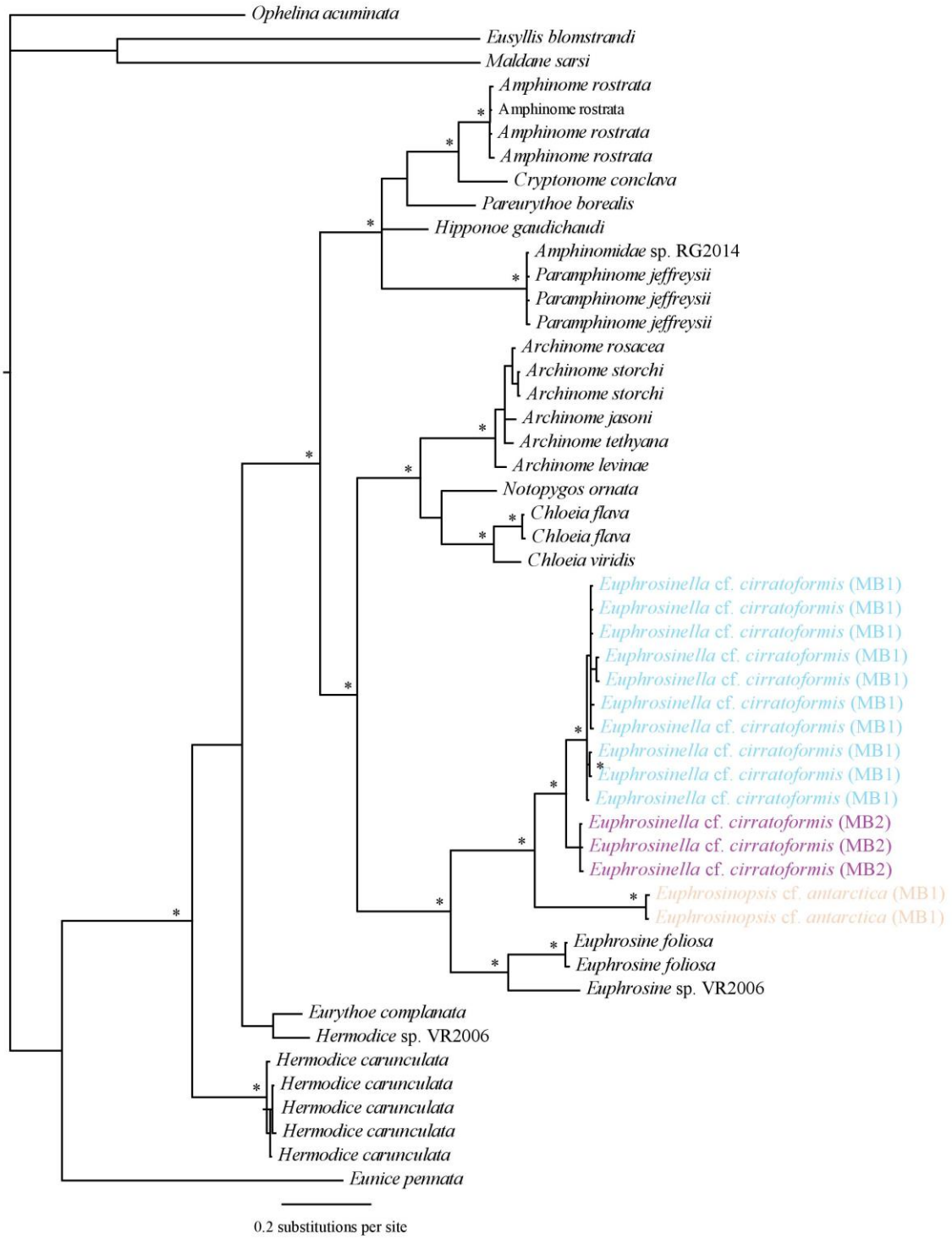

Figure 2-9 Phylogenetic tree of Euphrosinidae and Amphionomidae from Bayesian analysis using 16S (rDNA) only. Including sequences from BIOPEARL specimens and labelled (MB#). Outgroups: *Ophelina acuminata* (Ophelidae), *Eusyllis blomstrandi* (Syllidae), *Maldane sarsi* (Maldanidae) and *Eunice pennata* (Eunicidae), * indicates significant node values (>95%) for Bayesian posterior probabilities.

2.3.5 Paraonidae - *Aricidea simplex* Day, 1963

Paraonidae species commonly represent a dominant fraction of deep-sea polychaetes globally (Blake, 1996). There are currently about 90 described species, however it is thought that actual diversity of this family is considerably higher (Rouse and Pleijel, 2001, López, 2008). *Aricidea simplex* has been recorded from several locations worldwide including the Southern Ocean (IOBIS, 2015). No reference sequences for this species were publically available, thus the identification of *Aricidea simplex* is based purely on morphology. As only one COI sequence was obtained from the BIOPEARL and JR275 material, these results are based on the 16S sequences collected from 25 individuals.

Preliminary phylogenetic analyses suggested that cryptic species were present with two distinct clades emerging from the sequenced specimens. However, re-examination of material and comparison to the new description of another *Aricidea* species, *Aricidea belgicae* (Fauvel, 1936) showed that this was an artefact of misidentification. Out of the 25 *Aricidea* specimens sequenced, 13 individuals were assigned to the morphospecies *A. belgicae* and all bar one of the remaining specimens are still believed to be true *A. simplex*. The main morphological difference between the two is the lack of median antennae in *A. belgicae* (López, 2008). Given the delicate nature of soft bodied polychaetes and damage from sampling and sorting, it was considered that the median antenna had detached and individuals were thus identified as *A. simplex*. During re-examination one specimen with median antennae matched the species description for *Aricidea pulchra* Strelzov, 1973. Given the similar morphology between *A. simplex* and *A. pulchra* it could have been predicted that these two species are more closely related than *A. belgicae* and *A. pulchra*. However, the position of this specimen in the phylogenetic tree, *Aricidea* cf. *pulchra* (MB; Figure 2-10), shows the opposite result. This could suggest that the presence or absence of median antennae is not a phylogenetically important character, although further morphological and sequence analysis incorporating a greater range of taxa from the *Aricidea* genus and *A. pulchra* specimens would be needed to confirm this.

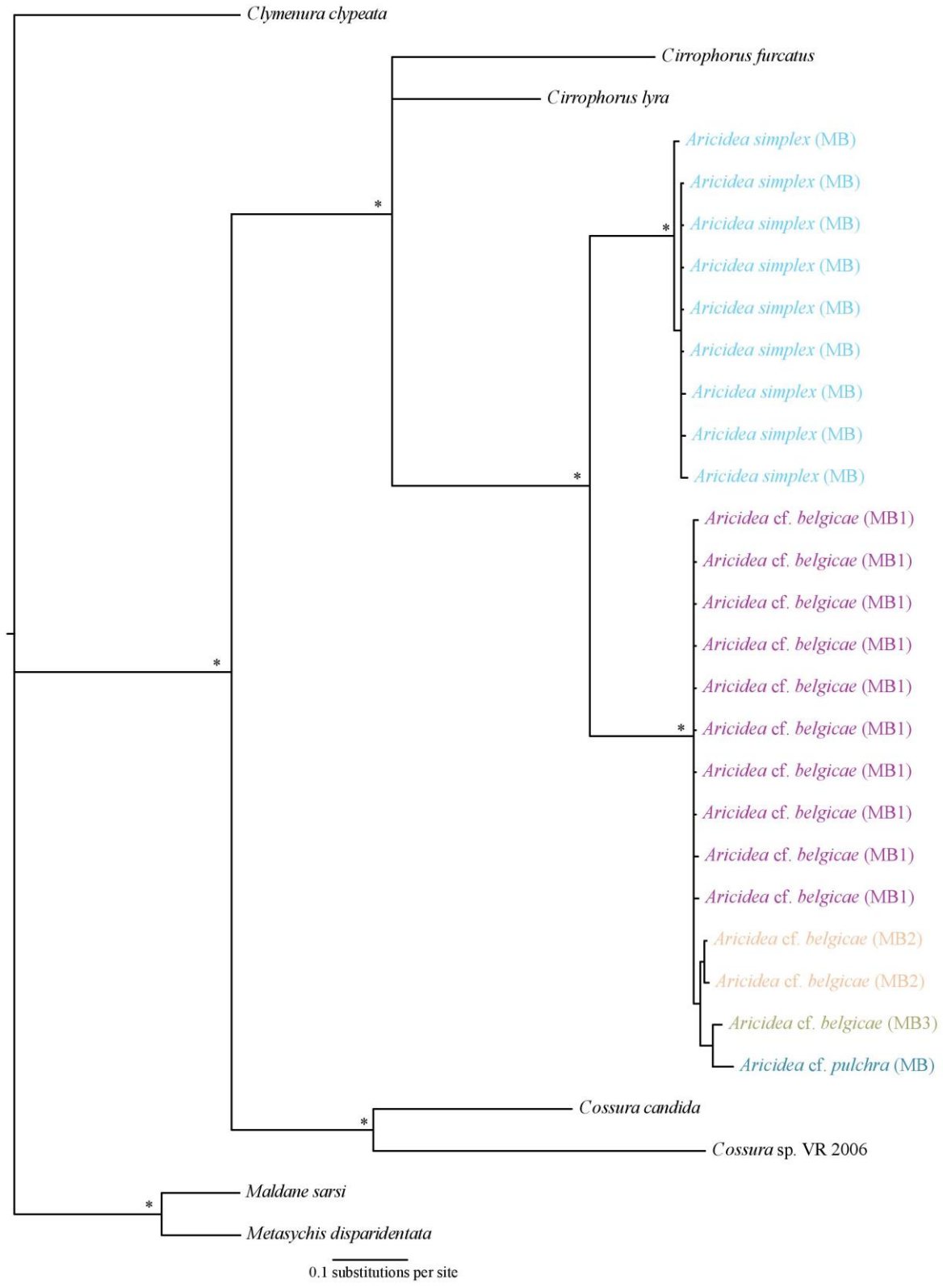

Figure 2-10 Phylogenetic tree of Paraonidae from Bayesian analysis using 16S (rDNA) only. Including sequences from BIOPEARL and JR275 specimens and labelled (MB#). Outgroups: *Clymenura clypeata, Maldane sarsi* and *Metasychis disparidentata* (all Maldanidae), * indicates significant node values (>95%) for Bayesian posterior probabilities.

Within *Aricidea simplex* there was no evidence for cryptic species. For *A. belgicae* however the clade formation and K2P distances indicate cryptic diversity. Sequence variation between *A. belgicae* MB2 and MB3 was ten times greater than the MB2 within clade variation (Table 2-5). However, some clade comparisons including *A. belgicae* MB1 and MB2 were only marginally greater than their within clade variation. In comparison to previous species investigated, for example *Glycera* sp. and *Scalibregma* sp. there is not an obvious 'barcoding gap' which could suggest a series of populations or a species complex. During secondary morphological examinations some differences in the number of branchae on the *A. belgicae* specimens were noted. Slight polymorphism with regard to variable numbers of branchae has been noted previously within the *Aricidea* genus (López, 2008). As discussed in Chapter 3, the *A. belgicae* clades were geographically restricted from one another. With this additional consideration the three clades are regarded as potential cryptic species.

***Aricidea* clades**	***Aricidea* cf. *belgicae* (MB1)**	***Aricidea* cf. *belgicae* (MB2)**	***Aricidea* cf. *belgicae* (MB3)**	***Aricidea* cf. *pulchra* (MB)**
***Aricidea* cf. *belgicae* (MB1)**	0.00-0.61%			
***Aricidea* cf. *belgicae* (MB2)**	0.82-1.54%	0.27		
***Aricidea* cf. *belgicae* (MB3)**	2.46-3.07%	2.45-2.78	*	
***Aricidea* cf. *pulchra* (MB)**	4.18-5.05%	3.89-4.18	3.05	*

Table 2-5 Minimum and maximum K2P pairwise distances (%) within and between *Aricidea belgicae*a (MB#) and *Aricidea* cf. *pulchra* (MB) clades in Figure 2-10 using 16S data. Single values indicate only one pairwise comparison * for clades represented by only one specimens so no within clade comparisons.

2.3.6 Spionidae - *Laonice weddellia* Hartman, 1978

Laonice weddellia is a well-documented presumed circum-Antarctic polychaete, thus it is perhaps not surprising that this morphospecies was identified in material from all BIOPEARL sites and in the additional Weddell Sea material. Assigning species to the genus *Laonice* based on morphological characteristics is not too problematic, however identifying species within *Laonice* has been more troublesome (Greaves et al., 2011, López, 2011). Prior to the re-examination of BIOPEARL voucher specimens and comparison with the most recent *Laonice* species descriptions, Bayesian analysis revealed two distinct clades for COI and three for 16S represented by multiple individuals with some outliers, indicating the potential existence of several cryptic species. However, by comparison with recent *Laonice* species descriptions, it was apparent that defining morphological characters had been overlooked, for example, the segment in which interparapodial pouches appear.

Following secondary morphological analysis the most sequenced clade represented by 23 individuals for 16S and 8 individuals for COI (Figure 2-11, Figure 2-12), is believed to be the true *Laonice weddellia*. The second most sequenced *Laonice* species from the BIOPEARL material most closely matched the *Laonice antarctica* (Hartman, 1973) description. The final clade containing multiple individuals, for which only 16S sequences were obtained, resembled the more recently described *Laonice vietezi* López, 2011. These clades are referred to as *Laonice* cf. *Antarctica* (MB) and *Laonice* cf. *vietezi* (MB) due to the quality of the preserved material and lack of reference sequences for comparison from type localities. There was no evidence of cryptic species within any of the *Laonice* specimens. K2P pairwise distances were all <2.0% for both genes within species and at least ten times greater between species (Table 2-6).

Given the phylogenetic positions of *Laonice* sp. MB1 (Figure 2-11) and *Laonice* sp. MB2 (Figure 2-11, Figure 2-12), they probably belong to separate *Laonice* species. Some specimens were incomplete and damaged and therefore could not be assigned to any currently named species. Genetic and morphological analysis of the *Laonice* species illustrate that when identifying a large numbers of specimens, many incomplete, it is easy to assign them to the most abundantly occurring taxa and overlook very subtle morphological characters.

***Laonice* clades**	***Laonice* cf. *antarctica* (MB)**		***Laonice weddellia* (MB)**		***Laonice* cf. *vietezi* (MB)**	
***Laonice* cf. *antarctica* (MB)**	0.17-1.04	0.00-1.70	14.84-17.28		16.10-17.92	
***Laonice weddellia* (MB)**	22.55-24.15		0.00-1.56	0.00-1.44	16.74-18.94	
***Laonice* cf. *vietezi* (MB)**	*		*		*	0.00-0.28

Table 2-6 Mean pairwise K2P distances (%) within and between COI (white cells) and 16s (grey cells) sequences of *Laonice* MB clades. * no COI sequences available for *Laonice* cf. *vietezi.*

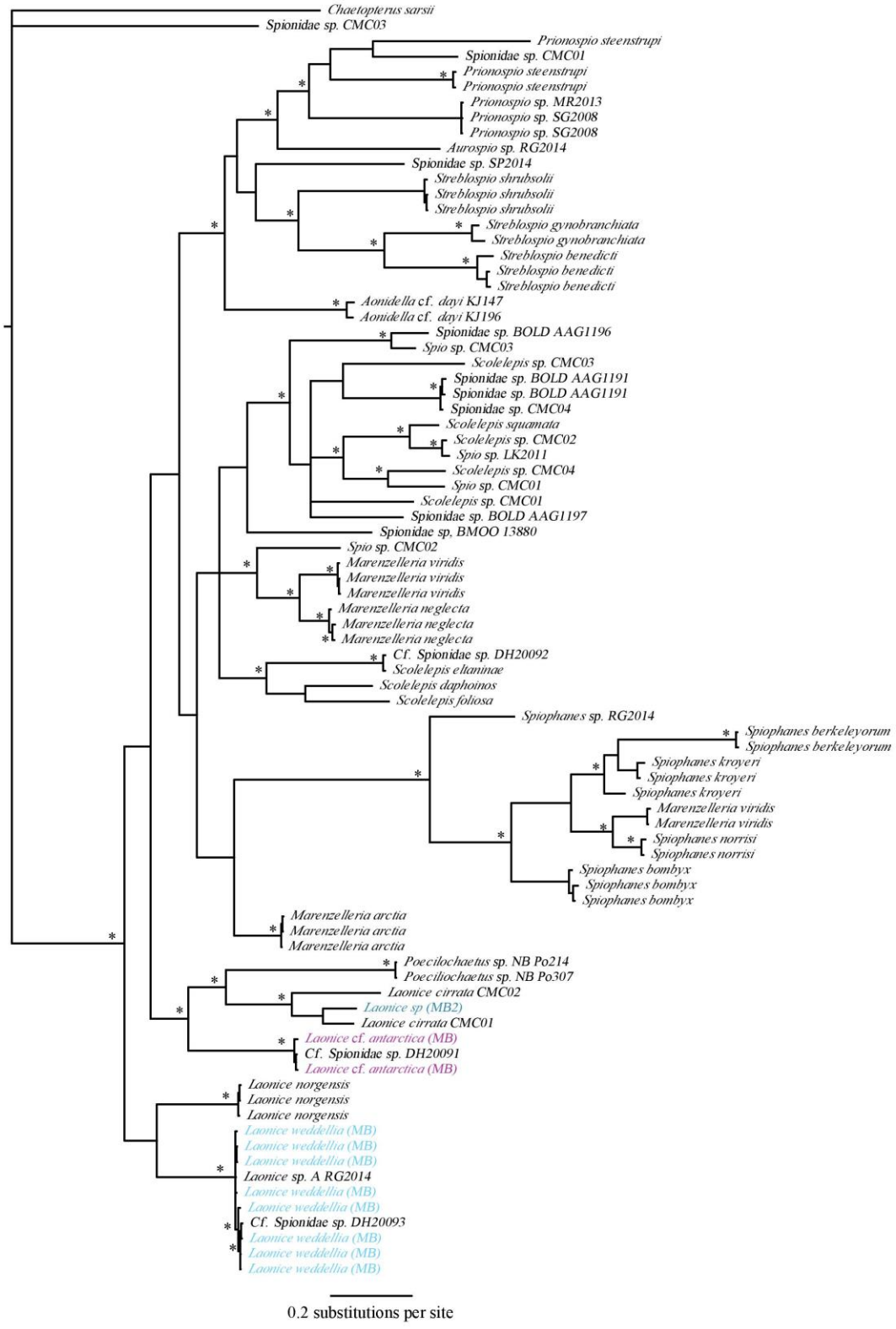

Figure 2-11 Phylogenetic tree of Spionidae from Bayesian analysis using COI (mtDNA) only. Including sequences from BIOPEARL and JR275 specimens and labelled (MB) or (MB#). Outgroup: *Chaetopterus sarsi* (Chaetopteridae), * indicates significant node values (>95%) for Bayesian posterior probabilities.

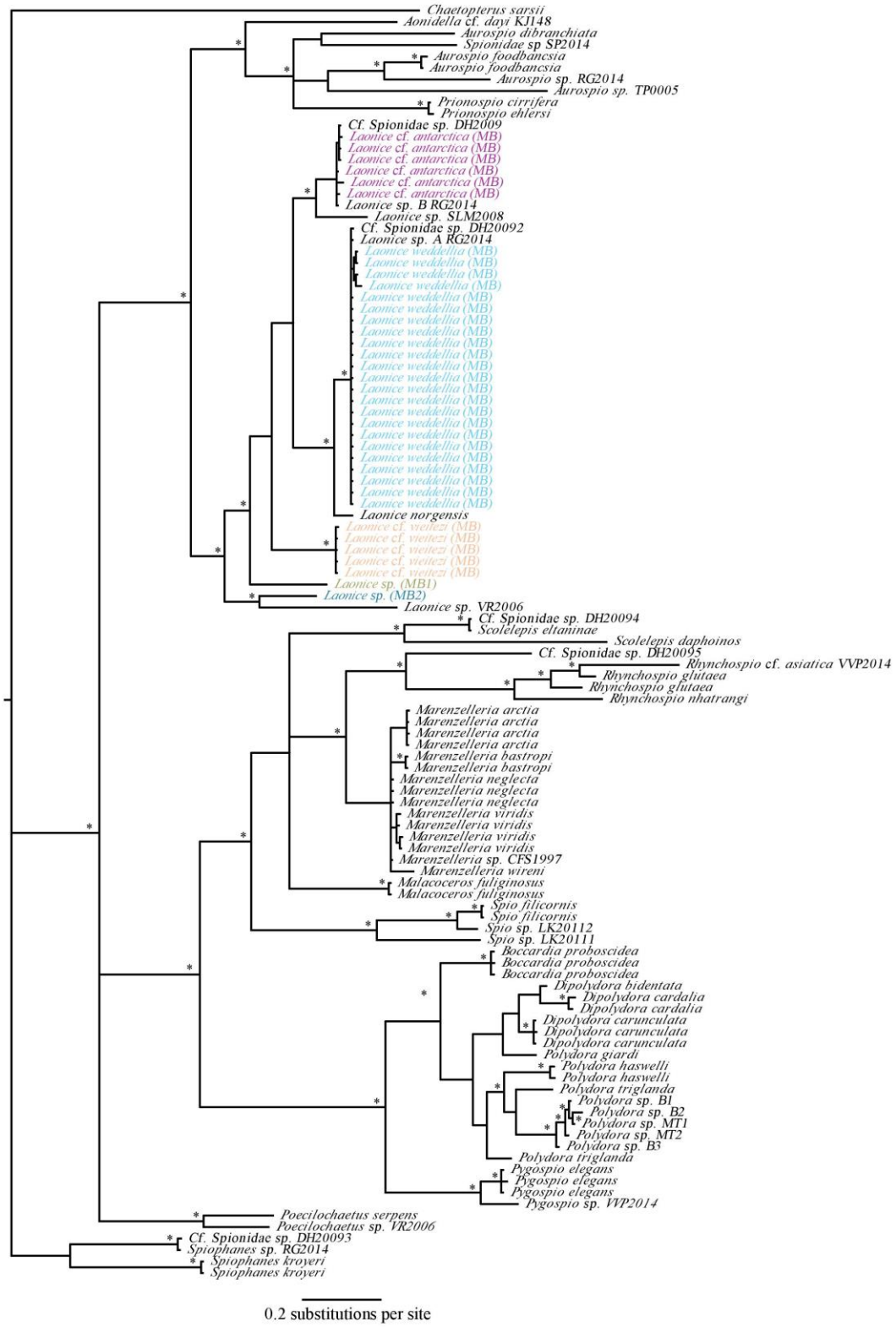

Figure 2-12 Phylogenetic tree of Spionidae from Bayesian analysis using 16S (rDNA) only. Including sequences from BIOPEARL and JR275 specimens and labelled (MB) or (MB#). Outgroup: *Chaetopterus sarsi* (Chaetopteridae), * indicates significant node values (>95%) for Bayesian posterior probabilities.

2.3.7 Acrocirridae – Spp. of *Flabelligena* Gillet, 2001

Acrocirridae is a relatively small polychaete family with nine described genera. The genus of interest in this study, *Flabelligena*, contains only six formally-described species (Aguirrezabalaga and Ceberio, 2006). As with many polychaete families this is not a true reflection of the diversity of this genus. Limited taxonomic and molecular analysis has been carried out on this genus and only a single COI and 16S sequence are available on GenBank, neither of which are assigned to known species. The abundance and diversity of Acrocirridae has been noted in several deep-sea sampling programmes (personal observation). Within the BIOPEARL II samples two *Flabelligena* morphospecies (sp. A and sp. B) were found, neither of which could be assigned to any described species. In total 20 16S and 8 COI sequences were collected from these two morphospecies. Taxa from the sister family to Acrocirridae, Flabelligeridae, were included in the phylogenetic analysis due to the limited number of Acrocirridae sequences publically available.

There did not appear to be any cryptic species in either *Flabelligena* species according to the COI and 16S Bayesian analysis (Figure 2-13, Figure 2-14). This was confirmed by the K2P distance analysis with <2.0% variation in COI and <1.5% variation in 16S for all pairwise comparison within each species. However, in the first analysis both species clades contained a mixture of specimens assigned to species A and B. This could have indicated multiple cases of polymorphic species. However the re-examination of specimen morphology uncovered that this was not a case of polymorphism in the *Flabelligena*, instead being a result of identification and/or labelling errors. Specimens were reassigned to the correct species as indicated by their DNA barcodes. However in one case the most noticeable morphological difference between the two species, the presence (*Flabelligena* sp. B) or absence (*Flabelligena* sp. A) of eyes was not enough to identify the specimen. Instead minor species-specific characters combined with the DNA results were needed to resolve the individual's identity. As to why the eyes on a single specimen were absent remains uncertain. Morphological characteristics can be damaged or lost when material is fixed in ethanol, more so than if it were in formaldehyde (Rouse and Pleijel, 2001). The existence of a polymorphic species should not be completely discounted however, as this trait was only noted in a single specimen, it is impossible to suggest that it would be a frequent occurrence rather than a one off.

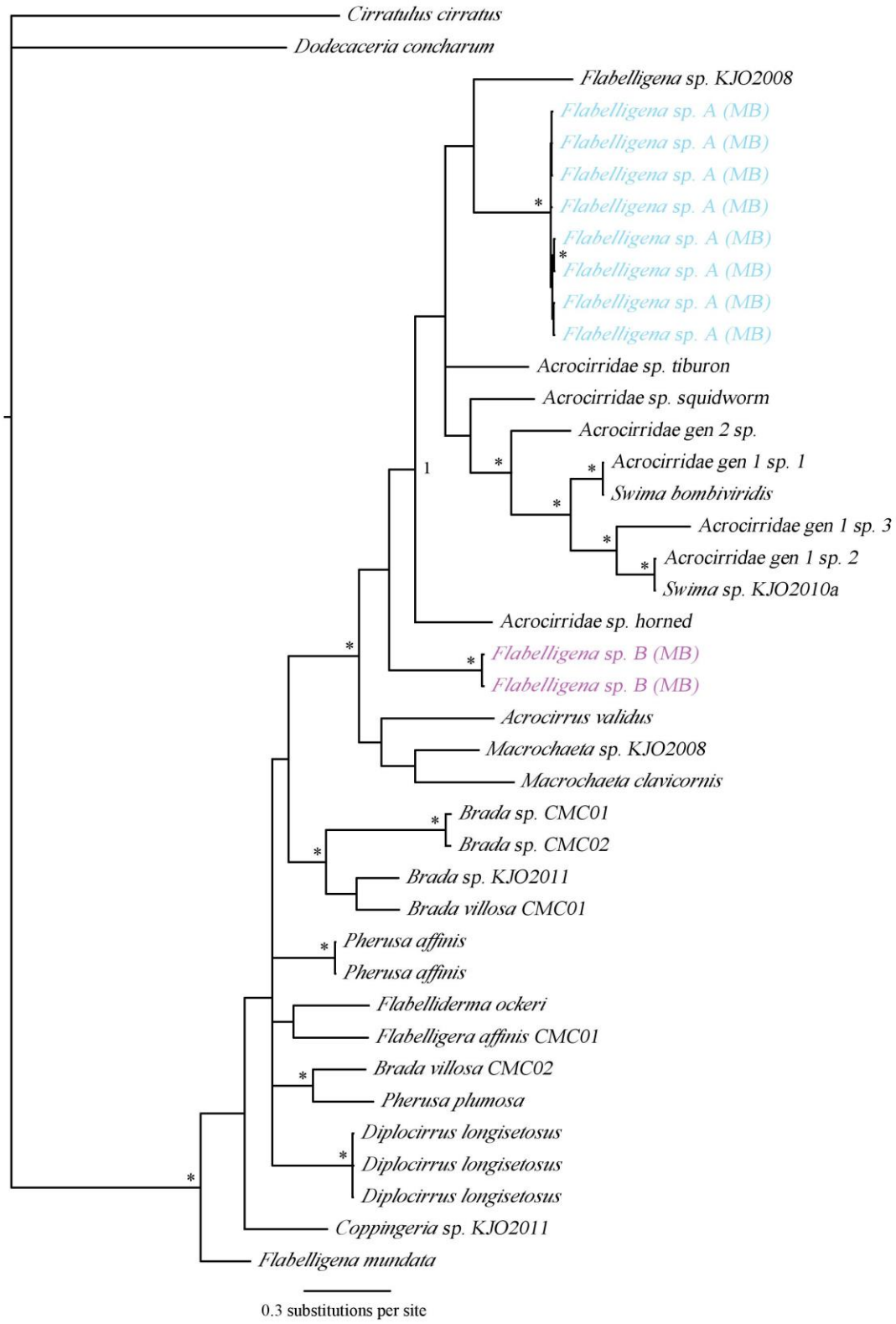

Figure 2-13 Phylogenetic tree of Acrocirridae and Flabelligeridae from Bayesian analysis using COI (mtDNA) only. Including sequences from BIOPEARL specimens labelled (MB#). Outgroups: *Cirratulus cirratus* and *Doecaceria concharum* (both Cirratulidae), * indicates significant node values (>95%) for Bayesian posterior probabilities.

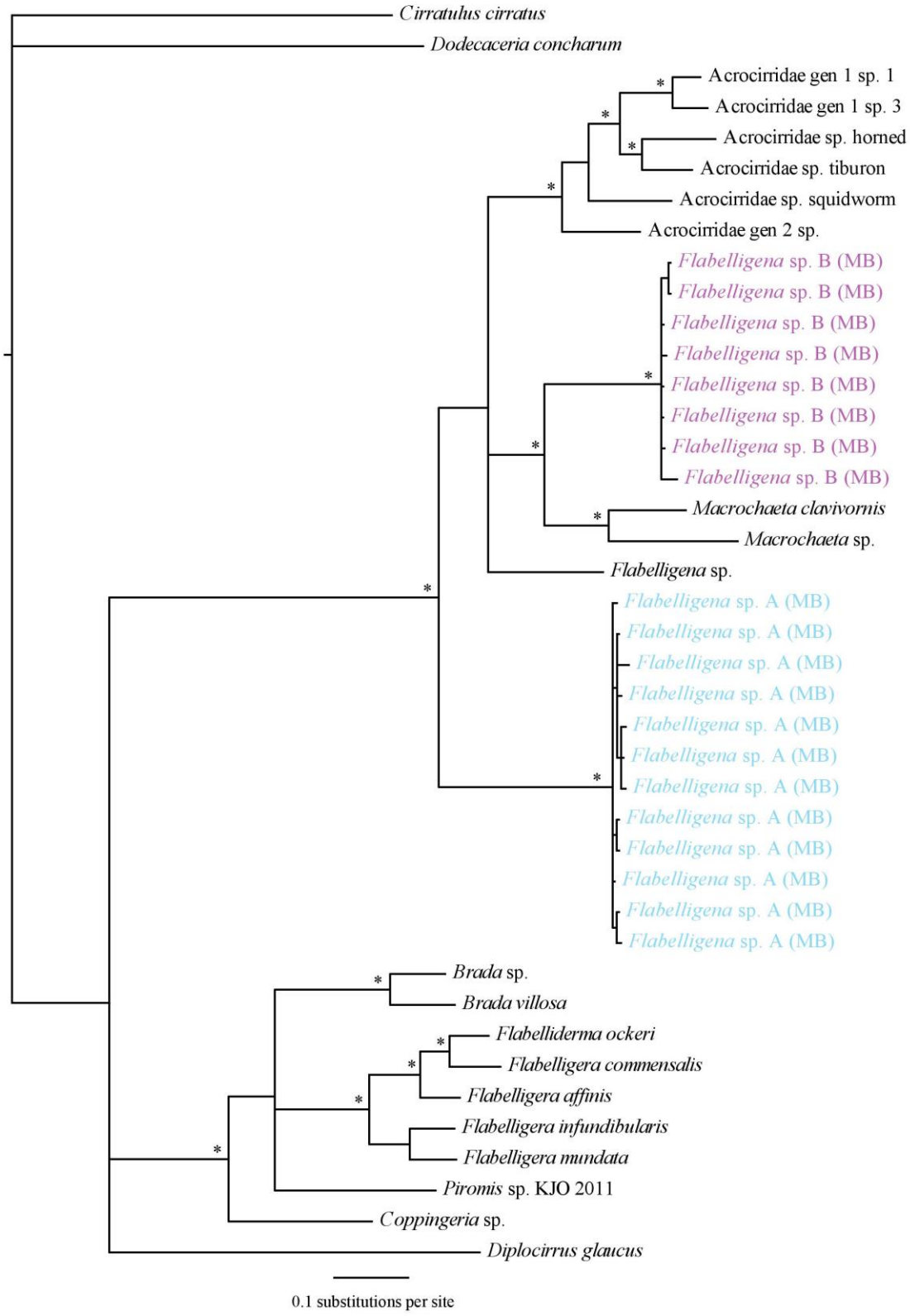

Figure 2-14 Phylogenetic tree of Acrocirridae and Flabelligeridae from Bayesian analysis using 16S (rDNA) only. Including sequences from BIOPEARL specimens labelled (MB#). Outgroups: *Cirratulus cirratus* and *Dodecaceria concharum* (both Cirratulidae), * indicates significant node values (>95%) for Bayesian posterior probabilities

2.3.8 Lumbrineridae – *Lumbrineris kerguelensis-cingulata*

Lumbrineridae are commonly found at continental shelf depths in muddy and sandy sediments. The family have a simplified body form compared to other polychaetes and their reduced morphological characteristics previously led to the assignment of most species into only a few genera (Carrera-Parra, 2001). One of the biggest genera within the family, *Lumbrineris*, was last revised by Carrera-Parra (2006), who questioned the classification of 21 *Lumbrineris* species. Out of the five Lumbrineridae morphospecies within the BIOPEARL, the most abundant was *Lumbrineris kerguelensis-cingulata*. As discussed in Orensanz (1990) this group includes a number of different morphological forms, which could be different species or subspecies within it. The group is distinguishable from other *Lumbrineris* based on several morphological features, however the different forms are extremely difficult to tell apart especially if individuals are juveniles or incomplete. Orensanz (1990) believed that the sub Antarctic material examined contained at least two known species; *L. cingulata* Ehlers, 1897 and *L. kerguelensis* Grube, 1978 as well as three other forms. The sequences obtained from the BIOPEARL *Lumbrineris kerguelensis-cingulata* specimens reveal that this group is genetically as well as morphologically diverse.

Several COI reference sequences are available for *L. cingulata* but did not match either of the two sequences obtained for this gene from BIOPEARL *Lumbrineris kerguelensis-cingulata* specimens. Unfortunately no 16S *L. cingulata* or *L. kerguelensis* sequences were publicly available and thus could not be used for species identification. Bayesian analysis of the 16 16S sequences obtained revealed relatively high levels of genetic variability, resulting in six different clades containing 1 to 5 individuals (Figure 2-15).

The two sequenced BIOPEARL individuals within *Lumbrineris kerguelensis-cingulata* (MB1f**)** matched a larval sequence from the Ross Sea (Heimeier et al., 2010). Mature *Lumbrineris* sp. have previously been documented in this area in Cantone et al. (2000), where five species were identified from morphological analysis, two of which could not be assigned to named species. Although this does not help decipher the true identity of the larva, nor the MB specimen, it highlights that the diversity of *Lumbrineris* species around Antarctica may be hugely underestimated. Secondary morphological analysis did not reveal any new information. Most of the specimens were juvenile and incomplete and the available morphology could not identify them beyond *Lumbrineris kerguelensis-cinuglata*. A single larger specimen matched the *Lumbrineris cingulata* redescription in Carrera-Parra (2006), however given the lack of

genetic comparison to known *Lumbrineris cinuglata* specimens and the degree of genetic variation presented, it is labelled *Lumbrineris kerguelensis-cinuglata* (MB1e). Given the inability to precisely examine these specimens morphologically, variable K2P distances between clades (Table 2-7) and the already discussed variability within this group, it is not possible to confidently distinguish between potential cryptic species.

***Lumbrineris kerguelensis-cingulata* MB clades**	**MB1a**	**MB1b**	**MB1c**	**MB1d**	**MB1e**	**MB1f**
***L. kerguelensis-cingulata* (MB1a)**	0.00-1.42					
***L. kerguelensis-cingulata* (MB1b)**	9.45-11.61	*				
***L. kerguelensis-cingulata* (MB1c)**	9.22-11.74	5.62-7.63	0.00-0.36			
***L. kerguelensis-cingulata* (MB1d)**	9.35-12.97	6.24-6.70	4.38-5.88	1.46		
***L. kerguelensis-cingulata* (MB1e)**	10.40-12.50	6.11	4.57-6.10	4.86-4.88	*	
***L. kerguelensis-cingulata* (MB1f)**	10.35-15.54	5.77-5.56	3.91-5.54	4.21-5.22	3.91-5.21	0.00-0.36

Table 2-7 Minimum and maximum K2P pairwise distances (%) within and between the *Lumbrineris kerguelensis- cingulata* MB clades based on 16S. * only one sequence available so no intraspecific comparison, bold indicates only one pairwise comparison available. Column headings refer the same MB# clade as listed in the row labels.

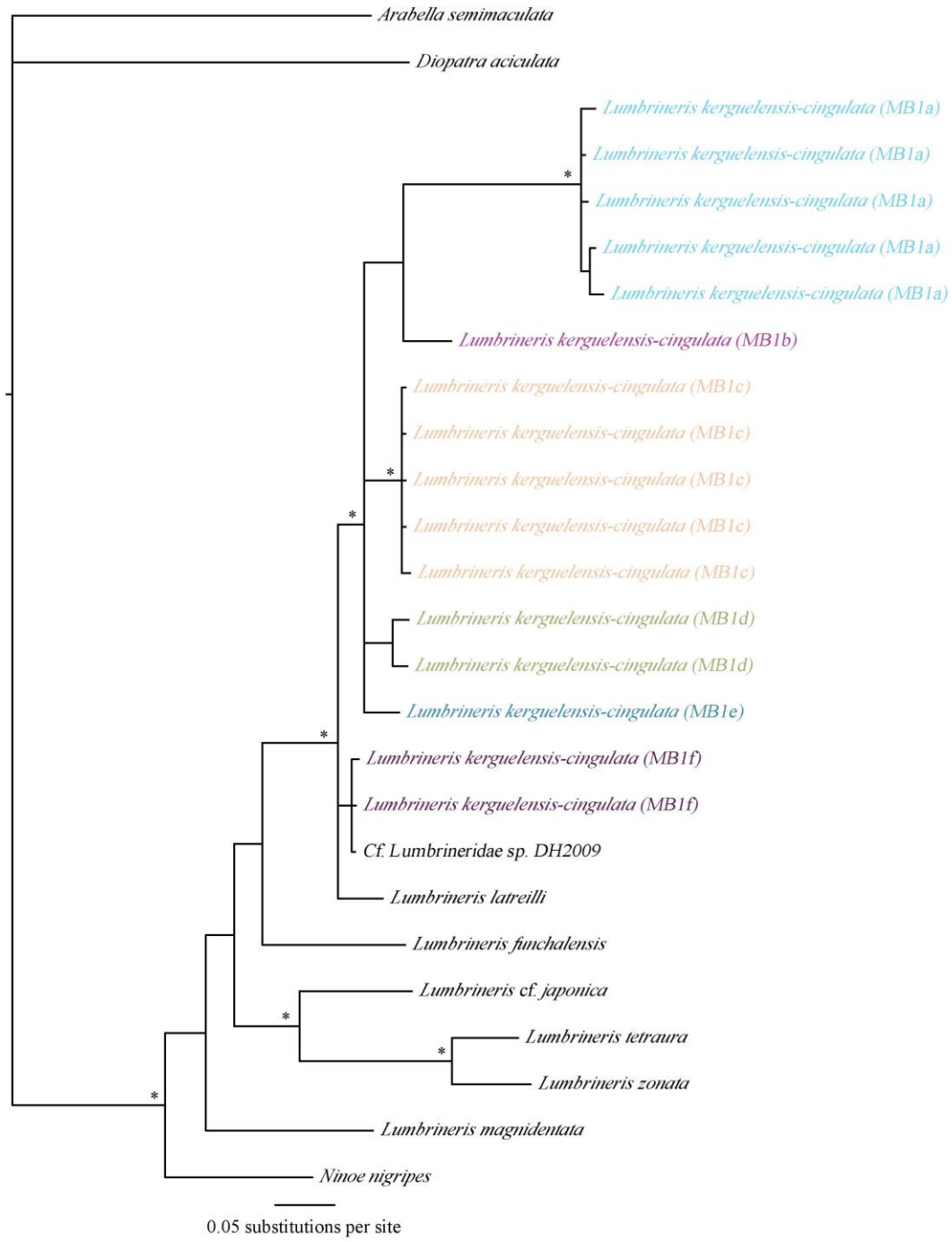

Figure 2-15 Phylogenetic tree of Lumbrineridae from Bayesian analysis using 16S (rDNA) only. Including sequences from BIOPEARL specimens labelled (MB#). Outgroups: *Arabella smimaculata* (Oenonidae) and *Diopatra aciculata* (Onuphidae), * indicates significant node values (>95%) for Bayesian posterior probabilities.

2.3.9 Maldanidae - *Maldane sarsi* Malmgren, 1865

Several Maldanidae species are known to occur in abundance within Antarctic waters especially in deeper soft sediments, such as those sampled during BIOPEARL (Cantone et al., 2000, Brueggeman, 1998). The species of interest in this study, *Maldane sarsi,* was originally described from the North Sea and has long been considered a cosmopolitan species. *Maldane sarsi* currently has three accepted subspecies listed on WoRMS including *Maldane sarsi antarctica* Arwidsson, 1911. The type locality of *M. sarsi antarctica* includes the Graham Coast of the Western Antarctic Peninsula and South Georgia (Fauchald, 2007). The concept of a subspecies causes confusion and disagreement amongst taxonomists. Mayr (1942) suggested that subspecies are likely to be genetically distinct, geographically separate populations of the same species interbreeding freely at zones of contact and can also include completely isolated populations. Here phylogenetic analysis shows genetic differences between Northern hemisphere *M. sarsi* sequences and those morphologically identified to the same species within the BIOPEARL material (Figure 2-16).

Given the limited availability of Maldanidae sequences on GenBank, those of their sister family, Arenicolidae, were also included in Bayesian analyses. The 16S data did not produce a monophyletic Maldanidae clade (Figure 2-16); furthermore the sequences obtained from the BIOPEARL specimens are distributed throughout the phylogeny within five separate clades. The Maldanidae family are particularly hard to identify to a species level when specimens are incomplete (De Assis and Christoffersen, 2011). Guided by the phylogenetic results, on re-examination the mostly incomplete BIOPEARL specimens were reidentified to different Maldanidae species. One specimen was assigned to the described species *Asychis amphiglyptus* (MB), two clades were confidently identified to a genus level labelled here as *Eupraxillella* cf. *antarctica* (MB) and *Praxillella sp.* (MB) and a single specimen could not be identified beyond family level and is labelled Maldanidae *sp.* (MB). These results highlight underestimated diversity levels of Antarctic Maldanidae and the difficulties in working with fragmented material. Two specimens were re-identified (and labelled) as the subspecies *Maldane sarsi antarctica* (MB). This decision was based on the clade formation containing both BIOPEARL and the *Maldane sarsi* sequences suggesting they are closely related. However a K2P distance of 4.67- 4.97% was recorded between the BIOPEARL sequences and the reference sequence. Further analysis with more specimens and COI data would be able to confirm whether *Maldane sarsi antarctica* is a subspecies or true species.

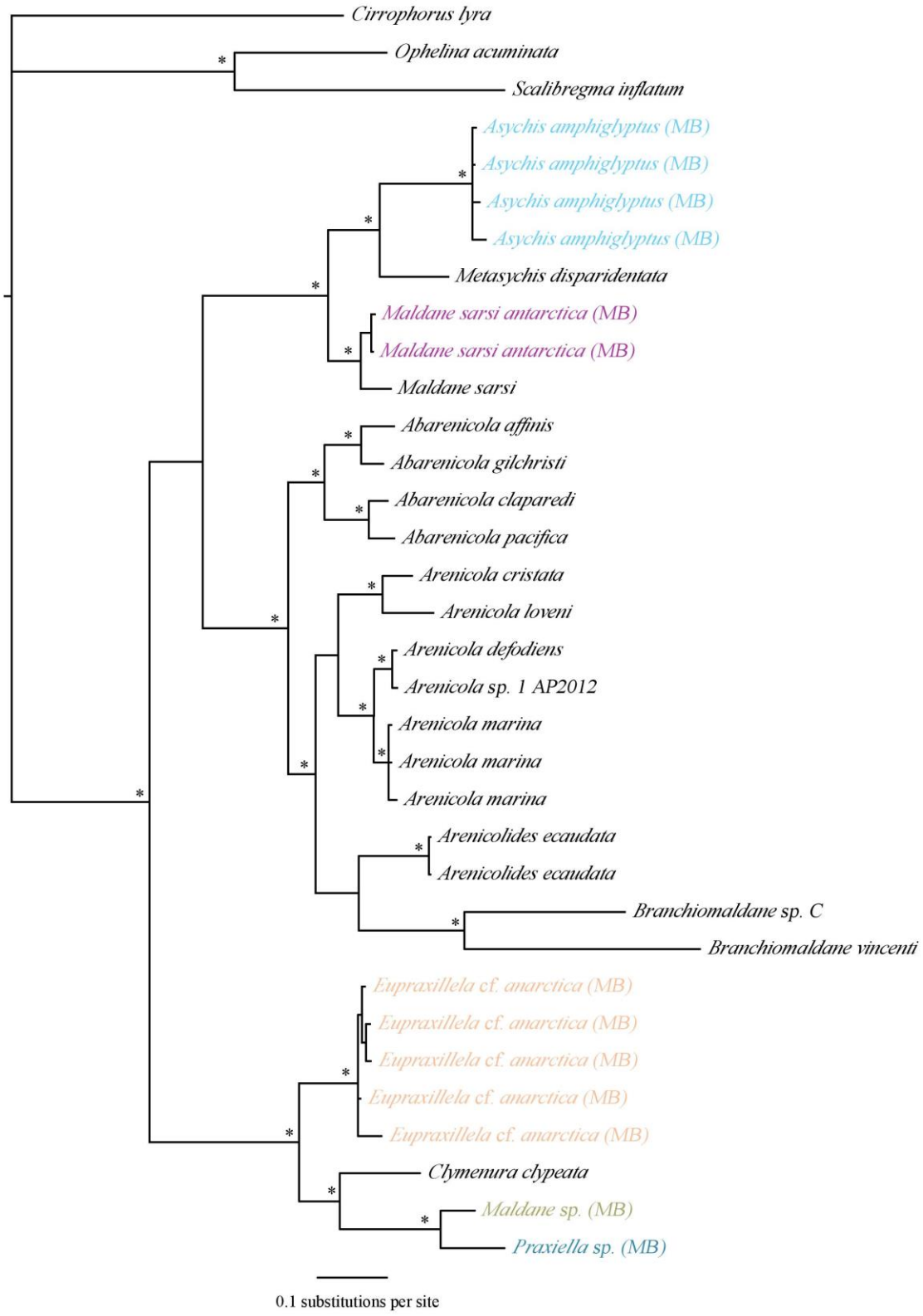

Figure 2-16 Phylogenetic tree of Maldanidae from Bayesian analysis using 16S (rDNA) only. Including sequences from BIOPEARL specimens labelled (MB#). Outgroups: *Cirrophorus lyra* (Paraonidae), *Ophelina acuminata* (Ophelidae) and *Scalibregma inflatum* (Scalibregmatidae), * indicates significant node values (>95%) for Bayesian posterior probabilities

2.3.10 Cirratulidae - *Chaetozone* cf. *setosa* Malmgren, 1867

The cirratulid species *Chaetozone setosa* was described from material collected in the waters surrounding Spitzbergen, Greenland, Iceland and Scandinavia. The species was previously considered cosmopolitan, found in soft sediments from the intertidal zone to the deep sea documented across the Arctic, Atlantic and Antarctic Oceans (Chambers, 2000). However, *Chaetozone,* for which *Chaetozone setosa* is the type species, lacks a generic genus description and some taxonomists have questioned this widespread distribution (Woodham and Chambers, 1994, Chambers and Woodham, 2003). Difficulties in distinguishing between *Chaetozone* species may have led to the false identification of many specimens and subsequently an overestimation of its biogeographic distribution (Blake et al., 1996, Petersen, 1999). Furthermore both morphological and reproductive differences have been identified between overlapping populations of *C. setosa* and it is now regarded as a species complex with an uncertain biogeographic distribution outside of Arctic and sub-Arctic regions (Christie, 1985, Blake et al., 1996). In Blake (2015) described eight new species of *Chaetozone* from Arctic waters and suggests that many of the older records of *C. setosa* are probably misidentified. Twelve *Chaetozone* species were identified within the BIOPEARL material, the most abundant of which matched the *C. setosa* description. However in light of the discussed uncertainties the specimens prior to DNA barcoding were labelled *Chaetozone* cf. *setosa*.

Only two COI sequences from clades MB1 and MB4 were obtained from the BIOPEARL individuals, comparison with *C. setosa* COI reference sequences on GenBank confirmed that these clades were definitely not *C. setosa.* It should be noted however that the reference sequences were not collected from *C. setosa* type locality (Spitsbergenin) but from Russian and Candian waters within the Arctic Ocean. Furthermore, there were two clades within the reference sequences suggesting the presence of additional cryptic species or taxonomic uncertainties within this morphospecies.

Bayesian analyses of the 18 16S BIOPEARL sequences resulted in the 5 clades of *Chaetozone* cf. *setosa*. With no 16S reference sequences for *C. setosa* it was not possible to determine whether any of the BIOPEARL clades were the same species. During morphological re-examination several differences between clades were noted and were thus considered to be different morphospecies. Given the combined morphological and genetic variation between individuals and the discussed misidentifications of *C. setosa*, it is perhaps inappropriate to

suggest that any of the other clades could be true *C. setosa* and for this reason these clades are hereon in referred to as *Chaetozone* sp. MB#.

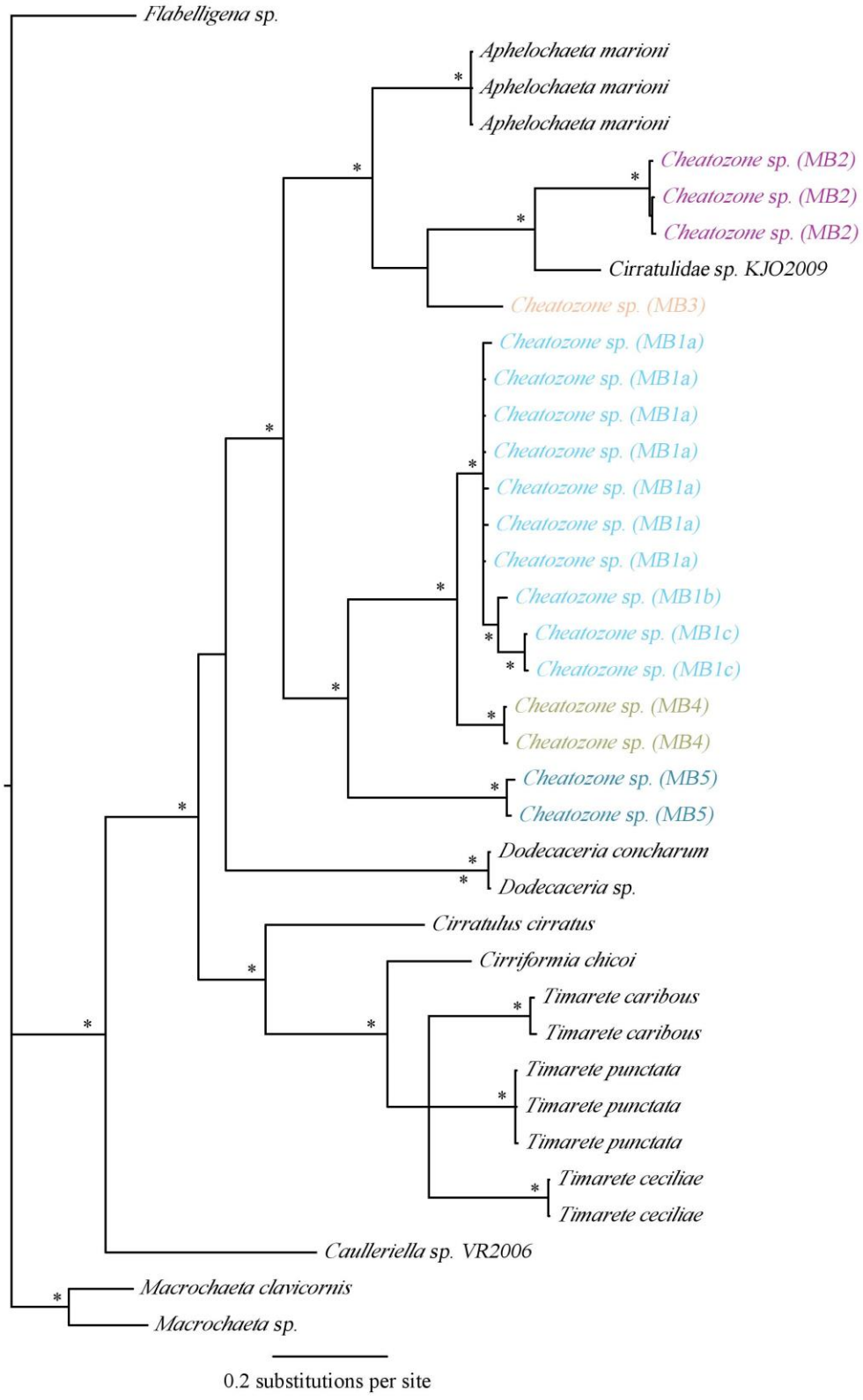

Figure 2-17 Phylogenetic tree of Cirratulidae from Bayesian analysis using 16S (rDNA) only. Including sequences from BIOPEARL specimens labelled (MB#). Outgroups: *Flabelligena* sp, *Macrochaeta clavicornis* and *Macrochaeta* sp. (all Acrocirridae), * indicates significant node values (>95%) for Bayesian posterior probabilities

The most abundant *Chaetozone* sp. sequenced from the BIOPEARL material, *Chaetozone* sp. (MB1) shows high intraspecific variability, indicated from the phylogenetic tree and K2P comparisons, which ranged from 0.00-7.22%. In comparison to other Cirratulidae species including MB clades, K2P distances within species were all less than 1.5%. The greater genetic variation in *Chaetozone* sp. (MB1) could indicate potential cryptic species. However, the pairwise comparisons within this clade lack clear distinction, and with a lack of COI data, a conserved approach will be taken. This also reflects taxonomic opinion within the literature. Christie (1985) noted subtle morphological differences between previously considered *C. setosa* within the coastal waters around Northumberland, after which three morphologically distinct populations were described. It is perhaps unsuitable to suggest they are cryptic species rather than distinct morphospecies without further examination of whole specimens.

2.3.11 Nephtyidae – Spp. of *Aglaophamus* Kinberg, 1866

The family Nephtyidae comprises five genera and over 100 species, generally found on soft sediment covering all ocean depths. The genus of interest in this study, *Aglaophamus,* is well documented throughout Antarctic waters (Brueggeman, 1998). Following successful PCR and DNA sequencing results, three morphologically identified *Aglaophamus* species were chosen as target species including *Aglaophamus trissophyllus* (Grube, 1877)*, Aglaophamus foliosus* Hartman, 1967 and *Aglaophamus digitatus* Hartman, 1967. The number of 16S sequences obtained from the BIOPEARL *Aglaophamus* species was almost 3 times greater than COI. Difficulty in obtaining COI from Nephtyidae species has been documented in previous molecular studies (Ravara et al., 2010). Here a Nephtyidae specific COI forward primer (Carr et al., 2011) was used with the universal HCO reverse COI primer (Folmer et al., 1994) (Table 2-8) however, in most cases only the reverse (non-specific) primer could be sequenced. The lack of family specific primer binding could suggest high genetic variability within the COI gene of Nephtyidae.

As recorded for Hesionidae sp. A, the phylogenies produced using COI and 16S data produced different results (Figure 2-18, Figure 2-19). The COI phylogeny produced five different clades with significant support values, whilst 16S produced unresolved clades most of which lack significant support values. The clades presented did not correspond to the primary morphological identification of the *Aglaophamus* species. With the exception of *Aglaophamus*

sp. (MB5), secondary morphological examination identified all BIOPEARL specimens, as *A. trissophyllus*. The *A. trissophyllus* COI reference sequence on the BOLD database collected from a Deception Island sample matched three BIOPEARL sequences labelled *A. trissophyllus* (MB1a) (Figure 2-18). The other *Aglaophamus* specimens were named *Aglaophamus* cf. *trissophyllus* (MB#) or *Aglaophamus* sp. (MB#) based on phylogenetic grouping and K2P distances.

A. trissophyllus (MB1a), (MB1b) and (MB1c) clades are not considered to represent potential cryptic species but might be a results of a *A. trissophyllus* species complex. This decision was made based on the relatively low between clade K2P distances which were comparable to the K2P distances between *Aglaophamus* sp. (MB3) sequences (Table 2-8). The lack of genetic difference between *A. trissophyllus* (MB1a), (MB1b) and (MB1c) is also shown in 16S groupings (Figure 2-19). The COI K2P distances between MB1 clades and MB2 and MB3 were much greater (>10.00%), suggesting that at least three cryptic species exist within the sample set. However, these cryptic speices would not have been identified from Bayesian and K2P analysis of 16S alone (Figure 2-19, Table 2-9).

The first phylogeny of Nephtyidae by Ravara et al. (2010) discussed several differences in the current characters used to identify Nephtyidae species amongst taxonomists. Ravara et al. (2010) suggests that previously considered morphological characters used for species discrimination may need some reconsideration based on genetic evidence. This study has reinforced that. In the case of these *Aglaophamus* species the false morphological identification of specimens was associated with the lack of morphological differences between *Aglaophamus* species with specimen size/life stage. Many of the 'smaller' BIOPEARL individuals identified as *A. foliosus* and *A. digitatus* may have been juveniles, which were only identifiable as *A. trissophyllus* after DNA barcoding and expert examination.

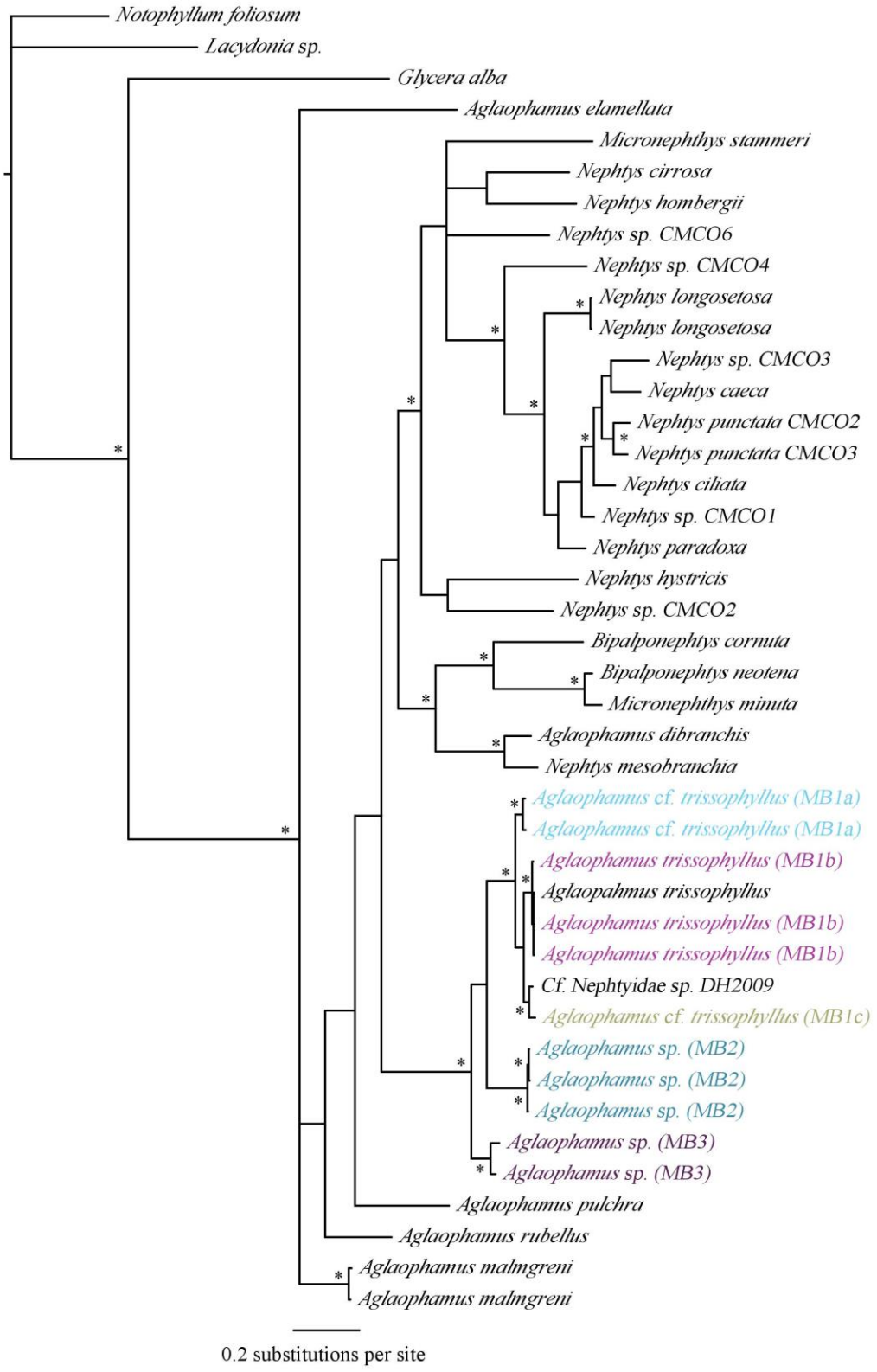

Figure 2-18 Phylogenetic tree of Nephtyidae from Bayesian analysis using COI (mtDNA) only. Including sequences from BIOPEARL specimens labelled (MB#). Outgroups: *Notophyllum foliosum*, *Lacydonia* sp. (both Phyllodocidae) and *Glycera capitata* (Glyceridae), * indicates significant node values (>95%) for Bayesian posterior probabilities.

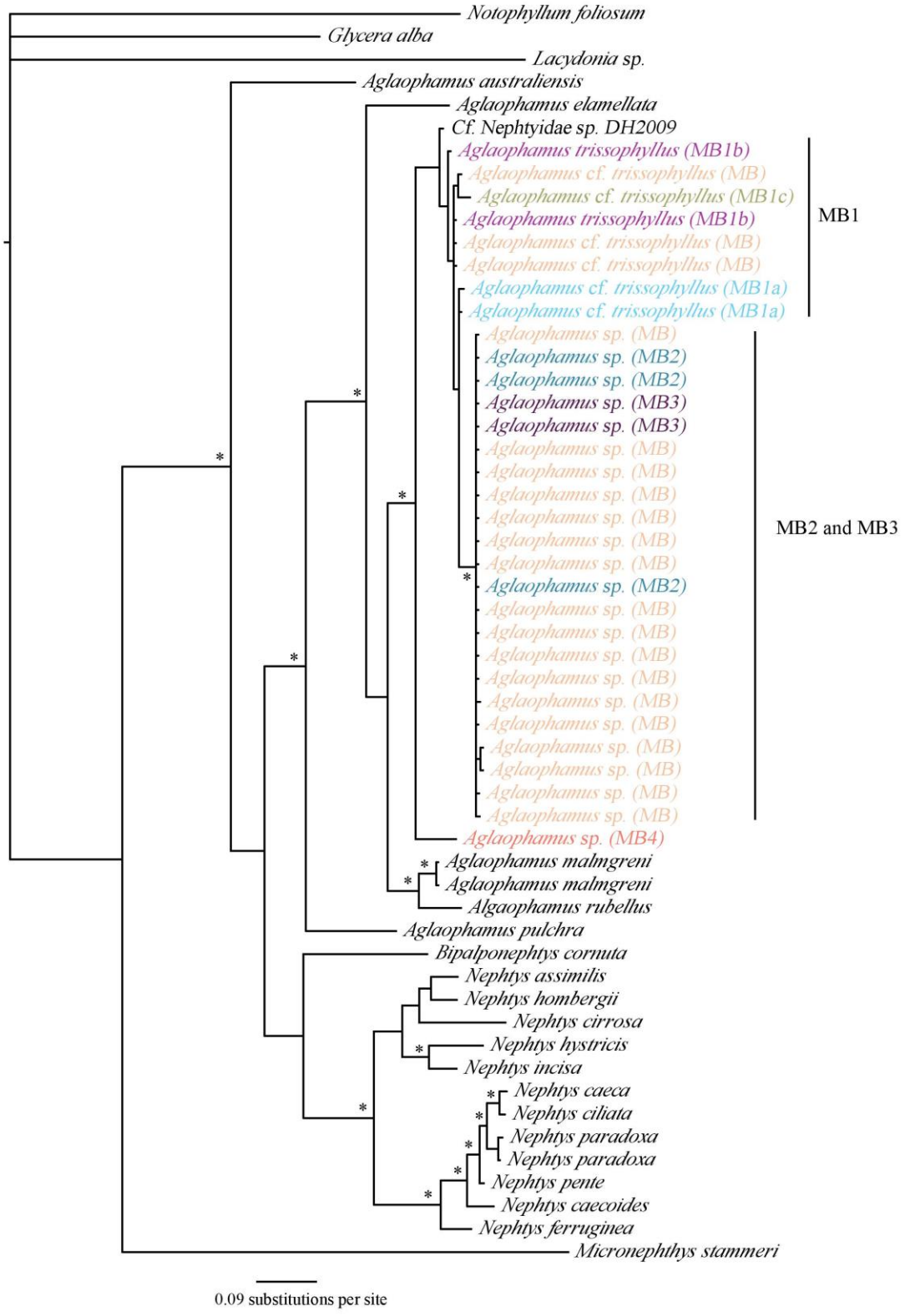

Figure 2-19 Phylogenetic tree of Nephtyidae from Bayesian analysis using 16S (rDNA) only. Including sequences from BIOPEARL specimens labelled (MB) and (MB#) dependant on their corresponding COI sequences in Figure 2-18. Outgroups: *Notophyllum foliosum*, *Lacydonia* sp. (both Phyllodocidae) and *Glycera capitata* (Glyceridae), * indicates significant node values (>95%) for Bayesian posterior probabilities.

Aglaophamus MB clades	MB1a	MB1b	MB1c	MB2	MB3
***Aglaophamus* cf. *trissophyllus* (MB1a)**	0.92				
***Aglaophamus trissophyllus* (MB1b)**	5.33-5.75	0.00-0.18			
***Aglaophamus* cf. *trissophyllus* (MB1c)**	5.34-6.13	3.77-4.74			
***Aglaophamus* sp. (MB2)**	13.08-13.34	13.56-14.41	12.89-13.53	0.16-0.18	
***Aglaophamus* sp. (MB3)**	11.52-12.97	11.56-13.01	10.69-13.51	9.64-13.31	2.92

Table 2-8 Mean pairwise K2P distances (%) within and between COI sequences of *Algaogphamus trissophyllus* (including the reference and MB sequences) and MB# clades as labelled in Figure 2-18. Bold indicates only values obtained from only one pairwise comparison. Column headings refer the same MB# clade as listed in the row labels.

Aglaophamus MB clades	MB1	MB2, MB3	MB4
***Aglaophamus* cf. *trissophyllus* (MB1)**	0.00-1.67		
***Aglaophamus* sp. (MB2), (MB3)**	1.63-3.38	0.00-0.80	
***Aglaophamus* sp. (MB4)**	5.58-6.90	5.53-6.13	*

Table 2-9 Maximum and minimum pairwise K2P distances (%) within and between 16S sequences of *Aglaophamus trissophyllus* and MB# clades as labelled in Figure 2-19. Column headings refer the same MB clade as listed in the row labels.

2.3.12 Polynoidae

The Polynoidae family consists of over 748 described species making it one of the most diverse polychaete taxa (Hutchings, 2000). They are found in almost all marine benthic environments and were the most diverse and abundant family with 23 morphospecies identified from over 5000 individuals within the BIOPEARL II samples. By number, this amounts to over a third of the current known Polynoidae species in the Southern Ocean (Neal et al., 2014). Potential cryptic species have already been recorded within the BIOPEARL Polynoidae; three clades of *Austrolaenilla antarctica* Bergström, 1916 were found when DNA barcoding was used to identify Polynoidae juveniles (Neal et al., 2014). Given the dominance of Polynoidae within BIOPEARL, three Polynoidae species were chosen for sequencing. The most abundant of which was *Harmothoe fuligineum* (Baird, 1865), collected from both BIOPEARL expeditions, as well as two undescribed species only collected on BIOPEARL II; *Macellicephala* sp. A and *Macellicephaloides* sp. B.

Reference sequences for *Harmothoe fuligineum* are publically available and were sequenced during Neal et al. (2014)'s Polynoidae study. All except one of the COI *H. fuligineum* sequences obtained for this project matched these reference sequences (Figure 2-20). The K2P pairwise distance between the single outlier, *Harmothoe* cf. *fuligineum* (MB), and the individuals within the *H. fuligineum* (MB) clade ranged from 5.84-7.53%. To compare, K2P

distances between previously identified, MOTUs of *Harmothoe imbricata* (Linnaeus, 1767) were calculated. The K2P distances between the *H. imbricata* CMC0# sequences in Mincks Hardy et al. (2011) ranged from 4.31-14.03%. As the distances between *H. fuligineum* (MB) and *H.* cf. *fuligineum* (MB) fall at the lower end of this range and, the 16s sequence for the *H.* cf. *fuligineum* (MB) individual was positioned amongst the other *H. fuligineum* (MB) sequences, it was not considered to be a separate species.

For *Macellicephala* sp. A, there are two clearly defined clades from the 12 16S and four COI sequences obtained from the BIOPEARL specimens (Figure 2-20, Figure 2-21). The K2P distances also suggest that there are two separate species present. The COI within clade variation for *Macellicephala* sp. (MB1) ranged from 0.14-0.73%, more than ten times lower than the pairwise distances between the *Macellicephala* sp. (MB1) and (MB2) clades at 12.57-12.84%.

The determination of cryptic species from the *Macellicephaloides* sp. sequences was difficult without COI data. A lack of genetic diversity in 16S has also prevented the determination of cryptic species in the Antarctic polynoid *Austrolaenilla antarctica* (Neal et al., 2014). The K2P distances between *Macellicephaloides* sp. (MB1b) sequences ranged from 0.00 to 2.36% in 16S. This value is comparable to the lower range of the K2P distances between the *Macellicephala* sp. (MB1) and (MB2) clades at 2.75-4.30%. As the identification of cryptic species works on the basis that genetic variation is greater between than within species, a conserved approach was taken and no cryptic species were suggested for *Macellicephaloides* and the sequences are labelled MB1a and MB1b (Figure 2-21).

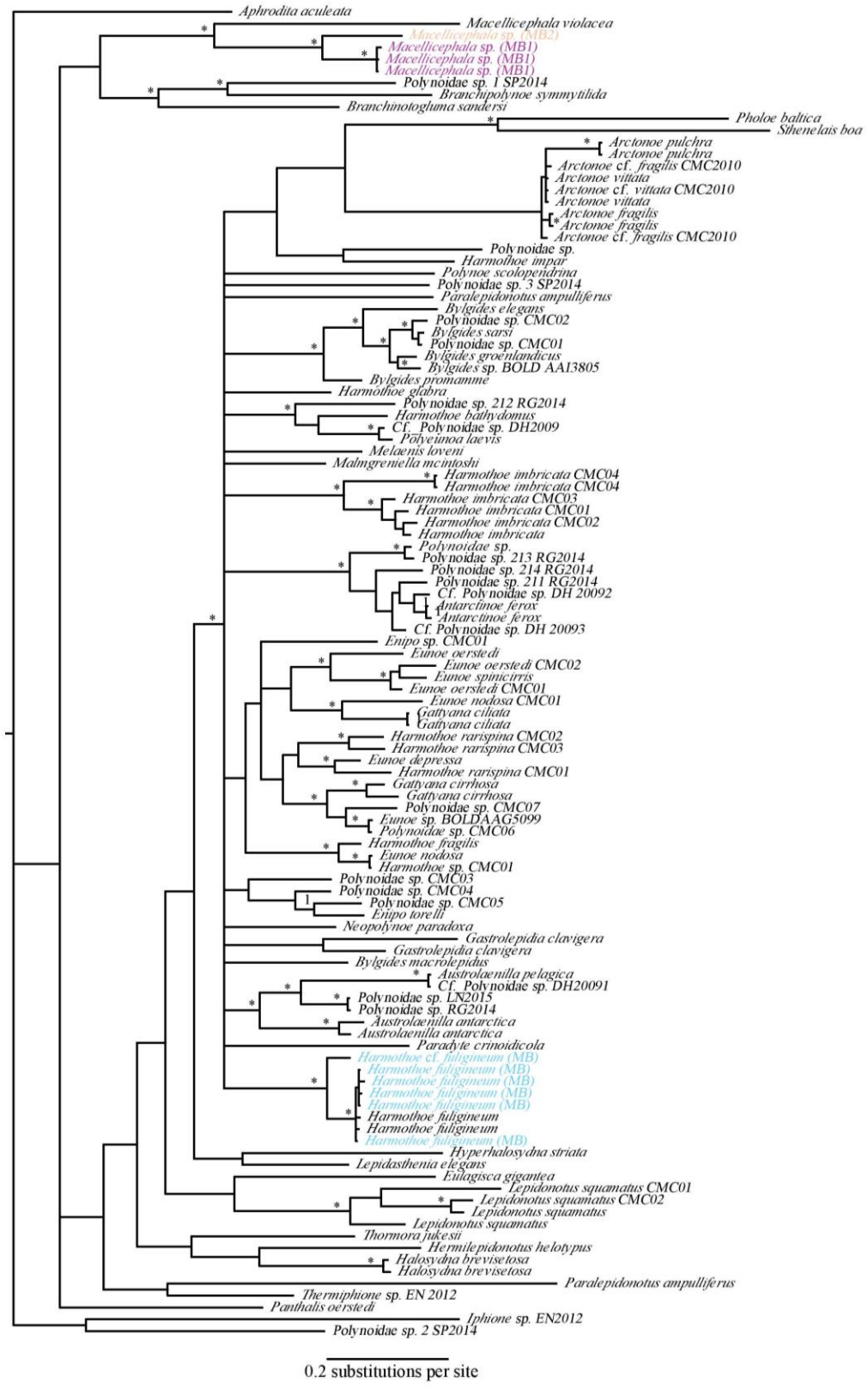

Figure 2-20 Phylogenetic tree of Polynoidae from Bayesian analysis using COI (mtDNA) only. Including sequences from BIOPEARL specimens labelled (MB#). Outgroups: *Aphrodita aculeate* (Aphroditidae), *Panthalis oerstedi* (Acoetidae) and *Iphione* sp. EN2012 (Iphnionidae), * indicates significant node values (>95%) for Bayesian posterior probabilities.

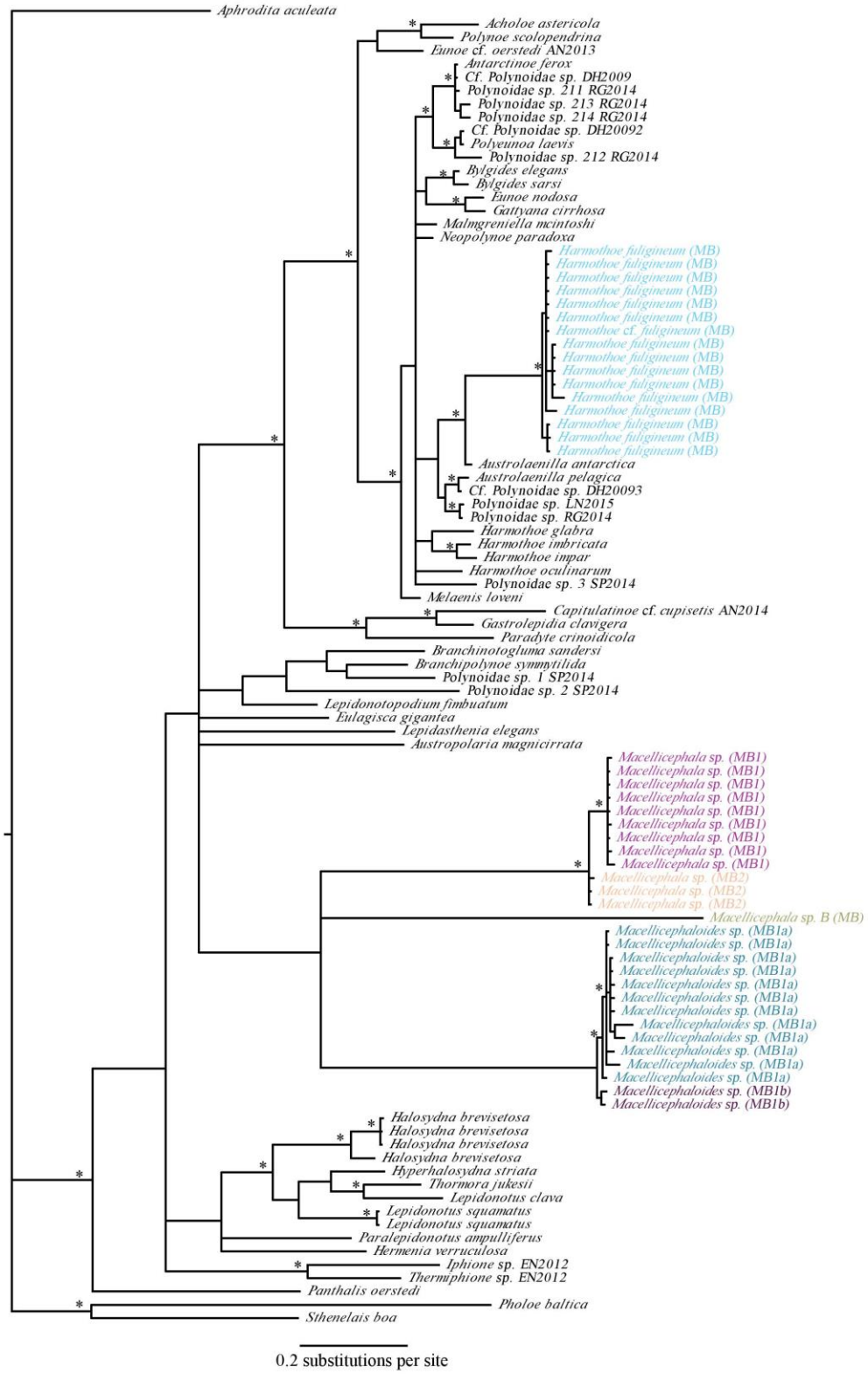

Figure 2-21 Phylogenetic tree of Polynoidae from Bayesian analysis using 16S (rDNA) only. Including sequences from BIOPEARL specimens labelled (MB#). Outgroups: *Aphrodita aculeate* (Aphroditidae), *Panthalis oerstedi* (Acoetidae) and *Iphione* sp. EN2012 (Iphnionidae), * indicates significant node values (>95%) for Bayesian posterior probabilities.

2.4 Summary and Discussion

2.4.1 Determination of cryptic diversity in Antarctic polychaetes using COI and 16S

The barcoding gene of choice within this study was COI. However, in one third of the target species investigated only sequences for the secondary barcoding gene, 16S, were collected. In the remaining target species, successful PCR and sequencing of 16S, was between 1.89 to 8.98% times greater than that of COI. This was despite trialling several primer combinations; including polychaete and family specific primers in some cases. Greater PCR success for 16S is probably associated with the more conserved nature of the 16S gene and a lack of mutations within the primer-binding site compared to COI.

The congruent results between these two mitochondrial genes in several species show that 16S can be used to discriminate between potential cryptic species. Thus despite the slower evolutionary rates 16S can fulfil the barcode criteria set out by Hebert et al. (2003a). Such results have also been observed in other taxa for example amphibians (Vences et al., 2005b), as well as Antarctic crustaceans (Held, 2003) and nudibranchs (Wilson et al., 2009). A greater abundance of 16S compared to COI sequences for Antarctic fauna was noted in Grant and Linse (2009), where 37% of all sequences available were 16S and only 15% were COI. Furthermore, within family level polychaete studies, the retrieval of 16S is often more successful (e.g. Ruta et al., 2007, Ravara et al., 2010). To conclude, 16S should not be initially viewed as an inferior barcoding gene to COI and may in some cases result in greater specimen coverage without underestimating species diversity. However, where possible both genes should be analysed to aid the discrimination between uncertain clades and potential species, especially when the difference between inter and intraclade variability is low.

2.4.2 The prevalence of cryptic species within Antarctic waters

Clear examples of cryptic diversity were uncovered in eight out of the 15 target species investigated, indicating that large numbers of species could be overlooked and that there is a limited understanding of species diversity within the Southern Ocean. DNA barcoding has not only uncovered that species diversity is being underestimated as a result of the presence of cryptic species, in addition it has shown that errors in morphological identification may also be a contributing factor. Within five of the morphologically identified target taxa, multiple morpho-species were later confirmed following DNA barcoding. The former misidentification of these species could be assigned to multiple factors, including damaged specimens, the publication of species descriptions following identification and incorrect taxonomic decisions

inherently associated with the processing of larger number of specimens in a limited time frame. This also highlights the importance of secondary morphological analysis to prevent false positive results and an overestimation of cryptic diversity. Together with other uncertainties associated with the detection of cryptic species including scientific opinion, intraspecific variability and phylogenetic understanding, it is perhaps impossible to suggest the total prevalence of cryptic species amongst Antarctic polychaetes. As discussed in Nygren (2014) there appears to be no patterns in cryptic diversity across families, however other factors remain to be investigated (biogeography and functional traits).

2.4.3 The importance of DNA databases in diversity studies

The comparison of DNA barcodes to those in genetic databases such as GenBank and BOLD can hugely accelerate and improve the accuracy of species identification. Within this study several cases of cryptic species were identified by comparison to publically available sequences, for example: *Glycera capitata, Scalibregma inflatum, Aglaophamus trissophyllus* and *Maldane sarsi*. The comparison of BIOPEARL nephtyid sequences with the public *Aglaophamus trissophyllus* sequence was able to confirm the presence of the named species as well as several cryptic species within the BIOPEARL specimens. However, when cryptic species are identified from the described morphological species if there are no reference sequences available for comparison this can create taxonomic uncertainty, as seen in *Aricidea belgicae* and *Euphsrosinella cirratoformis*. Furthermore, when cryptic species are uncovered these often remain undescribed such as the case of the Schüller (2011) *Glycera* clades. As well as time constraints and taxonomic uncertainties, this can be associated with a lack of material from type localities and therefore sequencing material from type localities should be a priority in future barcoding work to improve this issue. Fortunately, an increasing number of newly described species include DNA barcodes in their formal description including the Antarctic polychaete *Austropolaria magnicirrata* Neal et al., 2012.

Modern protocols for describing species are increasing, which could allow for increased cryptic species descriptions. For example, Summers et al. (2014) described 21 species of Mysostomida polychaetes using a turbo-taxonomy approach, which combines DNA sequencing with live photography and a brief morphological description. Improving and expanding our current sequence databases increases the opportunity for future biogeography, population connectivity and evolutionary studies. Thus identifying and describing cryptic species will also aid

biological investigations, management and conservation programmes, which often only consider named species.

2.4.4 Cryptic species and conservation

The primary aim of biological conservation is to preserve and manage global biodiversity (Rubinoff, 2006). The use of molecular taxonomy to measure species diversity has been discussed with relevance to Antarctic and deep-sea marine systems. However cryptic species have also been found in many keystone species from both freshwater and terrestrial habitats. Examples include bees (Murray et al., 2008), butterflies (Hebert et al., 2004a), bats (Davidson-Watts et al., 2006), Amazonian frogs (Funk et al., 2011) and freshwater shrimp and fish (Cook et al., 2008). Their abundance not only influences diversity measures but is important in the identification of endangered species as the conservation status of a species cannot be determined until its taxonomic status is described (Avise, 1989, Murray et al., 2008). Furthermore, if cryptic species are 'rarer' or have a more restricted distribution than their original species, these factors should be incorporated in to management practices for more effective conservation (Schönrogge et al., 2002, Bickford et al., 2007). The use of molecular taxonomy to define species distributions is discussed in Chapter 3 and its importance for marine management discussed again in Chapter 6.

2.5 References

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3 Genetic-level assessment of the biogeography of Antarctic Polychaetes

The data in this chapter were published in Brasier et al. (2017)'Distributional patterns of Polychaetes across the West Antarctic based on DNA barcoding and particle tracking analysis' in the journal, Frontiers in Marine Science.

3.1 Introduction

3.1.1 Marine Biogeography

Biogeographic studies investigate the spatial distribution of organisms within their environment (Crisci et al., 2009). To define the biogeography of a species requires geo-referenced taxonomic data; subsequently diversity and biogeographic investigations are often coupled or sequential to one another (O'Dor et al., 2010). Biogeographic analysis can be used to describe spatial patterns, identify biodiversity hotspots and detect impacts of environmental change. Additionally, when biogeographic patterns are compared to environmental data the potential drivers behind species distributions can be identified. This knowledge can then be used to model future distributions under changing scenarios (e.g. Robertson et al., 2003, Kearney and Porter, 2009, Woodin et al., 2013).

Whether a species can establish itself within a given environment or move between habitats is governed by multiple biological, ecological, chemical and physical factors that interact to determine a species range, or, biogeography (Krebs, 1972, Gaylord and Gaines, 2000, Crisci et al., 2009). Biological factors are generally taxon-specific; these have developed over evolutionary time and are a result of their physiological adaptations, life-history strategies and selection pressures (Somero and DeVries, 1967, Bradbury et al., 2008). Marine organisms are only able to maintain normal functioning and establish populations under certain physical and chemical conditions thus their physiological limits can constrain their distribution (Krebs, 1972). The tolerance of different species varies with some able to survive under a range of conditions and whilst others are highly sensitive to subtle changes. For example, if a species is sensitive to changes in temperature, natural changes in temperature with latitude may prevent species survival or dispersal beyond its limit (e.g Peck (2002) and Peck et al. (2004)). However, even if the physical and chemical properties of seawater are within the physiological niche of a given species, its ability to survive might be over ridden by other ecological factors including

predation or competition for space, food and resources (Paine, 1966). Potential physical factors that could also influence marine biogeography include ocean current systems and topographic features, both of which may act as connections between habitats or barriers to dispersal thus promoting or limiting species ranges (Cowen et al., 2006, Cowen et al., 2007, Cowen and Sponaugle, 2009).

Understanding the drivers and controls of species distributions and biogeography patterns are of current research interest due to the likely impacts of climate change and anthropogenic impacts on species ranges (Griffiths et al., 2017). To predict and manage the impacts of these changes on marine habitats requires a sound understanding of how populations and species distributions are established and maintained.

3.1.2 Biogeographic patterns in Antarctic marine fauna

The Southern Ocean is often described as one of the most isolated marine environments. The closest landmass to Antarctica is 960 km away and the ocean is enclosed by both the Antarctic Circumpolar Current (ACC) and the Polar Front, Figure 3-1. These two oceanographic features act as both a physical and biological barrier which, may have prevented population connectivity and species movement in and out of the Southern Ocean (Ekman, 1953). For shallow water organisms there are only three connections from the Antarctic continent to the north. These include the Scotia Arc islands and the Macquarie-Balleney and Kergulen-Gausberg ridges which rise to 1800 and 200 m below the surface respectively (Knox, 1994). For this reason early studies suggested that the majority of benthic fauna within Antarctic waters would be endemic to the Southern Ocean (Ekman, 1953, Hedgpeth, 1969).

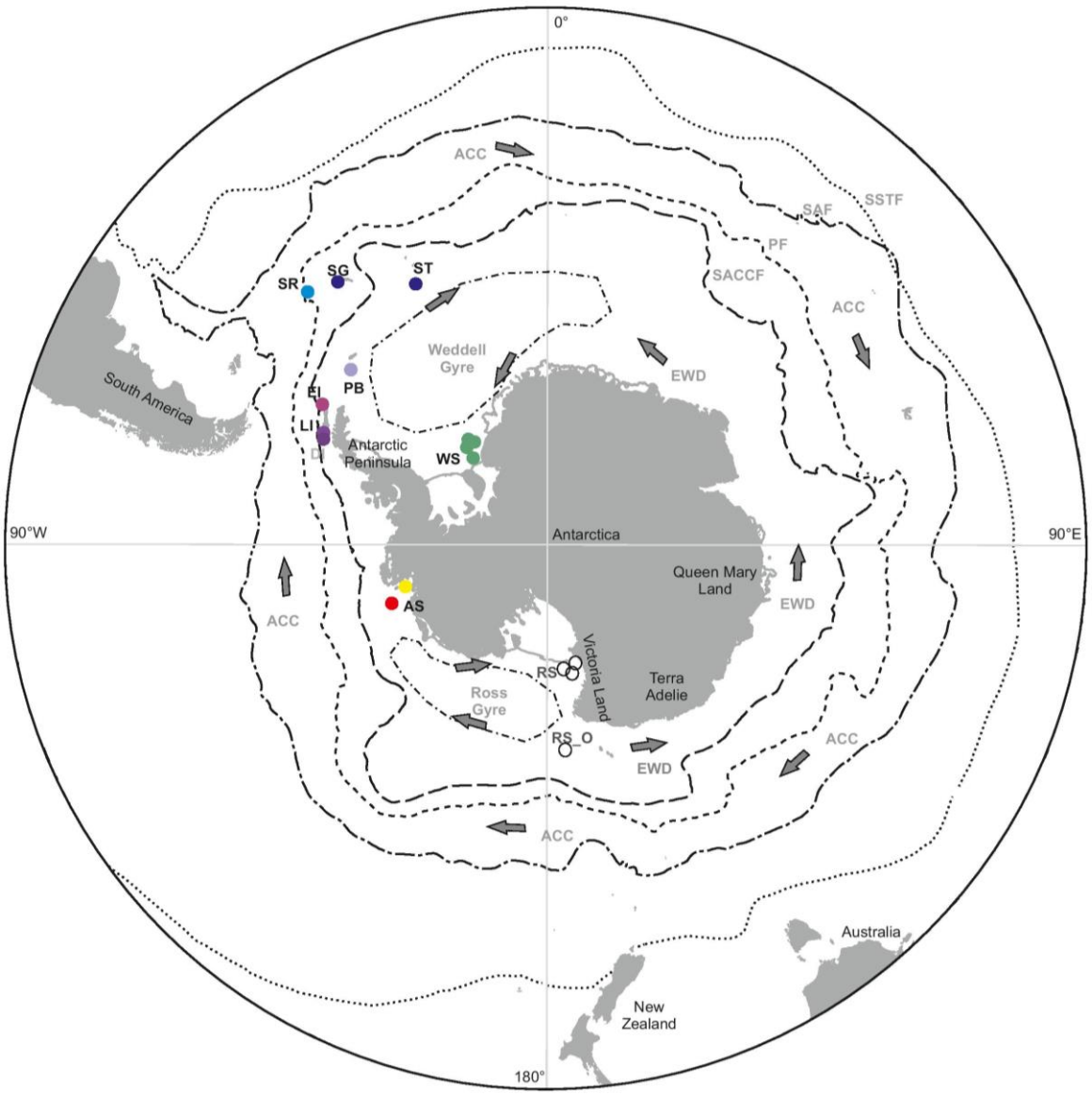


Figure 3-1 Collection location of polychaete DNA barcodes used in this study. Colours correspond to those of the haplotype networks. SR = Shag Rocks, SG = South Georgia, ST = Southern Thule, PB = Powel Basin, EI = Elephant Island, LI = Livingston Island, DI = Deception Island, AS = Amundsen Sea, WS = Weddell Sea, RS = Ross Sea and RS_O = Ross Sea offshore. Position and direction of oceanography currents including the Antarctic Circumpolar Current (ACC), East Wind Drift (counter current) (EWD) and the Weddell and Ross Sea Gyres. Position of the polar front (PF), the Southern Antarctic Circumpolar Current Front (SACCF), Subantarctic Front (SAF) and the Southern Subtropical Front (SSTF). Adapted from Brasier et al., (2017).

The concept of Antarctic endemism has been examined at different taxonomic levels and biogeographic scales. We now know that species can exist both sides of the Polar Front. However, research has found that endemism is a real feature within the Southern Ocean benthos (for reviews see Dell, 1972, Arntz et al., 1997, Clarke and Johnston, 2003, De Broyer and Danis, 2011, Brandt et al., 2012). Given the size of the Southern Ocean there have been several attempts to divide the region into smaller biogeographic provinces based on faunal diversity.

Ekman (1953) was the first to define biogeographic provinces within the Southern Ocean, dividing the continental shelf into sub-regions including South Georgia and West and East Antarctica. Similar suggestions were made by Powell (1965) and Hedgpeth (1969) which, as discussed in Griffiths et al. (2009), has remained the foundation of most Antarctic benthic biogeographic investigations.

Many Southern Ocean endemic species area also considered to have circum-Antarctic distributions (Arntz et al., 1997, Clarke and Johnston, 2003). These distributions could be a result of several factors having a homogenizing effect on Antarctic marine fauna. The continuous coastline around the continent itself provides connectivity between the different seas around Antarctica. Given the relatively uniform physical conditions across the continental shelf, individual settlement and survival is not restricted by their physiology (Arntz et al., 1994). Furthermore oceanographic currents including the ACC, its counter current, and the Weddell Sea gyre could aid the dispersal of larvae around the continent (Fahrbach et al., 1994, Orsi et al., 1995, 1999, Linse et al., 2007).

In comparison to other oceans, Antarctic fauna often have extended depth distribution or exhibit eurybathic traits (Brandt et al., 2007). Some of the first suggestions of an abundance of eurybathic species were made by Dell (1972) and Knox and Lowry (1977) for various taxa including sponges, corals, polychaetes and molluscs. Broad depth distributions of Antarctic fauna are thought to be associated with the advance and retreat of sea ice during interglacial cycles (Brey et al., 1996). During periods of glacial expansion some 'shelf fauna' were moved on to the slope where they could survive due to the isothermic water column and the deep nature of the Antarctic shelf. Following glacial retreat, if, the now 'slope fauna' recolonized the ice-free shelf areas eurybathic traits were established amongst the surviving species (Clarke and Johnston, 2003).

Although common, the degree of eurybathy appears to be variable between and within phyla and the data sets analysed (Brandt et al., 2009). For example within Antarctic sponges eurybathic tendencies are highly varied between classes. Downey et al. (2012) reported stenobathic distributions in calcareous sponges, some evidence of eurybathy in hexactinallid sponges and high degrees of eurybathy in the widely distributed demosponges. With an increased number of deep-sea sampling programmes structuring with depth has been recorded. For example in cumacean peracarids and polychaetes the degree of species similarity was greatest within the same depth zones, e.g. shelf regions and deep basins (Mühlenhardt-Siegel, 2011, Neal et al., 2017).

3.1.3 Deep-sea contributions to Antarctic biogeography

As a result of the isolated nature and inaccessibility of the deep Southern Ocean; our understanding of species diversity, biogeography and ecology has always lagged behind that of the Antarctic shelf and the other deep oceans (Kaiser et al., 2013). It was traditonally believed that the lack of environmental variability and geographical barriers to dispersal within the deep-sea would lead to large species ranges (McClain and Hardy, 2010). When considering the biogeography of deep-sea Antarctic species the ACC and Polar Front may not influence their distribution as much as shallow water species. As discussed in Clarke (2003) although the surface currents may affect pelagic species, it is no barrier to the benthos. Furthermore, it may actually assist the movement of individuals and species into and out of the Southern Ocean (Hunter and Halanych, 2010).

Another example of facilitated faunal connections is the northward movement of deep water formed in the Weddell Sea (Vinogradova, 1997). During the ANDEEP expeditions to the Weddell Sea, many taxa collected were shown to occur north of the Polar Front and in some cases, the equator (Brandt et al., 2007). This was particularly apparent for meiofaunal taxa including nematodes and hard-shelled foraminifera for which the majority of species were shared between the deep Southern Ocean and abyssal sites in North Atlantic (Cornelius and Gooday, 2004, Sebastian et al., 2007). Within the larger size classes, although cosmopolitan species may exist, there was still an overall dominance of endemic species (Brandt et al., 2014). Other studies have supported this (e.g McClain, 2007) suggesting that despite undersampling, regionally and locally-restricted species do exist. Subsequently, the deep sea is no longer perceived as a purely homogeneous environment. A combination of topographic features, oceanographic conditions and biological factors can limit a species movement in and out of certain habitats as in shallow water (Palumbi, 1992, 1994).

3.1.4 Antarctic biogeographic databases

Since the CAML and the development of taxonomic databases, there have been some attempts to revisit the biogeographic provinces suggested by Hedgpeth (1969). However, it is becoming increasingly apparent that generalised biogeographic schemes cannot be applied to all taxa. Biogeographic patterns may differ depending on the class of animals being considered (Griffiths et al., 2009, Brandt et al., 2012). Furthermore the creation of large biological databases such as the Scientific Committee on Antarctic Research Marine Biodiversity Information Network (SCAR-MarBIN) has highlighted several areas of Antarctica and the

Southern Ocean which are currently lacking taxonomic information (Griffiths et al., 2009). Prior to the BIOPEARL expedition the Amundsen and Bellingshausen Seas were mostly unsampled. This region is of biogeographic interest as it marks the West and East Antarctica divides in the biogeographic zones put forward by Hedgpeth (1969).

Another database that has become increasingly important especially for biogeographical studies is the Register of Antarctic Marine Species (RAMS). RAMS is a regional database within the World Register of Marine Species which compiles and manages an authoritative taxonomic list of all marine species (Jossart et al., 2015). As well as taxonomic data RAMS also links with several other initiatives including GenBANK and the Barcode of Life Database (BOLD). The combination of genetic and distributional data improves our ability to describe and compare the biogeographic distributions of individual taxa and observe large scale biogeographic patterns. This is important for both understanding ecosystems, monitoring biological change with natural or anthropogenic disturbance and advising management and conservation decisions.

3.1.5 The application of genetics to biogeographic investigations

The accumulation of homologous sequence data across a species range can provide new insights into species biogeography not available from traditional species identification. Using DNA to confirm species distributions takes our understanding of species biogeography to the next level as without taking cryptic species into account our understanding of biogeographical patterns will be severely limited (Nygren, 2014). The comparison of DNA barcodes from multiple individuals and locations can be used to genetically confirm the presence and distribution of species (Mincks Hardy et al., 2011). It can also be used to assess the degree of gene flow and population connectivity between localities, providing insight into the level of interaction between populations.

By comparison with environmental factors and ecological traits, genetic data can also be used to describe species dispersal including the direction, distance and potential barriers (Mincks Hardy et al., 2011). Thus molecular investigations into species biogeography can identify potential causes of species divergence in already-established cryptic species and those evolving today. Allopatric cryptic species could be separated by a physical oceanographic barrier, for example a frontal system, trough or strait (Knowlton, 1993, Palumbi, 1994). The lack of genetic exchange between the cut-off populations would, over evolutionary time, result in genetically distinct cryptic populations occupying different regions. However, in some cases cryptic

species or divergent populations co-exist within the same regions and the cause of genetic differentiation is unclear. It is possible that physical barriers did previously exist and are no longer present allowing species to disperse and coexist, or, there may be another biological factor which led to their divergence within the same habitat (Smith, 1966, Via, 2001).

Since the early 2000s there has been an increase in the number of Antarctic biodiversity and biogeography investigations using DNA across many taxonomic groups. Table 3-1 summarises the findings of 26 difference phylogenetic studies of benthic Antarctic invertebrates from sixtaxonomic groups (Crustacea, Echinodermata, Mollusca, Nemertea, Polycheata and Pycnogonida). Evidence for genetically distinct lineages and populations within species has been recorded in the majority of studies using COI or 16S genes. Many of these populations, often considered to be cryptic, will have different distribution patterns to those previously accepted for their species. Cryptic populations are often more restricted or separated from one another although the co-existence of genetically distinct groups within the same location has also been recorded. Prior to this investigation only a single investigation into the genetic diversity of polychaete species within the Southern Ocean had been published. Thus, these data make a considerable contribution to our current knowledge of the biogeography of Southern Ocean polychaetes.

Reference	Taxa	Genetic marker	Previously defined distribution(s)	Genetically distinct populations or linages	Observed distribution(s)
	Crustacea				
Baird et al. (2011)	*Eusirus perdentatus* *Eusirus giganteus* (Amphipoda)	COI CytB ITS2	Circum-Antarctic	Yes	One cryptic species deemed circum-Antarctic others more restricted
Brandão et al. (2010)	*Macroscapha* spp (Ostracoda)	COI ITS2	Circum-Antarctic	Yes	Most populations geographically and bathymetrically segregated.
Havermans et al. (2011)	*Orchomene* spp. (Amphipoda)	COI	Circum-Antarctic	Yes	Cryptic diversity in three species examined. Some clades appear circum-Antarctic, others more restricted.
Held (2003)	*Ceratiserolis trilobitoides* (Isopoda)	16S	Circum-Antarctic Sub-Antractic	Yes	One clade widespread in the Western Antarctic one restricted to the Antarctic Peninsula.
Held and Wägele (2005)	*Glyptonotus antarcticus* (Isopoda)	16S	Circum-Antarctic Sub-Antarctic	Yes	All geographically restricted one clade from the Antarctic peninsula, one clade from the Ross Sea and two clades from the Weddell Sea.
Leese and Held (2008)	*Ceratiserolis trilobitoides* (Isopoda)	Microsat.	Circum-Antarctic Sub-Antractic	Yes	One clade widespread in the Western Antarctic one restricted to the Antarctic Peninsula, as described in Held (2003).
Lorz et al. 2012	*Epimeria georgiana* (Amphipoda)	COI	Circum-Antarctic	Yes	Three clades from the southern Scotia Arc and one clade from the Weddell Sea.
Raupach and Wägele (2006)	*Acanthaspidia drygalski* (Isopoda)	16S	Circum-Antarctic	Yes	Genetically distinct populations within the Weddell Sea.
Raupach et al. (2007)	*Betamorpha fusiformis* (Isopoda)	16S 18S	Southern and Atlantic Ocean	Yes	All clades restricted; two clades restricted to the Weddell Sea, two to the Scotia Arc Islands and three clades restricted to South Atlantic Islands.

Raupach et al. (2010)	*Chorismus antarcticus* *Nematocarcinus lanceopes* (Decapoda)	COI 16S 28S	Circum-Antarctic	No	16S and 28S invariant between the Ross Sea, Antarctic Peninsula and the Weddell Sea. Some variation in COI but not significant enough to suggest cryptic species
	Echinodermata				
Hemery et al. (2012)	*Promachocrinus kerguelensis* (Crinoidia)	COI	Circum-Antarctic Sub Antarctic	Yes	Both clades identified had a circum-Antarctic and eurybathic one clade also found in the sub-Antarctic
Hunter and Halanych (2010)	*Ophionotus victoriae* (Ophuroidia)	COI 16S	Circum-Antarctic Sub-Antarctic	Yes	Genetically distinct eastern and western Anatrctic and Sub-Antarctic populations
Janosik et al. (2011)	*Odontaster* spp. (Asteroidia)	COI 16S	Circum-Antarctic Sub-Antarctic	No	One South American and Sub-Antarctic species, one species Antarctic and Sub-Antarctic, three Antarctic species one with evidence for circum-Antarctic distribution
O'Loughlin et al. (2011)	Holothurian spp	COI	Circum-Antarctic and Sub-Antarctic	Yes*	High inter-regional speciation and divergence 17/28 species demonstrated allopatric speciation
Wilson et al. (2007)	*Promachorinus kerguelensis* (Crinoidia)	COI Cytochrome b	Circum-Antarctic Sub-Antarctic	Yes	One Antarctic and sub-Antarctic clade, three clades restricted to the Antarctic Peninsula, two clades found along the Antarctic Peninsula and Scotia Arc Islands
	Mollusca				
Allcock et al. (2011)	*Pareledone* spp. (Octopoda)	COI	Circum-Antarctic	Yes	Five species endemic to the South Shetlands Six species with sxtended ranges One species cicrum-Antarctic
González-Wevar et al. (2011)	*Nacella concinna* (Gastropoda)	COI	Western Antarctic Peninsula and Sub-Antarctic	No	Unchanged
Linse et al. (2007)	*Lissarca notorcadensis* (Bivalvia)	COI 28S	Circum-Antarctic	Yes	Genetic difference between the Scotia Arc lineage and the Weddell and Ross Sea lineage
Wilson et al. (2009)	*Doris kerguelenensis* (Nudibranchia)	COI 16S	Circum-Antarctic Sub-Antarctic South American	Yes	Divergent lineages either side of the Drake Passage with evidence for allopatric groups within Antarctic waters
	Polycheata				

Schüller (2011)	*Glycera capitata*	COI 28S 18S	Cosmopolitan	Yes	Depth restricted distributions within the Weddell Sea
	Nemertea				
Mahon et al. (2010)	Multiple spp	16S	Na	Yes	Identified 19 new lineages with differing biogeographic distributions.
Thornhill et al. (2008)	*Paraborlasia corrugatus*	COI 16S	Circum-Antarctic Sub-Antarctic South American	Yes	Genetic difference between the Antarctic and sub-Antarctic, and South American populations.
	Pycnogonida				
Arango et al. (2011)	*Nymphon australe* (Pycnogonida)	COI 16S	COI 16S	No	Circum-Antarctic but evidence for genetically isolated populations
Weis et al. (2014)	*Pallenopsis patagonica* (Pycnogonida)	COI ITS	Circum-Antarctic Sub-Antarctic South American	Yes	Genetically distinct populations either side of the polar Front. Some clades well distributed within the Scotia Sea and Antarctic Peninsula, others restricted to the Weddell Sea.
Krabbe et al. (2010)	*Colossendeis megalonyx* (Pycnogonida)	COI	Cosmopolitan	Yes	One genetically distinct South American population, four clades associated with the Scotia Arc Islands, one clade from the South Sandwich and South Atlantic Island
Mahon et al. (2008)	*Nymphon australe* (Pycnogonida)	COI 16S	COI 16S	Yes	Two genetically distinct lineages, revisited in Arango et al (2011).

Table 3-1 Phylogeographic investigations of benthic Antarctic invertebrates. Including the detection of genetically distinct lineages or populations and their observed distributions with reference to the previously considered biogeography of each taxon.

3.1.6 Biogeography of Antarctic polychaetes: current status

In comparison to other taxa, Antarctic polychaetes appear to have some of the widest distribution ranges amongst the benthic macrofaunal invertebrates (Schüller and Ebbe, 2007). Within the ANDEEP samples more than half of the identified polychaetes matched species found north of the polar front, 20% of which have also been found in the northern hemisphere (Brandt et al., 2007). Since ANDEEP, Schüller and Ebbe (2014) collated all georeferenced occurrences of validated RAMS polychaete species within the SCAR-MarBIN database. The authors found that out of the 403 polychaete species, the majority, 273 species, appear to be locally restricted and 128 considered circumpolar species. Additionally a recent large-scale biogeographic review of Antarctic polychaetes found depth to be the main structuring factor of polychaete communities (Neal et al., 2017). As discussed in Chapter 2 early identification of Antarctic polychaetes is, in part, a reflection of species names taken from existing monographs, usually of European fauna (e.g. Hartman, 1964). Subsequently, many species believed to be cosmopolitan may actually consist of several cryptic species or clades with locally or regionally restricted distributions (Table 3-1). Although less common, genetic evidence for bipolar, cosmopolitan species including the polychaete *Sclerolinum contortum* does exist as shown by the comparison of COI barcodes collected from individuals in the Southern Ocean, Gulf of Mexico and the Arctic Ocean (Georgieva et al., 2015).

3.1.7 Polychaete reproduction

Polychaetes are known to exhibit a variety of reproductive traits. In general, most species contain separate sexes and reproduce by releasing eggs and sperm into the water column. Alternatively polychaetes can undergo epitoky, a process where a part of the body becomes fertile (epitoke) and is released into the water column to reproduce, leaving the sterile counterpart (atoke) on the seafloor. Epitoky is limited to free-living forms but is very common in some families such as Syllidae (Franke, 1999, Rouse and Pleijel, 2006). Polychaetes can also be parthenogenic or hermaphroditic (Petersen, 1999, Fauchald, 1974). External gestation and brooding have also been reported (e.g. Franke, 1999) but most polychaetes produce pelagic (trochophore) larvae.

The dispersal potential of pelagic larvae beyond their parent population is regulated by many interacting biological and physical factors. These include larval type, development time, physiological tolerance and settlement cues etc, and oceanographic features that control the

movement of larvae vertically and geographically in open marine environments. Given the methodological difficulties of studying the early life stages of marine species and the inclusion of undescribed species within this study, species-specific reproductive trait data are not avaliable. However, family level data are publically avaliable on the Polytraits database (http://polytraits.lifewatchgreece.eu). Family level reproductive traits which may influence species dispersal and subsequent biogeography are listed in Table 3-2.

From meroplankton studies we also know that polychaete larvae are abundant within the Antarctic water column. For example more than a third of MOTUs identified during a five year larval barcoding study in the Ross Sea were polychaetes (Heimeier et al., 2010). Furthermore, DNA barcodes from Ross Sea larvae matched Hesionidae sp. A, *Aglaophamus trissophyllus* and *Laonice weddellia* barcodes of adult specimens in this study (Gallego et al., 2014, Heimeier et al., 2010). In Heimeier et al. (2010) the larval specimens were collected via a hole in the sea ice at approximately 50 m depth in the Austral summer between December and January (Sewell, 2005). An abundance of polychaete larvae at this time of year has also been noted in other Antarctic locations at shallow water depths (less than 200 m) including the Bellingshausen Sea, the Antarctic Peninsula and the South Shetland Islands (Freire et al., 2006, Bowden et al., 2009, Sewell and Jury, 2011, Ameneiro et al., 2012). This is thought to be a biological response to the increased food availability during the annual phytoplankton bloom (Pearse et al., 1991).

The number and duration of larval stages is variable across polychaete families and within species. This is because larval development can be coupled with environmental factors that influence growth, development rates (Qian and Chia, 1991, Prevedelli and Vandini, 1999) and the feeding method (Blake and Arnofsky, 1999). Within the Ross Sea, Sewell and Jury (2011) recorded an increase in the abundance of nectochaete (early stage polychaete larvae) before and after the spring phytoplankton bloom. During the bloom there was a decline in later stage larvae, which could be indicative of settlement events. The dual peak in early larval stages could thus suggest there is a mixture of both faster developing larvae, which may settle during summer, and slower developing larvae, which remain in the water column until the following winter. On the Antarctic Peninsula Mincks and Smith (2007) noted an increase in juvenile polychaete abundance within the top 5 cm of sediment in late winter. This again could be indicative of a seasonal recruitment. At the family level recruitment may vary; there was significant evidence for seasonal recruitment in Acrocirridae, marginal seasonality in

Cirratulidae and Paraonidae and no seasonal recruitment in Lumbrineridae and Spionidae (Mincks and Smith, 2007).

Family	Fertilisation type	Development type	Larval mode	Larval feeding mode
Acrocirridae		*No data*		
Cirratulidae	Broadcast spawner	Indirect	Pelagic and benthic	Lecithotrophic
Euphrosinidae		*No data*		
Glyceridae	Broadcast spawner	Indirect	Pelagic and benthic	Planktotrophic
Hesionidae	Internal fertilization and broadcast spawners	Indirect	Pelagic and benthic	Planktotrophic and Lecithotrophic
Lumbrineridae	Broadcast spawner	Indirect	Pelagic and benthic	Lecithotrophic
Maldanidae	Internal fertilization and broadcast spawners	Direct and indirect	Pelagic when applicable	Lecithotrophic
Nepthyidae	Broadcast spawner	Indirect	Pelagic	Planktotrophic
Paraonidae	Internal fertilization and broadcast spawners	Direct and indirect	Pelagic when applicable	Lecithotrophic
Polynoidae	Broadcast spawner	Indirect	Pelagic and benthic	Planktotrophic
Scalibregmatiadae		*No data*		
Spionidae	Internal fertilization and broadcast spawners	Indirect	Pelagic	Planktotrophic and Lecithotrophic

Table 3-2 Reproductive traits of the 12 polychaete families containing species from which DNA barcodes were collected in this study. Traits obtained from the Polytraits database (http://polytraits.lifewatchgreece.eu). Trait definitions are based on those stated on Polytraits but reduced to the traits recorded here. Fertilisation type: fertilisation can take place internally (within the female body) or externally often by broadcast spawning. Development type: the mode of development from the larval to adult stage either indirect (one or more successive free living larval stages) or direct (no intermediate larval stages). Larval mode: position of larval development either pelagic (in the water column) or benthic (near or on the seafloor). Larval feeding mode: either planktotrophic (larvae capture their own food) or lecithotrophic (maternal derived nutrition).

3.1.8 Aims

In this chapter the COI and 16S barcodes presented in Chapter 2 are used to examine the biogeographic distribution of each species and cryptic species identified. The number of sequences available are too few for population genetics so instead this chapter is a more traditional biogeographic analysis using evidence of species occurrence from genetic evidence. The findings are compared to current distribution ranges documented within literature and the RAMS database. Any biogeographic separation or connectivity (indicated by matching

haplotypes) is discussed with relevance to their biological traits and oceanographic factors using insight from particle tracking model outputs in Brasier et al. (2017).

3.2 Methods

3.2.1 Specimen selection and presumed distribution

The species and specimen selection criteria were established prior to this biogeographic investigation during the biodiversity analysis. Following secondary species identification using DNA barcodes, (for detailed methods see Chapter 2) some of the original target species were reassigned to other known taxa or molecular operational taxonomic units (MOTUs) including their genus name followed by 'sp. (MB#)'. The species for which biogeography will be investigated are shown in Table 3-3. Note that not all species described in Chapter 2 are included. Species exclusion from biogeographic analysis was due to either taxonomic uncertainties, both morphological and genetic, or a lack of replicate sequences.

Prior to genetic-level biogeographic analysis all species were assigned to one of three possible distribution categories: cosmopolitan, circumpolar or restricted, Table 3-3. This was based on their distribution records on the Register of Antarctic Marine Species (RAMS) or, if undescribed, their distribution within the BIOPEARL and JR275 sites and any matches with georeferenced sequences on GenBank. To clarify, 'cosmopolitan' species are those that have been recorded throughout the majority of the world's oceans and both hemispheres. 'Circum-Antarctic' species are those that have been collected within different regions of the Southern Ocean and are considered to be widespread Antarctic species. This is consistent with the most recent biogeographic review of Antarctic polychaetes, Schüller and Ebbe (2014), who considered species circum-Antarctic if there were georeferenced RAMS records from at least the Weddell Sea, Antarctic Peninsula or the Scotia Arc as well as the Ross Sea or Eastern Antarctica. Species were considered 'restricted' if there were only identified from one region e.g. only present within the BIOPEARL II Amundsen Sea samples.

Morphological species identification	Current distribution status	Barcoded species identification	Scotia Arc							Amundsen Sea		Weddell Sea	Depth range (m)
			FT	SR	SG	ST	PB	EI	LI	OA	IA	WS	
Glycera capitata	Cosmopolitan	*Glycera* sp. (MB1) *Glycera* sp. (MB2)	0	5	10	5	4	4	0	209	640	Y	200-1500
Scalibregma inflatum	Cosmopolitan	*Scalibregma* sp. (MB1) *Scalibregma* sp. (MB2) *Scalibregma* sp. (MB3)	0	0	3	6	0	3	44	0	38	Y	200-1000
Chaetozone sp. A	Cosmopolitan	*Chaetozone* sp. (MB1)**	0	3	0	2	70	16	18	14	123		200-1500
Laonice weddellia	Circum-Antarctic	*Laonice weddellia* (MB)	0	11	51	25	18	89	11	74	171	Y	200-1500
Aricidea simplex	Circum-Antarctic	*Aricidea simplex* (MB) *Aricidea* cf. *belgicae* (MB1) *Aricidea* cf. *belgicae* (MB2) *Aricidea* cf. *belgicae* (MB3)	0	1	0	0	21	2	6	29	143	Y	200-1000
Euphrosinella cirratoformis	Circum-Antarctic	*Euphrosinella* cf. *cirratoformis* (MB1) *Euphrosinella* cf. *cirratoformis* (MB2)	0	85	0	0	28	0	0	67	46		200-1500
Lumbrineris kergulensis-cingulata	Circum-Antarctic	*Lumbrineris kergulensis-cingulata* (MB1)**	0	4	15	11	44	0	1	31	121		200-1500
Aglaophamus trissophyllus *Aglaophamus digitatus* *Aglaophamus foliosus*	Circum-Antarctic	*Aglaophamus trissophyllus* (MB1a) *Aglaophamus* cf. *trissophyllus* (MB1b) *Aglaophamus* cf. *trissophyllus* (MB1c) *Aglaophamus* sp. (MB2) *Aglaophamus* sp. (MB3)	1	6	21	5	0	0	0	12	353	Y	300-1000
Harmothoe fuligineum	Circum-Antarctic	*Harmothoe fuligineum* (MB)	0	0	45	10	5	0	0	158	534		200-1500
Macellicephala sp. A	Restricted* Restricted*	*Macellicephala* sp. (MB1) *Macellicephala* sp. (MB2)	0	0	0	0	0	0	0	177	124		500-1500
Macellicephaloides sp. B	Restricted*	*Macellicephaloides* sp. (MB1)	0	0	0	0	0	0	0	15	60		500-1500
Hesionidae sp. A	Circum-Antarctic*	Hesionidae sp. (MB1) Hesionidae sp. (MB2)	1	11	43	1	24	1	0	97	556		200-1500
Flabelligena sp. A	Restricted*	*Flabelligena* sp. A (MB)	0	0	0	0	0	0	0	47	1038		500
Flabelligena sp. B	Restricted*	*Flabelligena* sp. B (MB)	0	0	0	0	0	0	0	6	350		500

Table 3-3 Species/clades for which the biogeography will be investigated including their primary identification based on morphological analysis alone, their secondary identification based on both DNA barcodes of COI and/or 16S genes and morphological analysis and the presumed biogeography of the species based on distribution records of the species primary identification. *indicates apparent biogeographic distributions for unnamed taxa. ** Indicates species contained multiple clades considered to be a species complex, clades labelled with additional letters in results section. Sites in the Scotia Arc include FT = Falkland Trough, SR = Shag Rocks, SG = South Georgia, ST = Southern Thule, PB = Powel Basin, EI = Elephant Island, DI = Deception Island., For the Amundsen Sea OA = outer Amundsen and IS = inner Amundsen, and, WS = Weddell Sea.

3.2.2 Data analysis

Georeferenced haplotype networks of the sequenced BIOPEARL and JR275 specimens were used to visualise species distributions and decipher biogeographic patterns. If sequence matches within the GenBANK or BOLD databases were found these were also included in the networks (Table 3-4). To avoid problems with gaps, all sequences of the same species were trimmed in Mesquite (Version 2.75) to the same length following MAFFT (for 16S) or MUSCLE (for COI) sequence alignment in Geneious (R7). GPS coordinates were assigned to each sequence for its given sample location, and networks constructed using statistical parsimony (Templeton et al., 1992) and the TCS programme (Clement et al., 2000) in PopART (Leigh and Bryant, 2015) for editing in Corel Draw. Following preliminary analysis, for the presumed cosmopolitan and circum-Antarctic species, sites within the inner and outer Amundsen Sea would be combined, as were those within the Weddell Sea. This was decided because there was no observed biogeographic structuring within these regions between widespread cryptic species. Haplotype networks with depth referenced sequences were also created using the four depth bins; <500 m, 500 m, 1000 m, 1500 m and >2000 m.

As the recovery of 16S sequences was more successful in all species examined, providing a greater number of individuals and a larger biogeographic area, this gene was favoured over COI. If the COI data revealed cryptic species unidentifiable from the 16S data e.g. Hesionidae spp., the COI sequences were used, prioritising taxonomic accuracy at the expense of the number of individuals and biogeographic coverage.

MB species	Gene	GenBANK Name	GenBANK Accession No	Reference
Hesionidae sp. A	16S	Cf. Hesionidae sp. DH-2009	GU227022	Heimeier et al. (2010)
Lumbrineris kergulensis-cingulata	16S	Cf. Lumbrineridae sp. DH-2009	GU227023	Heimeier et al. (2010)
Aglaophamus trissophyllus	COI	Cf. Nepthyidae sp. DH-2009	GU227129	Heimeier et al. (2010)
Aglaophamus trissophyllus	16S	Cf. Nepthyidae sp. DH-2009	GU227024	Heimeier et al. (2010)
Harmothoe fuligineum	16S	*Harmothoe fuligineum*	KJ676609	Neal et al. (2014)
Laonice weddellia	16S	*Laonice* sp. A RG-2014	KF713471	Gallego et al. (2013)
MB species	Gene	BOLD Name	BOLD Database	Reference
Aglaophamus trissophyllus	COI	*Aglaophamus trissophyllus*	Private	Ravara (2015)

Table 3-4 Non MB sequences included in the haplotype networks, including details of both GenBANK and BOLD sequences.

3.2.3 Particle tracking models to estimate larval dispersal

The haplotype networks presented in this chapter were published alongside particle tracking analysis in a collaborative project with Dr James Harle (National Oceanography Centre) in Brasier et al. (2017). The model inputs were proposed, discussed and determined by all authors but the models themselves were coded and run by James. As I cannot deem ownership of the model data they are not presented within this chapter; however the genetic results will be compared to the model findings to help understand larval dispersal in the western Antarctic. By combining results from both genetic analysis and bio-physical models a greater insight into the role of larval dispersal in population connectivity is achieved. Genetic data can provide evidence of population connectivity, whilst models can help understand how they might maintain it (e.g. Gilg and Hilbish, 2003, Young et al., 2015).

3.3 Results

3.3.1 Presumed cosmopolitan species

During phylogenetic analysis discussed in Chapter 2, it was concluded that both of the presumed cosmopolitan species targeted, *Glycera capitata* and *Scalibregma inflatum,* are made up of several cryptic species. Nucleotide differences between publically available sequences obtained from specimens from the Northern Hemisphere, at or closer to their type locality, suggest that a cosmopolitan distribution is unlikely. Thus the biogeography of the sequenced

BIOPEARL and JR275 material is analysed to see if they are potentially circum-Antarctic or restricted within the Southern Ocean.

Both of the *Glycera* species identified were sequenced from all three of the sampled regions (Figure 3-2a). Of the 15 sequenced specimens identified as *Glycera* sp. (MB1) the majority, nine individuals, were collected in the Scotia Arc, including Livingston Island, South Georgia and Shag Rocks. Fewer haplotypes were recorded from the Amundsen Sea and Weddell Sea, with 2 and 4 individuals respectively. Similar numbers of *Glycera* sp. (MB2) were sequenced, 17 in total, contrastingly this species was more abundant in the Amundsen than the Scotia Arc with 12 and 2 individuals respectively. The depth distribution of these individuals was also variable between the two cryptic species. *Glycera* sp. (MB1) haplotypes were obtained from specimens at 500m depth or shallower where as *Glycera* sp. (MB2) haplotypes were only obtained from specimens at 500 m depth or deeper.

Of the three cryptic species identified from the *Scalibregma inflatum* morphospecies, *Scalibregma* sp. (MB1) had the largest distribution, collected from all three of the sampled regions at depths of 500 m or shallower. Nine of the 14 *Scalibregma* sp. (MB1) haplotypes were from specimens within the Amundsen Sea, four were from the Scotia Arc (Livingston Island and Elephant Island) and one from the Weddell Sea (Figure 3-2b). *Scalibregma* sp. (MB3) was restricted to Elephant Island with all four representatives collected from 500 m depth or shallower. The single representative of *Scalibregma* sp. (MB2) was collected from 1000 m depth at the Southern Thule site.

High sequence diversity within the morphospecies *Chaetozone* cf. *setosa* was noted in Chapter 2. Due to uncertainties in the identification and number of species only *Chaetozone* sp. (MB1) is included for biogeographic analysis. Subsequently, the potential for this species to be cosmopolitan seems unlikely and here, it is investigated for circumpolarity. The haplotype network shows the three clades identified in Chapter 2, *Chaetozone* sp. (MB1a), (MB1b) and (MB1c) (Figure 3-2c). With the sequences available two of these groups appear restricted. *Chaetozone* sp. (MB1a) haplotypes were collected from specimens at 1500 m in the Scotia Arc, from the Powell Basin and Elephant Island and *Chaetozone* sp. (MB1c) was represented by a single specimen from the outer Amundsen Sea from 500 m depth. Haplotypes of *Chaetozone* sp. (MB1b) were also collected from the same Scotia Arc sites as well as Livingston Island and the Amundsen Sea at depths of 200 to 1000 m. Thus *Chaetozone* sp. (MB31b) appears to be the most widespread and could potentially be circum-Antarctic possibly restricted to depths of 1000 m or shallower.

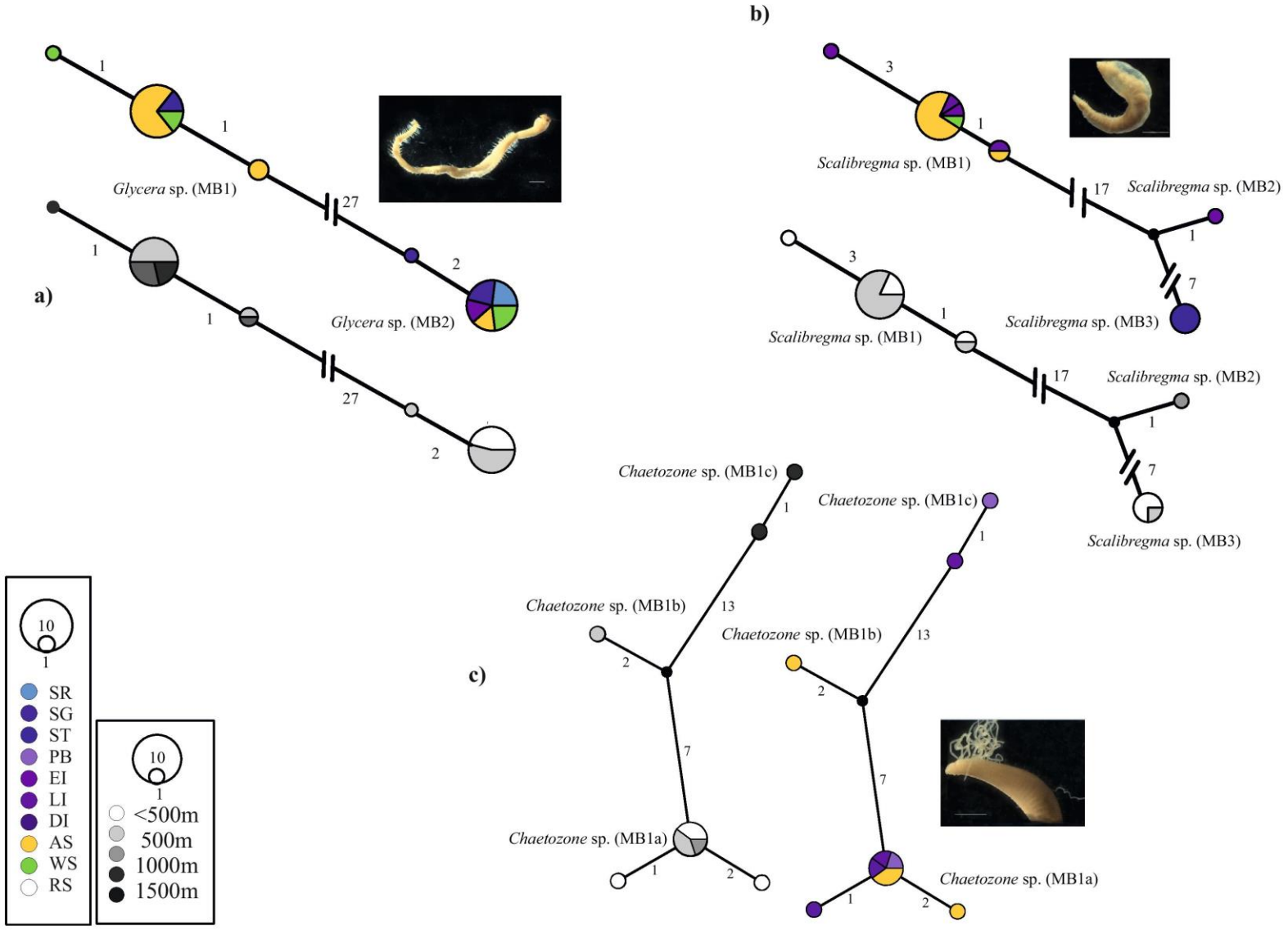

a)
Glycera sp. (MB1)
Glycera sp. (MB2)
b)
Scalibregma sp. (MB1)
Scalibregma sp. (MB2)
Scalibregma sp. (MB3)
c)
Chaetozone sp. (MB1c)
Chaetozone sp. (MB1b)
Chaetozone sp. (MB1a)
SR
SG
ST
PB
EI
LI
DI
AS
WS
RS
<500m
500m
1000m
1500m

Figure 3-2 Georeferenced and depth binned haplotype networks for a) *Glycera* spp, b) *Scalibregma* spp and c) *Cheatozone* sp. (MB3) all networks constructed using 16S sequences, numbers indicate the number of nucleotide differences between haplotypes, black circles indicate missing haplotypes. Coloured circles relate to sites where SR = Shag Rocks, SG = South Georgia, ST = Southern Thule, PB = Powel Basin, EI = Elephant Island, DI = Deception Island, AS = Amundsen Sea, WS = Weddell Sea and RS = Ross Sea, greyscale for depth binned networks.

3.3.2 Presumed Circum-Antarctic species

Of the presumed circum-Antarctic species there was no evidence for cryptic species within *Laonice weddellia, Aricidea simplex* and *Harmothoe fuligineum.* All three of these species appear to be widespread in the West Antarctic and potentially circum-Antarctic. *Laonice weddellia* (MB) haplotypes were obtained from all three of the sampled regions at depths of <500 to 1500 m (Figure 3-3a). Additionally a larval sequence from the Ross Sea matched one of the *Laonice weddellia* (MB) haplotypes extending the observed distribution of this species (Table 3-4).

Harmothoe fuligineum was the most abundant Polynoidae within the BIOPEARL samples however no individuals were identified from the Weddell Sea samples (Table 3-3). *Harmothoe fuligineum* (MB) sequences from South Georgia matched individuals collected within the Amundsen Sea (Figure 3-3b). Although additionally there were unique haplotypes from the Amundsen Sea. All sequenced specimens were collected at 500 m depth or shallower.

In Chapter 2 sequence and secondary morphological analysis found that the morphospecies *Aricidea simplex* also contained individuals from the species *Aricidea belgicae*. The biogeography of these two species, both presumed to be circum-Antarctic, is investigated separately (Figure 3-3c, d). The most frequent haplotype of *Aricidea simplex* (MB) was sequenced from the outer Amundsen Sea, Elephant Island and the Weddell Sea (Figure 3-3c). Additional haplotypes represented by single individual were found at the Weddell Sea, Amundsen Sea and the Shag Rocks sites. The depth distribution of the *Aricidea simplex* (MB) haplotypes ranged from <500 to 1000 m with.

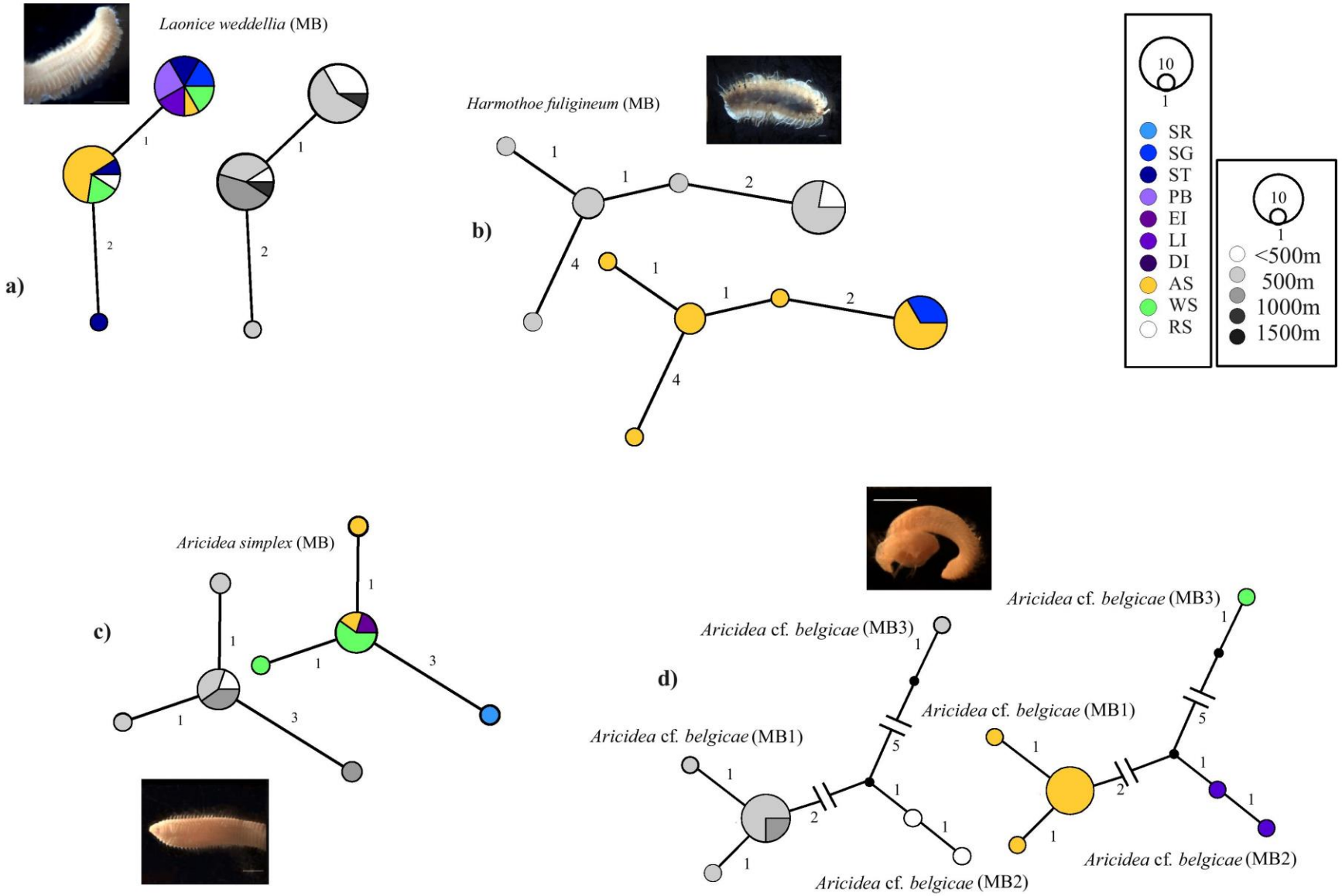

Laonice weddellia (MB)
a)
Harmothoe fuligineum (MB)
b)
10
1
SR
SG
ST
PB
EI
LI
DI
AS
WS
RS
<500m
500m
1000m
1500m
Aricidea simplex (MB)
c)
Aricidea cf. belgicae (MB3)
Aricidea cf. belgicae (MB1)
Aricidea cf. belgicae (MB2)
d)

Figure 3-3 Georeferenced and depth binned haplotype networks for a) *Laonice weddellia,* b) *Harmothoe fuligineum,* c) *Aricidea simplex* and d) *Aricidea* cf. *belgicae* spp. All networks constructed using 16S sequences, numbers indicate the number of nucleotide differences between haplotypes, black circles indicate missing haplotypes. Coloured circles relate to sites where SR = Shag Rocks, SG = South Georgia, ST = Southern Thule, PB = Powel Basin, EI = Elephant Island, DI = Deception Island, AS = Amundsen Sea, WS = Weddell Sea and RS = Ross Sea, greyscale for depth binned networks.

The specimens re-identified as *Aricidea* cf. *belgicae* (MB#) were collected from the Amundsen Sea, Scotia Arc (Livingston Island) and the Weddell Sea. The three genetically distinct clades (and potential cryptic species) of *Aricidea* cf. *belgicae* (MB#) were restricted to a single region (Figure 3-3d). *Aricidea* cf. *belgicae* (MB1) haplotypes were only obtained from the Amundsen Sea, *Aricidea* cf. *belgicae* (MB2) from the Weddell Sea and *Aricidea* cf. *belgicae* (MB3) from Livingston Island. The haplotypes of *Aricidea* cf. *belgicae* (MB1) had the greatest depth range of 500 to 1000 m where as *Aricidea* cf. *belgicae* (MB2) and *Aricidea* cf. *belgicae* (MB3) were only found at 500 and 200 m respectively.

Two cryptic species of *Euphrosinella cirratoformis* were identified in Chapter 2, the most abundant of which was *Euphrosinella* cf. *cirratoformis* (MB1). Two haplotypes of this species were found in the Amundsen Sea and two in the Scotia Arc (Figure 3-4a). For *Euphrosinella* cf. *cirratoformis* (MB2) all sequenced specimens belonged to one haplotype obtained from the Amundsen Sea at 500 to 1000 m depth. This was slightly deeper than the depth range for *Euprhosinella* cf. *cirratoformis* (MB1) which ranged from 200 to 500 m depth.

Despite its lack of formal description, Hesionidae sp. A was considered to be a circum-Antarctic species. This is based on the species presence in the Scotia Arc and Amundsen Sea sites (Table 3-3) and, the sequence matches with larval sequences from the Ross Sea (Table 3-4).

Both cryptic species, Hesionidae sp. (MB1) and Hesionidae sp. (MB2), contained haplotypes collected from the Amundsen Sea and the Powell Basin within the Scotia Arc (Figure 3-4b). Hesionidae sp. (MB1) was also sequenced from South Thule and an individual Hesionidae sp. (MB2) from South Georgia. The depth distribution of the two cryptic species is overlapping but Hesionidae sp. (MB2) was shallower, ranging from 200 to 500 m, than Hesionidae sp. (MB1) which ranged from 500 to 1500 m.

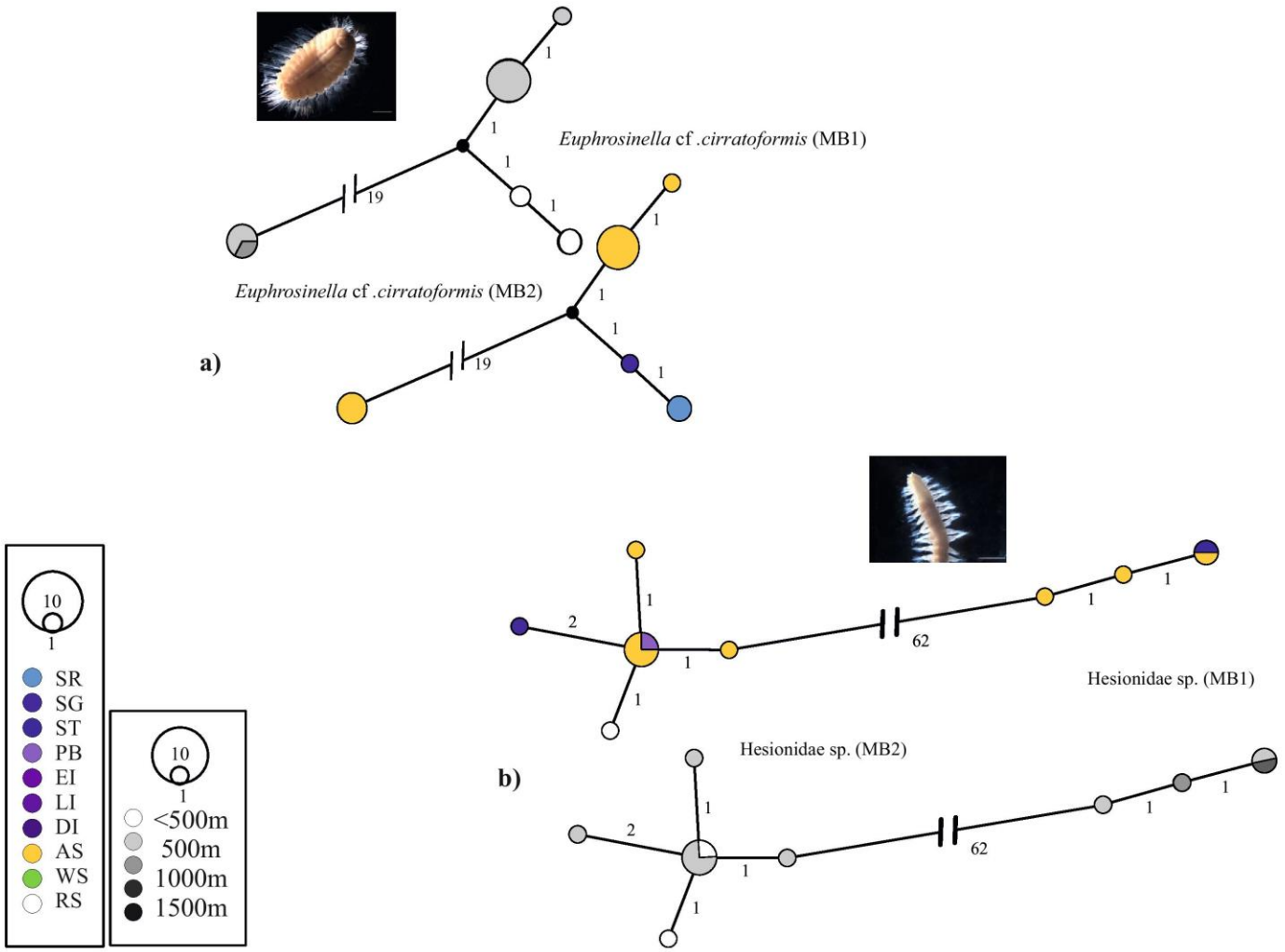
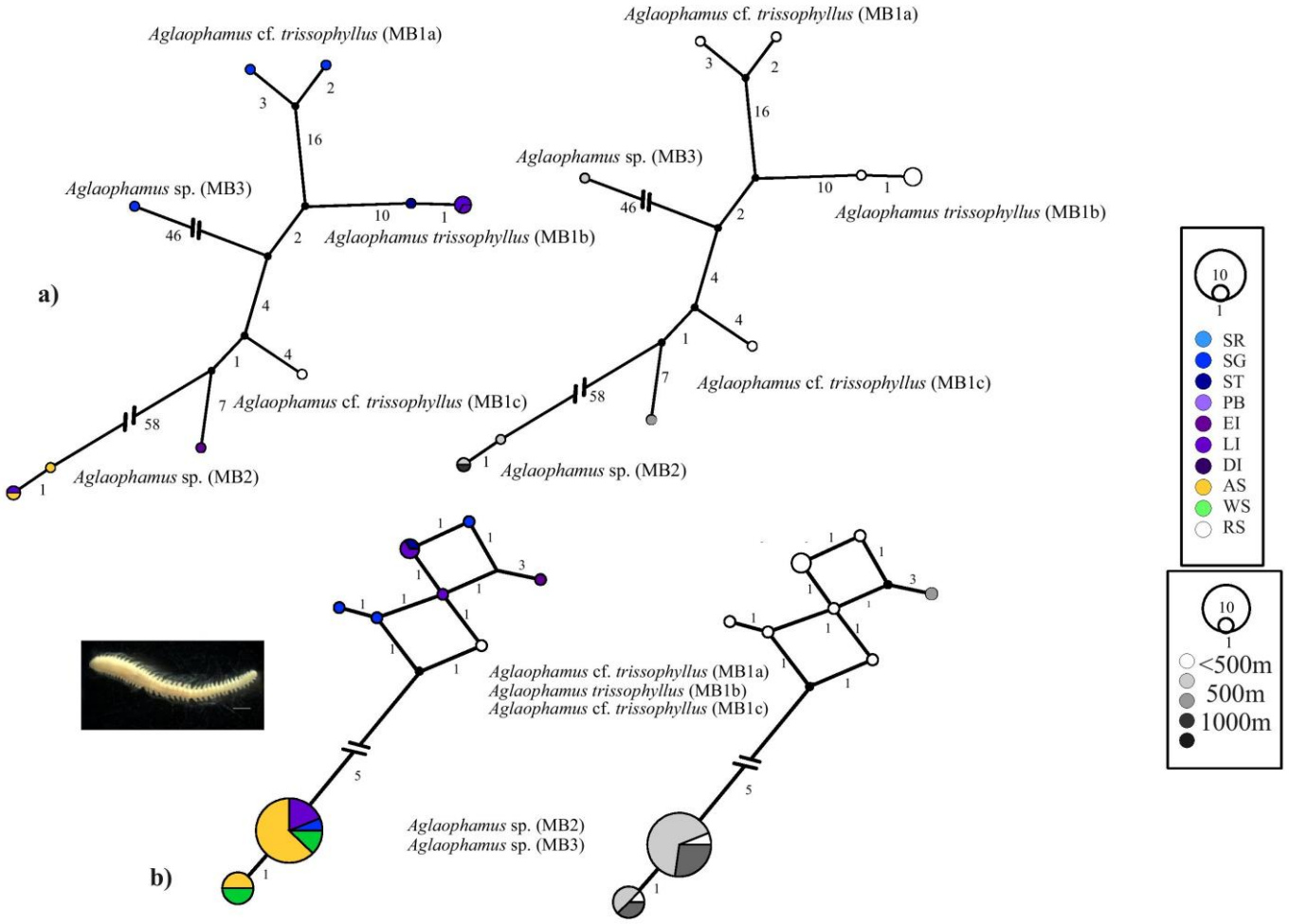

a)
Euphrosinella cf .cirratoformis (MB1)
Euphrosinella cf .cirratoformis (MB2)
b)
Hesionidae sp. (MB1)
Hesionidae sp. (MB2)
10
1
SR
SG
ST
PB
EI
LI
DI
AS
WS
RS
<500m
500m
1000m
1500m

Figure 3-4 Georeferenced and depth binned haplotype networks for a) *Euphrosinella* cf. *cirratoformis,* and b) Hesionidae spp. Networks were constructed using either 16S (a) or COI (b) sequences, numbers indicate the number of nucleotide differences between haplotypes, black circles indicate missing haplotypes. Coloured circles relate to sites where SR = Shag Rocks, SG = South Georgia, ST = Southern Thule, PB = Powel Basin, EI = Elephant Island, DI = Deception Island, AS = Amundsen Sea, WS = Weddell Sea and RS = Ross Sea, greyscale for depth binned networks.

The diversity results from COI and 16S phylogenetic analysis of *Aglaophamus trissophyllus* were variable. The COI results revealed five distinct clades, three of which (*Aglaophamus trissophyllus* (MB1b)*, Aglaophamus* cf. *trissophyllus* (MB1a) and (MB1c)) were considered to be a species complex. The other two clades, *Aglaopahmus* sp. (MB2) and *Aglaophamus* sp. (MB3) were identified as potential cryptic species of *Aglaophamus trissophyllus*. For 16S the genetic diversity was lower, the *Aglaophamus* cf. *trissophyllus* complex were contained within the same clade and *Aglaopahmus* sp. (MB2) and *Aglaophamus* sp. (MB3) formed a second clade. Both COI and 16S haplotype networks are shown here for comparison (Figure 3-5).

For the *Aglaophamus* cf. *trissophyllus* complex all COI and 16S sequences were obtained from specimens within the Scotia Arc (Livingston Island, Elephant Island, South Georgia and Southern Thule), as well as an additional larval sequence from the Ross Sea. All bar one of these specimens were collected at depths less than 500 m which was collected from 1000 m. The *A. trissophyllus* reference sequence on BOLD was also included in this network. The sequence was obtained from a specimen from Deception Island, also in the Scotia Arc, located just south of Livingston Island. The COI clade of *Aglaophamus* sp. (MB2) included individuals from both the Scotia Arc, Livingston Island and the inner Amundsen Sea at depths of 500 to 1500 m. The *Aglaophamus* sp. (MB3) COI clade was represented by a single individual collected from South Georgia at 500 m depth. A greater number of 16S sequences were obtained which extends the observed range the *Aglaophamus* sp. (MB2) and (MB3) to include Amundsen and Weddell Seas.

Seven MOTUs were assigned to the *Lumbrineris kerguelensis-cingulata* sequences in Chapter 2. Most clades were represented by only one or two individuals from the Scotia Arc (Figure 3-6). The exceptions to this include *L. kerguelensis-cingulata* (MB1a) containing specimens from the Amundsen Sea and *L. kerguelensis-cingulata* (MB1f) which included a larval sequence from the Ross Sea and Amundsen Sea. Other than *L. kerguelensis-cingulata* (MB1b), which was sequenced from the Powell Basin at 1500 m, all other haplotypes were sequenced from specimens from 200 to 500 m depth.


a)
Aglaophamus cf. trissophyllus (MB1a)
Aglaophamus sp. (MB3)
Aglaophamus trissophyllus (MB1b)
Aglaophamus cf. trissophyllus (MB1c)
Aglaophamus sp. (MB2)
b)
Aglaophamus cf. trissophyllus (MB1a)
Aglaophamus trissophyllus (MB1b)
Aglaophamus cf. trissophyllus (MB1c)
Aglaophamus sp. (MB2)
Aglaophamus sp. (MB3)
SR
SG
ST
PB
EI
LI
DI
AS
WS
RS
<500m
500m
1000m

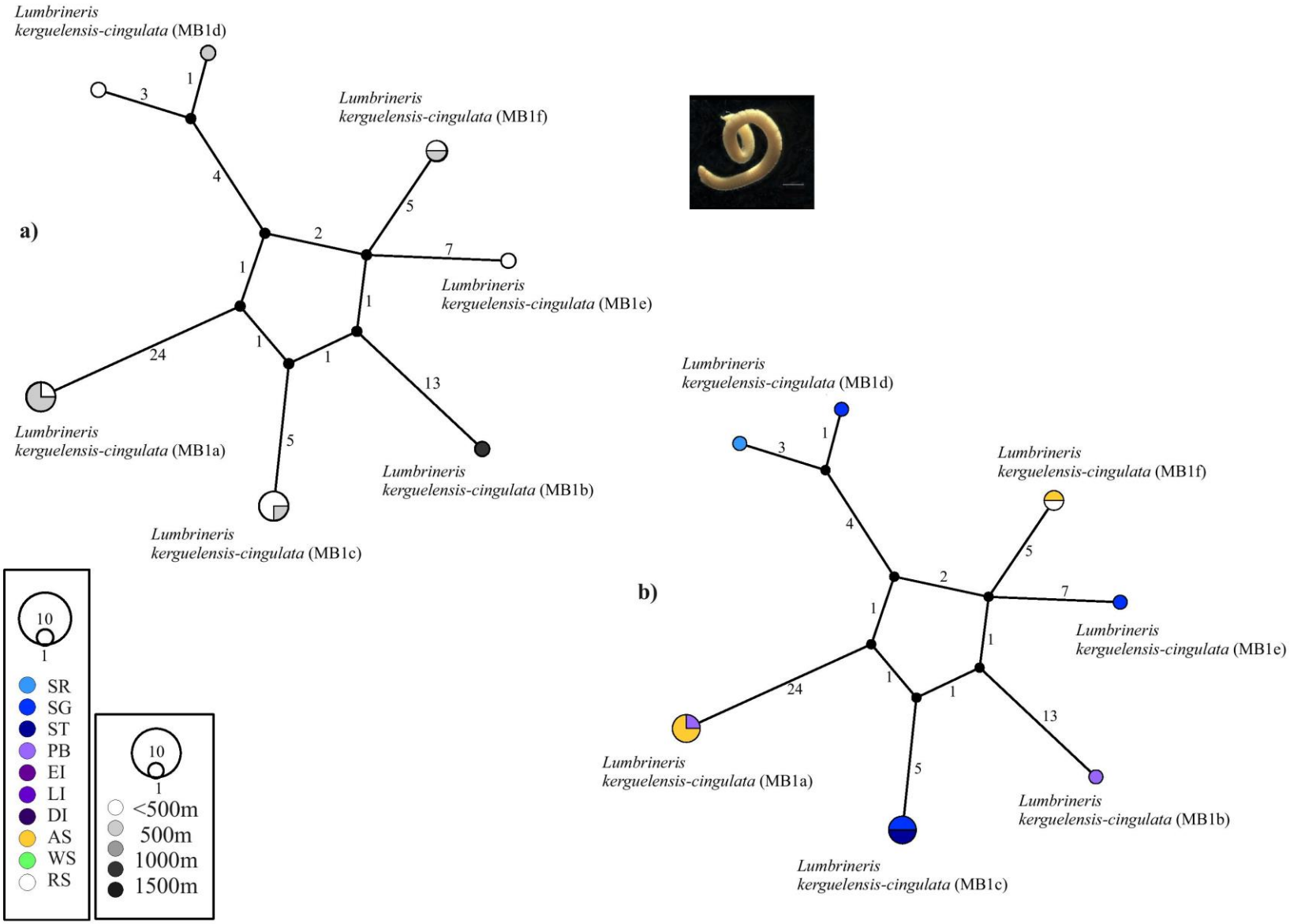

a)
Lumbrineris kerguelensis-cingulata (MB1d)
Lumbrineris kerguelensis-cingulata (MB1f)
Lumbrineris kerguelensis-cingulata (MB1e)
Lumbrineris kerguelensis-cingulata (MB1a)
Lumbrineris kerguelensis-cingulata (MB1b)
Lumbrineris kerguelensis-cingulata (MB1c)
1
3
4
5
2
7
1
1
24
1
1
13
5
10
1
SR
SG
ST
PB
EI
LI
DI
AS
WS
RS
10
1
<500m
500m
1000m
1500m
b)
Lumbrineris kerguelensis-cingulata (MB1d)
Lumbrineris kerguelensis-cingulata (MB1f)
Lumbrineris kerguelensis-cingulata (MB1e)
Lumbrineris kerguelensis-cingulata (MB1a)
Lumbrineris kerguelensis-cingulata (MB1b)
Lumbrineris kerguelensis-cingulata (MB1c)

Figure 3-5 Georeferenced and depth binned haplotype networks for *Aglaophamus* spp constructed using COI (a) and 16S (b) sequences numbers indicate the number of nucleotide differences between haplotypes, black circles indicate missing haplotypes. Note that for 16S the clades within *Aglaophamus trissophyllus, Aglaophamus* cf. *trissophyllus* (MB1) and *Aglaophamus* cf. *trissophyllus* (MB2) as well as *Aglaophamus* sp. (MB) and (MB4) could not be resolved from sequence analysis. Coloured circles relate to sites where SR = Shag Rocks, SG = South Georgia, ST = Southern Thule, PB = Powel Basin, EI = Elephant Island, DI = Deception Island, AS = Amundsen Sea, WS = Weddell Sea and RS = Ross Sea, greyscale for depth binned network.

Figure 3-6 Georeferenced and depth binned haplotype networks for *Lumbrineris kergulensis-cingulata* constructed using 16S sequences, numbers indicate the number of nucleotide differences between haplotypes, black circles indicate missing haplotypes. Coloured circles relate to sites where SR = Shag Rocks, SG = South Georgia, ST = Southern Thule, PB = Powel Basin, EI = Elephant Island, DI = Deception Island, AS = Amundsen Sea, WS = Weddell Sea and RS = Ross Sea, greyscale for depth binned networks

3.3.3 Restricted species

All species that were considered restricted were undescribed morphospecies identified from material collected within the Amundsen Sea sites. This restriction was based on their absence within the Scotia Arc or Weddell Sea samples and no sequence matches with other localities on GenBank. These four species included the two polynoids *Macellicephala* sp. A and *Macellicephaloides* sp. B and two Acrocirridae species *Flabelligena* sp. A and sp. B.

There was no evidence of cryptic species present within either of the *Flabelligena* morpho-species. *Flabelligena* sp. B (MB) had greatest number of nucleotides variations, with up to nine variable sites between haplotypes, but lacked any obvious divergence or group formation (Figure 3-7b). *Flabelligena* sp. A (MB) had more haplotypes overall, eight in total, but all haplotypes radiated from a central haplotype by one nucleotide difference (Figure 3-7a). No depth networks were constructed for this species as they were only collected from the 500 m samples.

Two cryptic species were identified from phylogenetic analysis of the *Macellicephala* sp. A sequences. *Macellicephala* sp. (MB2) was only sequenced from the inner Amundsen Sea whilst *Macellicephala* sp. (MB1) from both the inner and outer sites (Figure 3-7c). The two cryptic species had the same depth range from 500 to 1000 m. The *Macellicephaloides* sp. B network (Figure 3-6d) highlights the genetic diversity discussed in Chapter 2, with the ten haplotypes sequenced from the twelve individuals. The network lacks any obvious divergence or groupings which complicated the determination of any cryptic species or restricted populations. *Macellicephaloides* sp. (MB) haplotypes were obtained from individuals within the inner and outer Amundsen Sea sites covering a depth range of 500 to 1000 m.

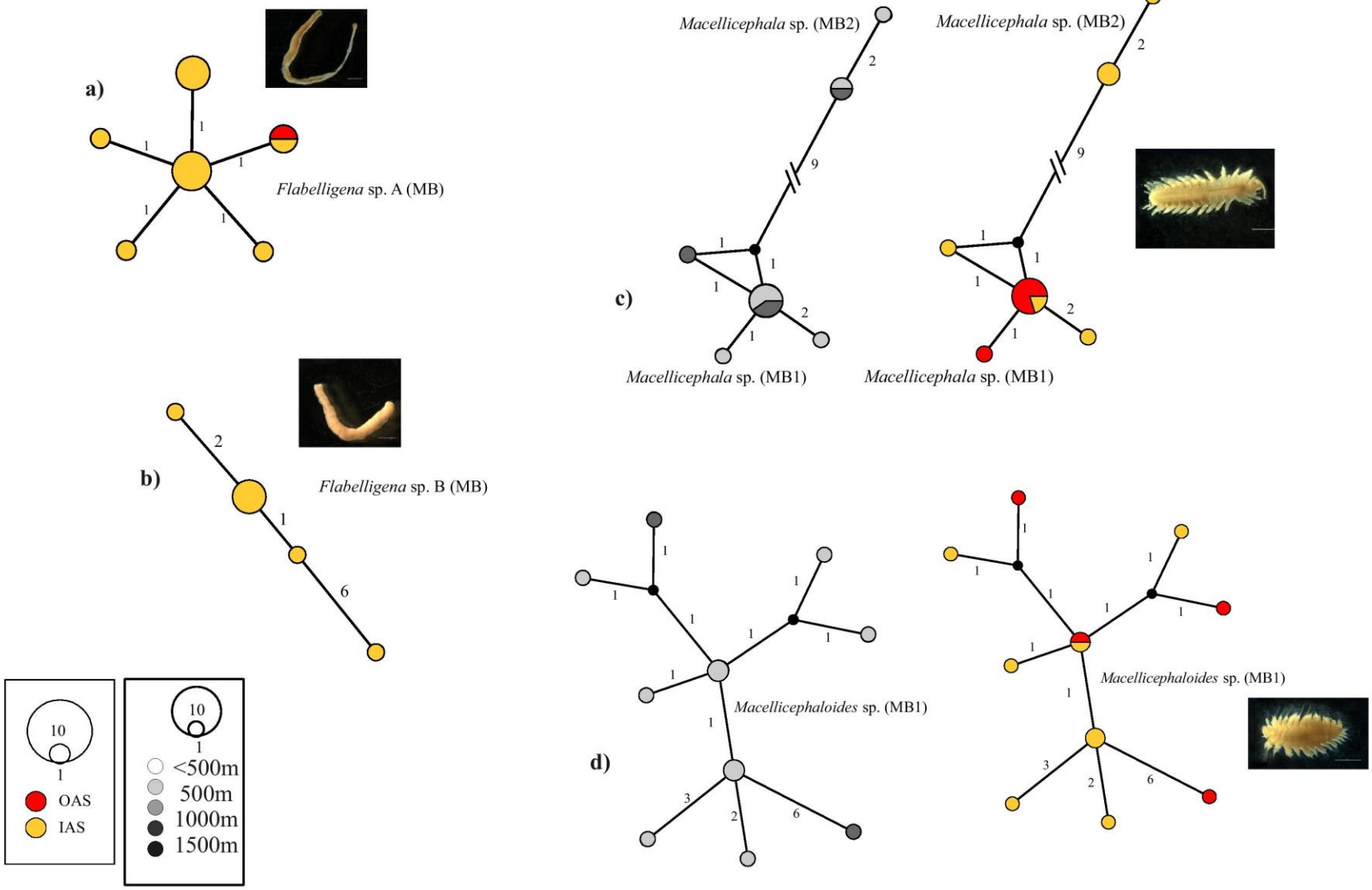

a)
Flabelligena sp. A (MB)
b)
Flabelligena sp. B (MB)
c)
Macellicephala sp. (MB2)
Macellicephala sp. (MB1)
Macellicephala sp. (MB2)
Macellicephala sp. (MB1)
d)
Macellicephaloides sp. (MB1)
Macellicephaloides sp. (MB1)
OAS
IAS
<500m
500m
1000m
1500m

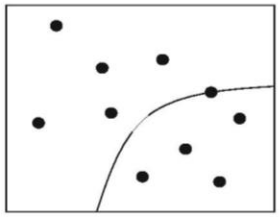
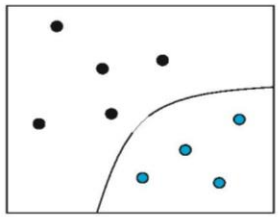
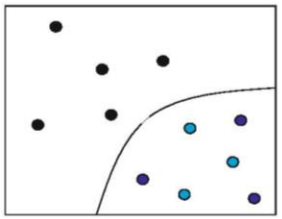
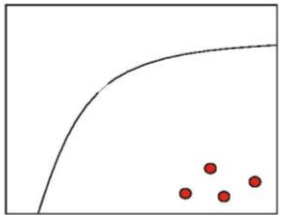
Figure 3-7 Georeferenced and depth binned haplotype networks of a) *Flabelligena* sp. A, b) *Flabelligena* sp. B, c) *Macellicephala* spp and d) *Macellcepahloides* sp. B, all networks constructed using 16S seuqences, numbers indicate the number of nucleotide differences between haplotypes, black circles indicate missing haplotypes. Coloured circles relate to sites where OAS = outer Amundsen Sea and IAS = inner Amundsen Sea, greyscale for depth binned networks.

3.4 Discussion

3.4.1 Revaluating 'cosmopolitan species' and Antarctic endemism

Prior to DNA barcoding three of the targetted morphospecies, *Glycera capitata*, *Scalibregma inflatum* and *Chaetozone setosa*, were considered to be globally cosmopolitan. Here we deem them genetically distinct from Northern populations and more likely cryptic circum-Antarctic species (Table 3-5). The existence of cosmopolitan species is often based on early identifications. Additionally it was previously accepted that marine environments lacked barriers to species dispersal (Palumbi, 1992, 1994). We now know that species dispersal is limited by ecological factors including physiological tolerance, competition and life history as well as biogeographical barriers. Other examples of previously accepted cosmopolitan polychaete species found to contain more restricted cryptic clades were identified in Carr et al. (2011)'s tri-oceanic investigation. These included *Lepidonotus squamatus, Alitta virens, Pectinaria granulata, Nereis pelagica* and *Harmothoe rarispina.*

In the Southern Ocean the formation of the ACC led to the isolation of Antarctic fauna around 41 million years ago (Rogers, 2007). For a species to establish populations across the ACC, organisms need to be capable of traveling or dispersing over great distances (>850km), over considerable depths (>4000m), across temperature inclines of 3-4^{o}C and against strong prevailing currents (Eastman, 1993). Thus it is perhaps not surprising many Antarctic marine fauna are endemic, current estimates range from 50 to 97% amongst taxa (De Broyer et al., 2014). Many morphologically identical species either side of the Antarctic Polar Front are genetically distinct cryptic clades with restricted biogeographic distributions (Strugnell and Allcock, 2013).

Previous distribution	Observed distribution	Schematic	Observed in
Cosmopolitan	One cosmopolitan species		-
	Cryptic species identified, endemic to the Southern Ocean		*Chaeotzone* cf. *setosa*
	More than one cryptic species identified, all endemic to the Southern Ocean existing sympatrically		*Glycera* spp. *Scalibregma* spp.
Restricted	Restricted population within the Southern Ocean		*Macellicephala* spp. *Macellicephaloides* sp. B *Flabelligena* spp.

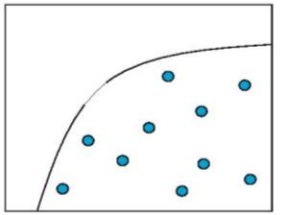
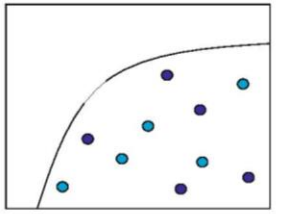
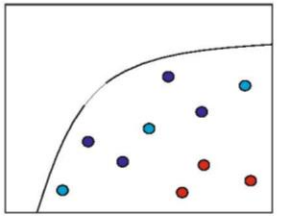
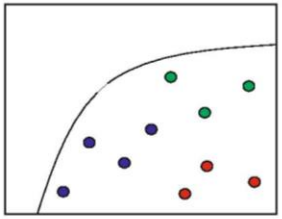
Previous distribution	Observed distribution	Schematic	Observed in
Circum-Antarctic	One cicrum-Antarctic species		*Laonice weddellia* *Aricidea simplex* *Harmothoe fuligineum*
	Two circum-Antarctic cryptic species existing sympatrically		Hesionidae sp. A *Lumbrineris cingulata-kergulensis* *Aglaophamus* spp.
	One or more circum-Antarctic cryptic species and one restricted population existing sympatrically		*Euphrosinella* spp.
	Two or more cryptic species within the Southern Ocean existing allopatrically		*Aricidea* cf. *belgicae* spp.

Table 3-5 Descriptions of the observed distributions with schematics of the examined moprhospecies compared to their previous distribution prior to DNA barocding. Within the schematics the curved line indicates the position of the Polar Front (the northern boundary of the Southern Ocean) and the dots species existence. Different colours indicate genetically distinct clades/species.

Despite the physiological and biogeographic barriers some eurybathic species may be able to disperse across the deeper areas of the Drake Passage. This could be aided by dispersal via thermohaline circulation and subsequently some species may maintain genetic connectivity and large species distributions across the Polar Front (Strugnell et al., 2008). An example of this includes the nemertean *Parborlasia corrugatus.* However, Thornhill et al. (2008) compared 16S sequences from individuals either side of the ACC and although a single well-mixed lineage was identified between Antarctic and sub-Antarctic regions, there was no evidence of recent gene flow between these populations and those of South America. It is now thought that *P. corrugatus* contains at least two cryptic species with reduced biogeographic ranges. Similar results have also been found for the nudibranch *Doris kerguelensis* (Wilson et al., 2009) and the pynogonid *Colossendeis megalonyx* (Krabbe et al., 2010).

3.4.2 Circum-Antarctic species

In both cryptic and non-cryptic species there was an abundance of potentially circum-Antarctic species as defined by Schüller and Ebbe (2014). Shared haplotypes were found between the Scotia Arc, the Amundsen Sea and, where available, the Weddell and Ross Seas. This sequence similarity may indicate genetic connectivity between these regions (Arango et al., 2011). Seven of the ten circum-Antarctic morphospecies contained cryptic representatives. In each case at least one of their cryptic clades contained specimens from all three sampled regions or matched sequences from other Antarctic localities. Furthermore these cryptic clades exist sympatrically, covering the same or overlapping regions of the western-Antarctic (Table 3-5).

The abundance of circum-Antarctic sympatric cryptic species questions how this distribution was established. It is now generally accepted that the majority of cryptic species within Antarctic water arose from physically-separated populations during glaciations (Allcock et al., 2001, Thatje et al., 2005). Thus the existence of sympatric species could suggest they evolved due to another method of isolation, for example differences in reproductive traits (Palumbi, 1994), responses to competition (Alizon et al., 2008) or predation (Wilson et al., 2013).

An alternative explanation is that their circum-Antarctic distribution was established after they evolved, possibly during post-glacial re-colonisation. Given the limited size and mobility of these benthic polychaetes, it is unlikely that adult specimens migrate between the three regions

sampled. Instead, connectivity between adult populations could be maintained by larval dispersal. Although the reproductive modes of different polychaete species vary, most polychaetes families, including those within this study, produce free-living larvae (Blake and Arnofsky, 1999, Faulwetter et al., 2014) (Table 3-2). It is possible that the dispersal of free-living polychaete larvae via circumpolar currents is a key driver of these circum-Antarctic distributions, maintaining genetic connectivity between the populations sampled.

In Brasier et al. (2017) particle tracking models are used to speculate the direction and potential distances of passive larval dispersal within the Southern Ocean. With inputs based on our current knowledge of polychaete reproductive traits, the models demonstrate that free-living larvae could travel substantial distances throughout the West Antarctic. Particles released in the Weddell, Bellinghausen and Ross Seas were transported westward with the Antarctic Counter Current flowing over the continental shelf. The importance of the counter current is discussed in Thorpe et al. (2007). The ACC is often considered the dominant current in maintaining circumpolar connections; however its counter current has been shown to connect many regions of high krill density. The ACC does still appear to have a role as particles released at sites within the Scotia Arc regions and off the Western Antarctic Peninsula were transported both westward and to the northeast. This eastward movement is likely to be driven by the ACC, meandering as they are transported further off the continental shelf. Similar particle movements have been observed in tracking models used to estimate krill connectivity (Hofmann and Murphy, 2004, Piñones et al., 2013).

The passive movement of particles around Antarctica indicates the possibility that larvae may be recruited into non-parent populations substantial distances from their origin. Only the sites sampled were used as particle release locations in Brasier et al. (2017). It is likely that there are several unsampled 'stepping stone' populations in-between these locations that may also promote genetic connectivity. Similar insights into larval dispersal have also been obtained from oceanographic observations in conjunction with genetic analysis. For example, Matschiner et al. (2009) observed lack of genetic structuring in the notothenioid, *Gobionotothen gibberifrons* throughout the Scotia Sea. Using surface drifter trajectories the authors assigned this to the passive transport of pelagic larvae by the ACC.

The maintenance of genetic connectivity by larval dispersal and recruitment may not be applicable to all polychaetes. As recorded in the literature, not all species produce free-living larvae (Table 3-2). Furthermore, in general, Antarctic taxa are considered to lack free-living larval stages (Pearse et al., 1991, 2009). With more limited dispersal, the existence of circum-

Antarctic brooding species is highly unlikely (Lörz et al., 2009). However, genetic connectivity in brooding species or those lacking pelagic larvae could be maintained by the passive rafting of larvae or adults on floating substrate or ocean debris (Waters, 2008). Leese et al. (2010) suggested that this method maintained connection between shallow water isolated populations of the isopod *Septemserolis septemcarinata* in the sub-Antarctic. Direct evidence of rafting on kelp has also been observed in the widespread sub-Antarctic brooding bivalve *Gasimardia trapesina* (Helmuth et al., 1994), the sea slug *Onchidella marginata* (Cumming et al., 2014) and two species of sub-Antarctic amphipods (Nikula et al., 2010). These additional mechanisms should be considered when interpreting population connectivity and species biogeography.

The two species complexes described in Chapter 2 including *Chaetozone* sp. (MB1) and *Lumbrineris cingulata-kergulensis* (MB) were distributed throughout the BIOPEARL sites. Additionally, *L. cingulata-kergulensis* matched larval sequences from the Ross Sea. With the uncertainty as to whether they contain true cryptic species makes it hard to determine whether these species are truly circum-Antarctic. *Lumrbineris* is considered a widespread genus from morphologically identified material collected over large spatial scales. However, this is now considered to be a bi-product of mistakes in identification. In updated taxonomic works more restricted distributions have been recorded at the species level (Carrera-Parra, 2001, Carrera-Parra, 2006). Complications and inaccuracies in species determination is an important consideration in biogeographic studies as individual taxonomic decisions will also influence biogeographic patterns (Brandão et al., 2010).

3.4.3 Restricted species

Flabelligena sp. A, *Flabelligena* sp. B, *Macellicephala* sp. A and *Macellicepaloides* sp. B appeared to be geographically restricted within the Amundsen Sea. Given that these species were also only sampled from 500 m this could suggest that their reproductive biology and other aspects of their ecology such as physiological tolerance or competition do not promote large-scale distributions. From these four morphospecies evidence of cryptic species was only identified in *Macellicephala* sp. A, which were sympatrically distributed in the Amundsen Sea.

Some of the cryptic clades of presumed circum-Antarctic species were only found within a limited number of sampled stations within the Scotia Arc. This was the case for *Scalibregma* sp. (MB2) and (MB3), and *Euphrosinella* cf. *cirratoformis* (MB2) that were more restricted than their broadly distributed, potentially circum-Antarctic, sister cryptic clades. These

restricted clades all contained fewer than 4 individuals. Although the restricted distribution could be an artefact of undersampling, the Scotia Arc is known for particularly high biodiversity within Antarctica (Allcock et al., 2011, Linse et al., 2007). Previous genetic studies of widely distributed species have found high genetic diversity associated with this region. This pattern has been recorded in the bivalve *Lissarca notorcadensis* (Linse et al., 2007), isopods *Glyptonous antarcticus* (Held and Wägele, 2005) and *Ceratoserolis trilobitoides* (Held, 2003), and the cephalopod genus *Pareledone* (Allcock et al., 2011). The high diversity could be associated with the fragmented nature and limited accessibility of habitats in this region. This favours speciation by population fragmentation, especially in species with limited dispersal capacities (Allcock et al., 2011, Strugnell and Allcock, 2013).

The three potential cryptic species of *Aricidea* cf. *belgicae* (MB1), (MB2) and (MB3) were each restricted to a single sampled area; Scotia Arc (MB1), Amundsen Sea (MB2) and Weddell Sea (MB3). Within this study this is the only example of allopatric cryptic species. Again this could be an artefact of undersampling as clades (MB2) and (MB3) were only represented by 2 and 1 individual respectively. Alternatively it could be isolated with their evolution and species specific traits. Restricted species are often considered more vulnerable to extinction or less likely to recover from physical disturbances as their population may not be resupplied by others (Chown et al., 2015).

3.4.4 Eurybathic species

Earlier studies of eurybathy suggested that polychaetes did not conform to the general 'eurybathic' characteristics assigned to Antarctic taxa (Brandt et al., 2009). In most oceans there is a noticeable change in faunal composition on the shelf break (Gage and Tyler, 1991). However in Antarctica where the continental slope is much deeper, the change in species composition occurs at about 2000m depth (Brandt et al., 2007). Sequenced specimens from BIOPEARL I and II were collected from 500 to 1500 m depth and the additional samples from the Weddell Sea were collected between 400 and 2000 m. Compared to Brandt et al. (2007) that sampled to over 6000 m depth, the depth range sampled here may not be great enough to visualise depth dependant changes. Recently, Neal et al. (2017) found the greater similarity in polychaete community composition between 500 m stations on the inner and outer shelf of the Amundsen Sea than the communities from the same station at 1000 and 1500 m depths.

The depth distribution of cryptic clades was variable. Like their geographic distribution, many potential cryptic species appear to exist sympatrically or have overlapping depth distributions.

For example, Hesionidae sp. (MB1) was sampled from depths of 500 m or deeper whereas Hesionidae sp. (MB2) was only collected at depths of 500 m or shallower. This segregation may be related to species specific traits, whereby each species is more suited to slightly different depths and outcompetes the other.

Although depth related patterns have been recorded both here and in other studies, the true absence of species at any given depth is very difficult to confirm. Even when species appear to be depth restricted from comprehensive sampling programs, this can be disproved by future sampling in other regions. For example, Schüller (2011) described three cryptic clades of *Glycera capitata* from the Weddell Sea, two clades were thought to be restricted to 2000 m. However in this study one of these clades matched *Glycera* sp. (MB2) specimens that were sequenced from stations 500 m and shallower. Like restricted species, the absence of species from certain depths should be treated with care.

3.4.5 Summary of biogeographic patterns and wider implications

The biogeographic distribution of one third of the nine morphospecies examined was different to their original description. This was in part a result of the abundance of cryptic species of formerly cosmopolitan species but also a result of some restricted species within Antarctica. Most of the cryptic species appear to exist sympatrically with at least one widespread clade. There is potential that these different clades dominate different localities that could be related to their functional traits.

The abundance of circum-Antarctic species could be explained by their larval dispersal between populations facilitated by passive transport by the ACC and its counter current around Antarctica. However, the lack of consistent biogeographic patterning across cryptic clades within this study and others demonstrates the complexity of Southern Ocean biogeography (Brandão et al., 2010, Strugnell and Allcock, 2013, Chown et al., 2015). This may well be a result of variable biological responses and ecological interactions within and between species under past and present physical conditions.

Similar to the Southern Ocean, the Mediterranean Sea and the Arctic Ocean, are also isolated marine habitats. Additionally, both these regions have contrasting physical properties to their surrounding basins and their marine communities have experienced cyclic fragmentation and isolation events over evolutionary time (Borsa et al., 1997, Mincks Hardy et al., 2011). Within the Arctic there is genetic evidence of species divergence from refugia populations (Mincks Hardy et al., 2011). As found in the Antarctic, whilst some cryptic linages have restricted

distributions, others exist sympatrically over larger areas (Luttikhuizen et al., 2003, Carr et al., 2011). Such differences may in part be a result of species-specific dispersal capabilities and physiological tolerance. Within the Mediterranean however, despite high dispersal capabilities many species have genetically distinct east and west populations associated with limited connectivity via the Sicilian Channel (Calvo et al., 2009). Thus as discussed for Antarctic polychaetes, the local oceanography as well as species-specific traits must be considered in biogeographic interpretation.

The results presented in this chapter have valuable implications; they improve our understanding of the drivers of biogeography and their implications for marine management under changing environmental conditions. Diversity studies can highlight valuable and vulnerable regions that can then be monitored or protected. These could include diversity "hotspots" or regions with an abundance of rare species (Neal et al. 2017). Additionally model data may be able to identify "source" locations that influence the biodiversity in neighbouring regions by larval dispersal (Botsford et al. 2009). The combination of multiple data sets such as diversity, biogeographic and genetic data, together with model outputs, provide valuable tools for designation of effective data driven marine management practices such as MPA designations and fishing restrictions (Robinson et al., 2017).

3.5 References

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4 Investigating the effects of preservation on the bulk and amino acid $\delta^{15}N$ signatures of *Arenicola marina*

The data presented in this chapter were combined with a long term study by Rachel M Jeffreys and are in preparation for submission in Limnology and Oceanography entitled 'Effect of preservation on compound-specific amino acid $\delta^{15}N$ patterns in marine invertebrates: implications for time-series records'.

4.1 Introduction

Stable isotope analysis (SIA) has long been used to study trophic relationships within marine ecosystems. The isotopic composition of nitrogen ($\delta^{15}N$) has typically been used to determine the trophic position of species or individuals within food webs. When studying isolated environments such as the deep sea or the Antarctic Ocean, the opportunities to collect samples are limited. However, a substantial amount of preserved material is contained within museum collections. The ability to use preserved material for SIA would enhance our understanding of the trophic traits of different species and how they might vary through space and time. To avoid false conclusions or misinterpretation of data, and to determine whether different datasets are comparable, an understanding of how both preservation methods and preservation time effect tissue sample $\delta^{15}N$ values is essential.

In Chapter 5, compound specific stable isotope analysis is used to determine the trophic traits of Antarctic polychaetes. These polychaetes were collected and preserved in ethanol for up to 10 years prior to analysis. Before preparing the limited biological material available, the effects of preservation method and preservation time on the total nitrogen content, bulk $\delta^{15}N$ ($\delta^{15}N_{Bulk}$) and $\delta^{15}N$ of amino acids ($\delta^{15}N_{AA}$) of *Arenicola marina* (Linnaeus, 1958), the British lugworm, was investigated.

There are several studies that examine the effect of preservation method on stable isotope ratios in trophic ecology, although few using deep-sea specimens (Table 4-1). Fanelli et al. (2010) quantified the effects of formalin-ethanol preservation on the $\delta^{15}N_{Bulk}$ values of five deep-sea species, including two polychaetes, from the North Atlantic. No significant effect of formalin or ethanol preservation on $\delta^{15}N_{Bulk}$ values after 6 months was recorded in comparison to a frozen control in most species. Similar studies on shallow water marine and freshwater fauna found that ethanol preservation did not significantly alter the $\delta^{15}N_{Bulk}$ values in the majority of

species (e.g. Kaehler and Pakhomov, 2001, Sarakinos et al., 2002). However, there is a level of inconsistency between studies; Fanelli et al. (2010) compared the results of 11 freshwater and marine studies incorporating over 30 species preserved in ethanol and formalin for up to 2 years. For ethanol preserved material, as many species had enriched or depleted $\delta^{15}N_{bulk}$ signatures as those that were unchanged. In the case of formalin preserved material, only 25% of $\delta^{15}N_{bulk}$ values were significantly affected by preservation method. Over longer time scales of up to 15 years, Rennie et al. (2012) found that the $\delta^{15}N$ values from formalin preserved material from several taxonomic groups were no different to those of the frozen control samples (Table 4-1). As explained in Chapter 5, $\delta^{13}C$ can be used to determine food sources, in most cases investigated preservation method (especially formalin) had a significant effect on $\delta^{13}C$ values (Table 4-1). The recorded variation in the effects of preservation on stable isotope signatures could result from multiple factors associated with individual species, preservation time and differences in control samples (i.e. frozen or dried material) (Table 4-1). Thus this study will provide further insight into the potential effects on polychaetes as a taxonomic group and reduce the potential for false results or misinterpretation in the following chapter.

Arenicola marina lives in a U-shaped burrow in soft sediment. The species feeds by ingesting sediment within its burrow, thus it is classed as a subsurface deposit feeder (Riisgard and Banta, 1998). It is generally believed that most of the sediment ingested is derived from the surface containing organic matter from which the feeder obtains energy (Fauchald and Jumars, 1979). The diet of *A. marina* is varied and includes bacteria, meiofauna and diatoms (Retraubun et al., 1996). There is some evidence to suggest that *A. marina* is a selective feeder, e.g. a lack of detritus in its gut and in contaminated sediments, rejection of coal particles based on size (Retraubun et al., 1996, Hyslop and Davies, 1999). Within the PolyTraits database (http://polytraits.lifewatchgreece.eu) there are records of herbivorous, omnivorous and scavenger feeding behaviours for *A. marina* (Faulwetter et al., 2014). Hence CSIA will also provide further insight into the trophic biology of this species.

The primary aims of this study were:

1. To determine how much *Arenicola marina* tissue (mg) is needed to obtain consistent $\delta^{15}N$ values from bulk and compound specific stable isotope analysis and if this changes with increased preservation time
2. To determine whether the preservation method of *Arenicola marina* affects $\delta^{15}N$ values obtained from bulk and compound specific stable isotope analysis and whether this changes with increased preservation time

Species	Environment	Preservation time	Control	Ethanol Preserved		Formalin Persevered		Reference
				$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	
Arius felis, Cynoscion nebulosus, Dorosoma cepedianum, Mugil cephalus (fish)	Estuarine	2/6 weeks	Frozen	No effect	Depletion	No effect	Depletion	Arrington and Winemiller (2002)
Perca fluviatilis (fish)	Freshwater	3/6 months	Dried	No effect	No effect	No effect	Depletion	Akın et al. (2011)
Blicca bjoerkna (fish)	Freshwater	3/6 months	Dried	No effect	No effect	No effect	Depletion	Akın et al. (2011)
Percidae (fish)	Freshwater	Up to 15 years	-	No effect	Depletion	No effect	Depletion	Edwards et al. (2002)
Salvelinus alpinus (fish)	Freshwater	10 months	Dried	No effect	No effect	No effect	Depletion	Kelly et al. (2006)
Pseudogastromyzon myersi, *Liniparhomaloptera disparis*, *Ctenogobius duospilus* (fish)	Freshwater	Up to 1 year	Frozen	No effect	Depletion	No effect	Depletion	Lau et al. (2012)
Brotia hainanensis (gastropod	Freshwater	Up to 1 year	Frozen	No effect	No effect	No effect	No effect	Lau et al. (2012)
Caridina cantonensis, Macrobrachium hainanense (decapod)	Freshwater	Up to 1 year	Frozen	No effect	No effect	No effect	No effect	Lau et al. (2012)
Gammarus sp. (amphipod)	Freshwater	15 years	Frozen			Enriched	Depletion	Rennie et al. (2012)
Ephemeroptera (aquatic insect)	Freshwater	15 years	Frozen			Depletion	No effect	Rennie et al. (2012)
Heptageniidae (aquatic insect)	Freshwater	15 years	Frozen			Enriched	Depletion	Rennie et al. (2012)
Gonoiobasis sp. (gastropod)	Freshwater	15 years	Frozen			No effect	Depletion	Rennie et al. (2012)
Physidae (gastropod)	Freshwater	15 years	Frozen			No effect	Depletion	Rennie et al. (2012)
Hydropsychidae (aquatic insect)	Freshwater	15 years	Frozen			No effect	Depletion	Rennie et al. (2012)
Leptoceridae (aquatic insect)	Freshwater	15 years	Frozen			No effect	Depletion	Rennie et al. (2012)
Corbicula fulminea (bivalve)	Freshwater	3 days/6 months	Frozen	Depletion	Depletion	Depletion	Enrichment	Sarakinos et al. (2002)
Catostomus occidentalis (fish)	Freshwater	3 days/6 months	Frozen	Enriched	No effect	No effect	Depletion	Sarakinos et al. (2002)
Hydropsyche sp. (aquatic insect)	Freshwater	3 days/6 months	Frozen	No effect	No effect	No effect	Depletion	Sarakinos et al. (2002)
Zooplankton sp.	Freshwater	2 weeks/12 months	Dried	No effect	No effect	No effect	No effect	Syväranta et al. (2008)
Macroinvertebrates (*Asellus aquaticus* (isopod), aquatic insects and oligochaetes)	Freshwater	2 weeks/12 months	Dried	No effect	No effect			Syväranta et al. (2008)
Corbicula uminea (mollusc)	Freshwater	12 months	Dried	Enriched	Enriched	Enriched	Depletion	Syväranta et al. (2011)
Chelonia mydas, *Caretta caretta*, *Trachemys scripta elegans* (turtles)	Marine	1-60 days	Dried	No effect	No effect			Barrow et al. (2008)
Pleuronectes americanus (fish)	Marine	2/4 months	Frozen	Enriched	Depletion	Enriched	Depletion	Bosley and Wainright (1999)
Crangon septemspinosa (decapod)	Marine	2/4 months	Frozen	No effect	No effect	No effect	Depletion	Bosley and Wainright (1999)
Anemonia sulcata (anemone)	Marine	6/24 months		Depletion	Depletion			Carabel et al. (2009)
Mytilus galloprovincialis (bivalve)	Marine	6/24 months	Frozen	Enriched	Depletion			Carabel et al. (2009)
Himanthalia elongata (seaweed	Marine	6/24 months	Frozen	No effect	No effect			Carabel et al. (2009)

Patella vulgata (gastropod)	Marine	6/24 months	Frozen	Enriched	No effect			Carabel et al. (2009)
Hoplostethus mediterraneus, Hymenocephalus italicus, Nezumia aequalis (fish)	Marine	6/24 months	Dried			No effect	Depletion	Fanelli and Cartes (2010)
Abra longicallus (bivalve)	Marine	Up to 12 months	Frozen	No effect	No effect	No effect	Depletion	Fanelli et al. (2010)
Molpadia musculus (holothurian)	Marine	Up to 12 months	Frozen	No effect	Enriched	No effect	Depletion	Fanelli et al. (2010)
Sipunculus norvegicus (sipunculid)	Marine	Up to 12 months	Frozen	Depletion	No effect	No effect	Depletion	Fanelli et al. (2010)
Chirimia biceps (polychaete)	Marine	Up to 12 months	Frozen	No effect	No effect	No effect	Depletion	Fanelli et al. (2010)
Nephthys hystricis (polychaete)	Marine	Up to 12 months	Frozen	No effect	No effect	No effect	Depletion	Fanelli et al. (2010)
Zooplankton sp	Marine	-		Enriched	Enriched	Enriched	Enriched	Feuchtmayr and Grey (2003)
Aurelia aurita (Scyphozoa)	Marine	6 months	Dried	Enriched	No effect			Fleming et al. (2011)
Argyrosomus hololepidotus (fish)	Marine	1/12 weeks	Dried	Enriched	Enriched	No effect	Depletion	Kaehler and Pakhomov (2001)
Octopus vulgaris (cephalopod)	Marine	1/12 weeks	Dried	No effect	Enriched	No effect	Depletion	Kaehler and Pakhomov (2001)
Ecklonia radiata (kelp)	Marine	1/12 weeks	Dried	No effect	Enriched	Depletion	Depletion	Kaehler and Pakhomov (2001)
Gadus morhua (fish)	Marine	1-21 days	Dried	Depletion	Enriched	Depletion	Depletion	Sweeting et al. (2004)

Table 4-1 Reported differences in stable isotope signatures of aquatic organisms between preservation treatments and controls over time in the literature (adapted from Fanelli et al. (2010)).

4.2 Methods

4.2.1 Sensitivity and preservation tests on *Arenicola marina* (Lugworm)

Live *Arenicola marina* were supplied by Online Baits UK and were left in artificial water in a temperature controlled oxygenated tank (T 5°C; 48 h) to extrude their gut contents. The water was changed after 24 hours to avoid reingestion of gut contents. These specimens were used for both sensitivity and preservation experiments. A total of 110 specimens were used, 20 for the sensitivity tests and 90 for the preservation tests. For the sensitivity tests all specimens were preserved in 70% ethanol and stored at room temperature. Four specimens were assigned to each of the following time treatments: time zero, one week, one month, six months and one year. Of the 90 specimens used for the preservation tests, 18 specimens were assigned to each of the time treatments with equal numbers (six) preserved in 70% ethanol, 10% formalin or frozen at -80°C. Note that after 24 hours, the formalin preserved samples were rinsed in de-ionized water (18.2 MΩ cm^{-1}; Milli-Q) and transferred into ethanol for the remainder of their treatment time. This treatment of preserved material is consistent with most deep-sea regimes of animal sampling in which specimens are fixed and then preserved.

The formalin and ethanol preserved material were removed from their treatment after the given treatment time and rinsed in Milli-Q water, weighed wet and frozen at -80°C. A wet weight of all specimens was recorded prior to overnight freeze-drying (-50 °C; 10^{-2} Torr) after which a final dry weight was recorded. All specimens were frozen with liquid nitrogen and were ground to a fine powder in a pestle and mortar, with N_2 (l) to aid homogenistation.

4.2.2 Total Nitrogen and $\delta^{15}N_{Bulk}$ analysis

From each specimen 1.0mg of tissue was used to measure total nitrogen (TN) content using a Carlo Erba NC 2500 CHN Elemental Analyser. Analyses were carried out in duplicate and Chitin Organic Analytical Standard was used in each set of analyses to determine instrument precision (< 0.1%) and accuracy (< 0.1%).

Single specimens with a total nitrogen content of ~10% were chosen for SIA from each of the sensitivity time treatments. Duplicate $\delta^{15}N$ analyses were carried out using a Costech Elemental Analyser coupled to a Delta V advance mass spectrometer (EA/IRMS) for the following tissue weights:- 0.05mg, 0.1mg, 0.2mg, 0.3mg, 0.5mg, 0.6mg, 0.7mg, 0.8mg, 0.9mg, 1.0mg, 1.25mg, 1.5mg, 1.75mg and 2.0mg in order to determine the linearity of the $\delta^{15}N$ measurements. For all

other analyses 1.0mg of tissue was used. At this weight $\delta^{15}N$ was consistent and thus chosen as the optimum sample weight (Figure 4-3). Samples were weighed into silver cups, within the elemental analysers the samples were combusted within a Costech Elemental Combustion System at 1000ºC and diluted with N_2 prior to entering ECS 4010 CH/N/CN reaction tube. USGS40 and USGS41 were used to determine the accuracy (< 0.5‰) and precision (< 0.7‰) of the EA/IRMS and correct the $\delta^{15}N$ values obtained from *Arenicola marina* samples. The values obtained for these standards were plotted against their known $\delta^{15}N$ values and the line equation was used for data correction. A local standard (Holothuridaea; *Psychropotes longicauda*; Iken et al. (2001)) was also used to monitor the precision of biological samples (< 0.9‰).

4.2.3 $\delta^{15}N_{AA}$ analysis

Tissue samples (0.5 mg from 4 of the 6 individuals analysed in bulk) were analysed for CSIA following Chikaraishi et al. (2007). This method is modified from Metges et al. (1996) and Jeffreys et al. (In prep).

Briefly, additional tissue samples (0.05, 1.0, 2.0, 3.0, 4.0, 5.0 and 7.5 mg) from a single time zero specimen preserved in ethanol were used to determine the linearity/sensitivity for CSIA. Average $\delta^{15}N$ values of source and trophic amino acids were used to calculate trophic level for each sample analysed (see section 4.2.5).

4.2.3.1 $\delta^{15}N_{AA}$ hydrolysis and derivatisation

Freeze-dried tissue was placed in a Reacti-vial (1 mL) together with internal standard (IS), L-Norleucine supplied by Sigma-Aldrich, suitable for amino acid analysis. For each 1 mg of tissue 4 µL of L-Norleucine (4.8ng/ml) was added (i.e. 20 µL for 5 mg). 6M HCL (0.2 mL) was added to the Reacti-vial and the sample was hydrolysed in an oven (110°C, 24 h).

Once at room temperature the hydrolysate was filtered through a 45 µm nylon filter within a Nanosep centrifuge tube at 10,000 rmp for 60 seconds. Samples were then de-fatted by adding 3:2 *n*-hexane:DCM, and mixed by shaking and removing the upper layer of organic solvent (repeated x3). To ensure no organic solvent remained the samples were blown under N_2 (ca. 1 min.) prior to being frozen and later freeze-dried.

Dried hydrosylates were esterified by addition of thionyl chloride:2-propanol (1:4 v/v; 0.2 mL) and heated in an oven (100°C; 2 h). Once cool, the sample was dried under N_2, DCM added (0.5 mL) and dried again twice. Samples were acetylated by addition of 1:4 pivaloyl

chloride:toulene (0.2 mL) and heated (110°C; 2h) for pivaloylation.

To extract the amino acids, Milli-Q water (0.2 mL) followed by 3:2 *n*-hexane:DCM (0.5 mL) was added to each sample. Samples were shaken (10 s) and the upper amino acid layer removed and filtered through a Pasteur pipette plugged with glass wool and anhydrous magnesium sulphate. This step was repeated (x2), the pipette rinsed with a small amount of DCM and the sample dried up under N_2.

4.2.3.2 Gas chromatography combustion isotope ratio mass spectrometry (GC/C/IRMS)

Derivitised samples and standards were stored at -20°C and diluted in DCM. Samples were analysed by a trace 1300 Series gas chromatograph (splitless Triplus RHS injector; coupled with a Thermoquest Scientific ISQ-LT mass spectrometer) prior to analysis by GC/C/IRMS to check the concentration of each of the eight amino acids within the standard (Figure 4-1) and those present within each sample (Figure 4-2). The area for phenylalanine obtained from the trace was used to decipher the dilution for Gas Chromatography Isotope Ratio Mass Spectrometry full method. Sample dilutions were adjusted for analysis by GC/C/IRMS. Two GC/C/IRMS methods were used, a full-AA method and a phenylalanine method, as reproducible data for the source amino acid required the samples to be more concentrated and the phenylalanine method prevented overloading the MS and reactor.

Stable nitrogen isotopic compositions of individual amino acids of *Arenicola marina* were determined in duplicate using a Thermo Trace Ultra gas chromatograph linked by a ConFlo IV interface to a Delta V Advantage isotope ratio monitoring mass spectrometer (irmMS; Thermo Fisher Scientific). Samples were injected in splitless mode onto a Restek Stabilwax-DA column (30 m, 0.32 mm ID, 1 μm film thickness). The injector temperature was 220ºC. The GC temperature was held at 50ºC for 2 minutes followed by ramp to 200ºC at 10ºC min^{-1} and 240ºC at 6ºC min^{-1} and held for 26.4 minutes. The carrier gas was ultra-high purity grade helium (flow: 1.4 mL min^{-1}) and Cu/Ni combustion reactor held at 1000°C. The same program was used for both the full and phenylalanine methods, the difference being that for the phenylalanine method the blackflush remained on until 1720 seconds rather than 1050 seconds in the full method to isolate the phenylalanine peak.

The software (Thermo Isodat 3) automatically computed the $^{15}N/^{14}N$ ratios of each compound peak, referenced to a standard gas (N_2) of known composition. The results are reported in per mil (‰) relative to the VPDB international standard. Standards containing eight amino acids of known isotopic composition (alanine, valine, leucine, norleucine, glycine, aspartic acid,

glutamic and phenylalanine) were measured after every 4-6 GC/C/IRMS analyses and used to determine instrument precision (< 0.7‰) and accuracy (< 0.9‰) and ensure the reproducibility of the data. $\delta^{15}N_{AA}$ measurements were corrected using the line equation of the known $\delta^{15}N_{AA}$ values and the measured $\delta^{15}N_{AA}$ values in the standard mixture following Chikaraishi et al. (2007). If necessary, for example if there was a noticeable shift in retention time or if low amplitude was recorded, the sample was re-analysed. Mean (n=4) $\delta^{15}N$ values for each amino acid were calculated from each combination of the preservation time and method.

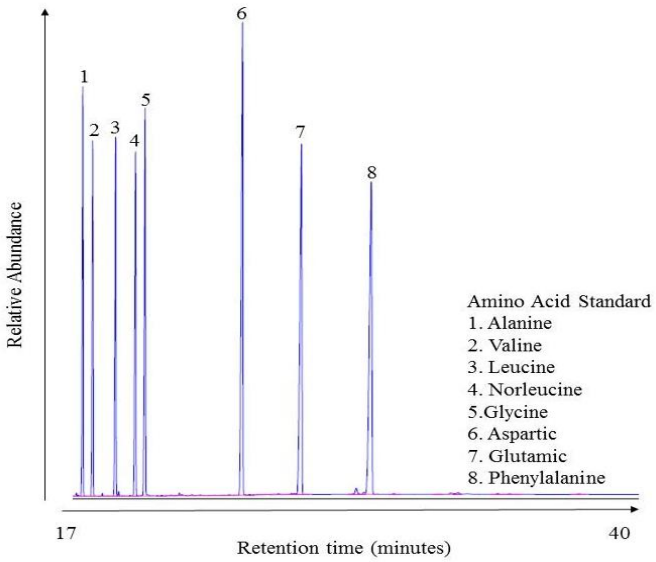

Figure 4-1 Gas chromatogram showing the retention time and relative abundance of the eight derivitised standard amino acids.

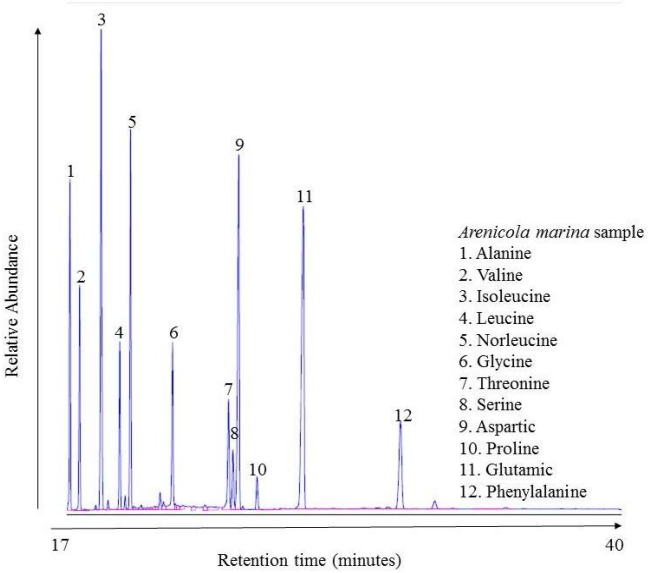

Figure 4-2 Gas chromatogram showing retention time and relative abundance of the 12 major amino acids of *Arenicola marina*.

4.2.4 Statistical analyses

Spearman's rank correlation was used to test for any relationship between tissue mass and $\delta^{15}N_{AA}$ values and, trophic level estimates within the sensitivity samples. Preservation $\delta^{15}N_{AA}$ data were tested for normality, using Shapiro-Wilk, and equal variance, using Brown-Forsythe. Two-way ANOVA was used to test for significant differences between preservation methods and times and for any interactive effect of the two variables. The interactive effect was tested for incase time and/or preservation method alone did not account for any significant differences between the $\delta^{15}N$ values obtained but together they had an accumulative combined effect. For example formalin may have no effect of on $\delta^{15}N_{AA}$ values unless samples were preserved for more than 6 months i.e. the effect of formalin is only seen when time is considered.

All analyses were conducted using SigmaStat. In two cases, outliers, values outside of the inter-quartile range, were removed from the dataset for statistical analysis. These included one $\delta^{15}N_{bulk}$ value obtained from a specimen after 1 week of formalin treatment and a phenylalanine $\delta^{15}N$ value obtained from a specimen from the 6-month frozen treatment. If significant ANOVA results were found a Tukey Test was used for post hoc analyses to identify significant pairwise comparisons.

4.2.5 Calculating trophic level

Trophic level was estimated using the $\delta^{15}N$ values of source and trophic amino acids in Equations 4-1 to 4-3, according to Chikaraishi et al. (2009).

$$TL_{Glu/Phe}: (\delta^{15}N\ Glu - \delta^{15}N\ Phe - 3.4)/7.6 + 1 \qquad \text{(Equation 4-1)}$$

$$TL_{Ala/Phe}: (\delta^{15}N\ Ala - \delta^{15}N\ Phe - 3.2)/5.7 + 1 \qquad \text{(Equation 4-2)}$$

$$TL_{Val/Phe}: (\delta^{15}N\ Val - \delta^{15}N\ Phe - 4.6)/4.6 + 1 \qquad \text{(Equation 4-3)}$$

Where Glu = glutamic acid, Ala = alanine, Val = valine and Phe = phenylalanine. The mean isotope difference between the respective trophic amino acid (i.e. Glu, Ala, Val) and source amino acid phenylalanine was 3.4‰ (Equation 4-1), 3.2‰ (Equation 4-2) and 4.6‰ (Equation 4-3) and the ^{15}N enrichment factor per increasing trophic level at 7.6‰ (Equation 4-1), 5.7‰ (Equation 4-2) and 4.6‰ (Equation 4-3).

On passing normality and equal variance tests, the effects of preservation time and method on the calculated trophic level was tested using two-way ANOVA.

4.3 Results

4.3.1 $\delta^{15}N_{Bulk}$ and total nitrogen

4.3.1.1 Sensitivity

$\delta^{15}N_{Bulk}$ values for all of the sensitivity samples ranged from 11.3 to 23.7‰ (Figure 4-3). The highest value was obtained from 0.8 mg of tissue at time zero which together with a value of 14.3‰ obtained from 0.9 mg of tissue, also for a time zero sample, was considered to be an outlier. Excluding these outliers, the $\delta^{15}N$ values were lighter and more variable (standard deviations >1.0‰, Table 4-1) for lower tissue weights (<0.4 mg). The largest $\delta^{15}N$ range was observed at a tissue mass of 0.1 mg from 11.9 to 18.4‰. When tissue mass exceeds 0.4mg, mean $\delta^{15}N$ values were less variable ranging from 19.0 to 19.6‰ across all preservations (Table 4-2, Figure 4-3).

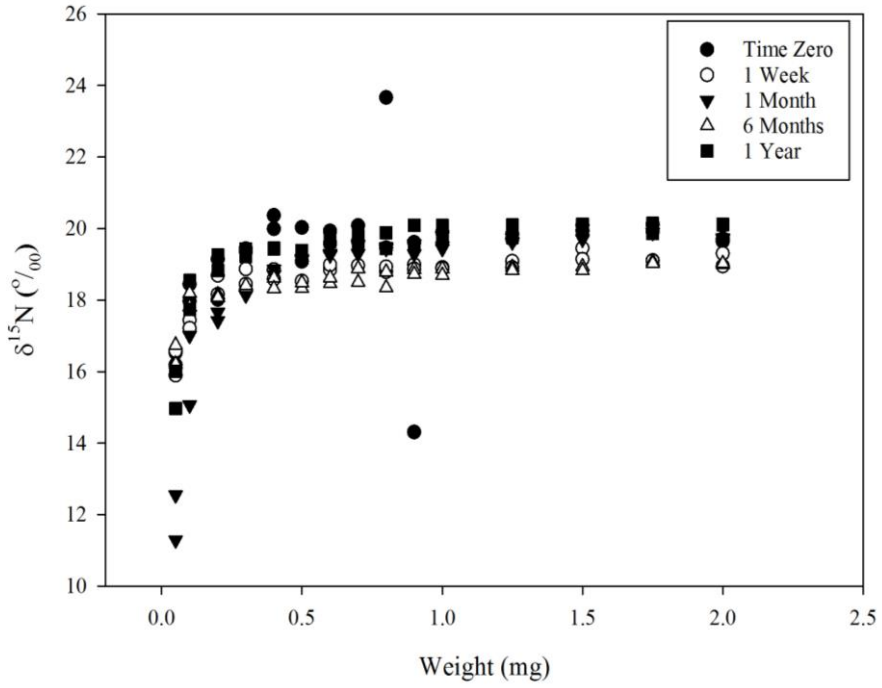

Figure 4-3 Duplicate $\delta^{15}N$ values against weight (0.05 – 2.0mg) of *A. marina* tissue for each preservation time, all specimens were preserved in ethanol.

Tissue weight (mg)	Mean ± $\delta^{15}N$ (‰)
0.05	15.3± 1.87
0.1	17.5 ± 1.0
0.2	18.3 ± 0.6
0.3	18.8 ± 0.5
0.4	19.1 ± 0.7
0.5	19.0 ± 0.5
0.6	19.4 ± 0.5
0.7	19.4 ± 0.5
0.8	19.2 ± 0.5
1.0	19.3 ± 0.5
1.25	19.4 ± 0.5
1.5	19.4 ± 0.5
1.75	19.6 ± 0.5
2.0	19.5 ± 0.4

Table 4-2 Mean $\delta^{15}N$ bulk values by weight (0.05 – 2.0mg) of *A. marina* tissue

4.3.1.2 Preservation

The total nitrogen content was variable, the only consistent observation was that formalin preserved samples, on average, had a greater total nitrogen content than those preserved in ethanol or frozen at each preservation time (Figure 4-4). Overall there was a significant difference in mean total nitrogen between preservation methods (Table 4-3). No continuous increase or decrease with increasing preservation time was observed in total nitrogen content in formalin or frozen preserved samples. Total nitrogen also varied within time periods between individuals of the same preservation treatment. For ethanol this difference was greatest after 1 week (2.5 to 10.3%), for formalin at one month (1.2 to 11.0%) and frozen at 6 months (5.5 to 10.3%). The highest recorded total nitrogen was from an individual that was in formalin for 6 months at 11.3%.

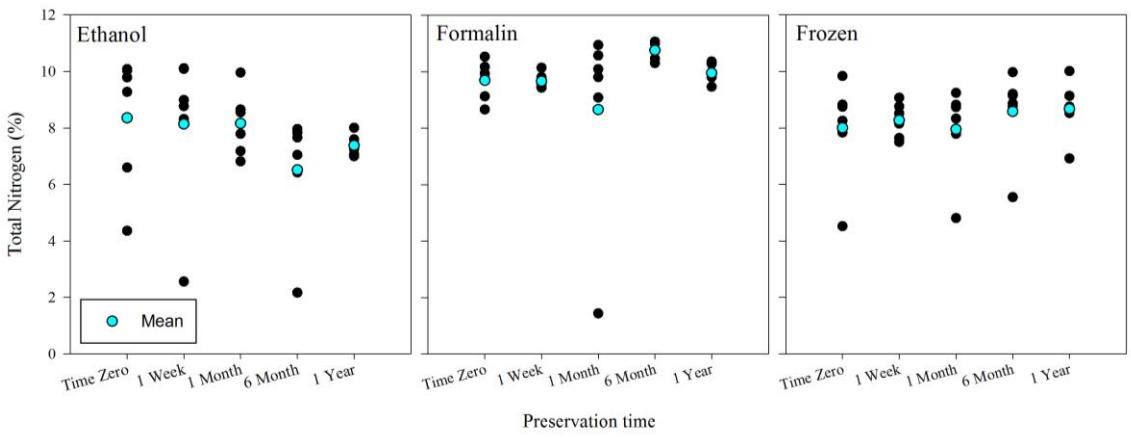

Figure 4-4 Total nitrogen contents (%) for each of the 6 specimens preserved in ethanol, formalin and frozen over the 5 preservation time periods.

For each of the preservation methods $\delta^{15}N$ values were, on average, enriched with time from 18.9 ± 0.5‰ to 19.8 ± 0.3‰, 19.0 ± 0.4‰ to 19.9 ± 0.4 ‰ and 18.0 ± 0.4‰ to 19.1 ± 0.6‰ between time zero and 1 year for ethanol, formalin and frozen samples respectively (Figure 4-5, Table 4-3). Two-way ANOVA results showed that there was a significant difference in the $\delta^{15}N$ values over time and across different preservation methods; there was also a significant interaction effect between the two variables.

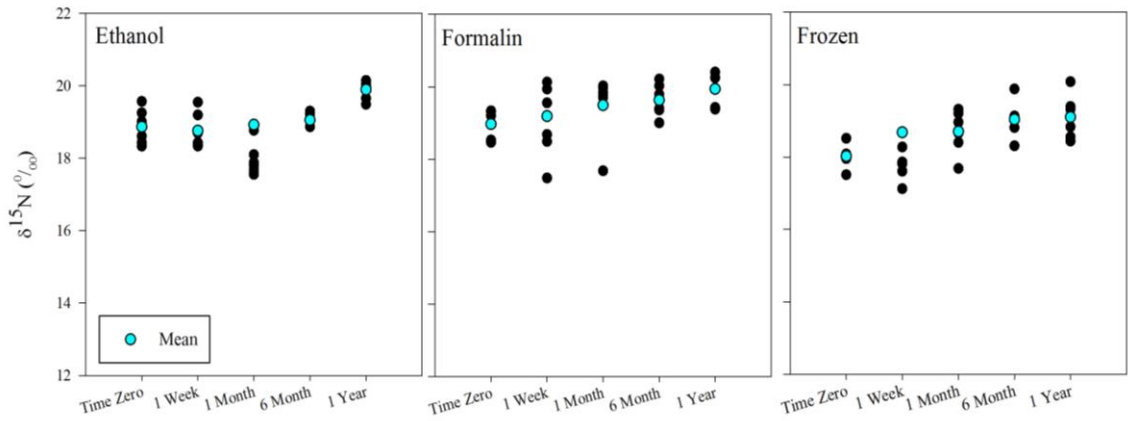

Figure 4-5 $\delta^{15}N$ values (‰) for each of the 6 specimens preserved in ethanol, formalin and frozen over the preservation time periods.

	Time	Preservation	Time*Preservation
Total Nitrogen	$F_{(4,75)} = 0.214$, P = 0.930	**$F_{(2,75)} = 11.352$, P < 0.001**	$F_{(8,75)} = 1.202$, P = 0.310
Bulk	**$F_{(4,75)} = 13.937$, P <0.001**	**$F_{(2,75)} = 20.367$, P < 0.001**	**$F_{(8,75)} = 3.295$, P = 0.003**
Alanine	$F_{(4,45)} = 2.080$, P = 0.099	$F_{(2,45)} = 1.116$, P = 0.337	$F_{(8,45)} = 1.084$, P = 0.392
Valine	$F_{(4,45)} = 1.233$, P = 0.310	$F_{(2,45)} = 1.477$, P = 0.239	$F_{(8,45)} = 1.777$, P = 0.107
Leucine	$F_{(4,45)} = 0.495$, P = 0.739	$F_{(2,45)} = 1.637$, P = 0.206	$F_{(8,45)} = 0.898$, P = 0.527
Glycine	$F_{(4,45)} = 0.083$, P = 0.987	$F_{(2,45)} = 2.575$, P = 0.087	$F_{(8,45)} = 0.489$, P = 0.858
Aspartic	$F_{(4,45)} = 1.236$, P = 0.309	$F_{(2,45)} = 0.352$, P = 0.705	$F_{(8,45)} = 1.371$, P = 0.235
Glutamic Acid	$F_{(4,45)} = 1.410$, P = 0.246	$F_{(2,45)} = 1.211$, P = 0.307	$F_{(8,45)} = 1.222$, P = 0.309
Phenylalanine	$F_{(4,45)} = 1.352$, P = 0.266	$F_{(2,45)} = 0.668$, P = 0.518	$F_{(8,45)} = 0.301$, P = 0.962

Table 4-3 Two-way ANOVA results of different amino acids including trophic amino acid; glutamic acid, alanine, valine, source amino acid; phenylalanine, and leucine, glycine and aspartic acid, bulk $\delta^{15}N$ values measured as well as total nitrogen (%). All amino acids passed both normality (Shapiro-Wilk) and equal variance (Brown-Forsythe) tests, bulk $\delta^{15}N$ and total nitrogen values were found non-normally distributed but passed equal variance. Significant values are highlighted in bold.

The Tukey Test results showed that the significant differences in total nitrogen values and $\delta^{15}N_{Bulk}$ values were obtained from several pairwise comparisons of preservation method within each time period. The most common pattern was a significant difference between mean $\delta^{15}N_{Bulk}$ values of the frozen and formalin samples (Table 4-4). For time, significant effects on $\delta^{15}N_{Bulk}$ values were mainly recorded between the shorter and longest time periods. For example a significant difference in the mean $\delta^{15}N_{Bulk}$ between 1Y vs. 1M and 1Y vs. 1W was recorded in all preservation methods (Table 4-5).

	TN	Bulk				
		TO	1W	1M	6M	1Y
Formalin vs. Frozen	P<0.050	P<0.050	P<0.050	P<0.050		P<0.050
Formalin vs. Ethanol	P<0.050			P<0.050		
Ethanol vs. Frozen		P<0.050	P<0.050			P<0.050

Table 4-4 Post hoc Tukey Test results from two-way ANOVA analyses on total nitrogen and bulk $\delta^{15}N$ data. Significant pairwise comparisons indicated by P<0.050, tested between preservation method for total nitrogen (TN) and within time periods for bulk $\delta^{15}N$ where TO = time zero, 1W = 1 weeks, 1M = 1 month, 6M = 6 months, 1Y = 1 year.

	Ethanol	Formalin	Frozen
1Y vs. 1M	P<0.050	P<0.050	P<0.050
1Y vs. 1W	P<0.050	P<0.050	P<0.050
1Y vs. TO	P<0.050		
1Y vs. 6M			
6M vs. 1M	P<0.050		
6M vs. 1W			P<0.050
6M vs. TO			P<0.050
TO vs. 1M	P<0.050		
TO vs. 1W			
1W vs. 1M			

Table 4-5 Post hoc Tukey Test results from two-way ANOVA analyses on bulk $\delta^{15}N$ data. Significant pairwise comparisons indicated by P<0.050, tested between time periods for each preservation method.

4.3.2 Amino acids

4.3.2.1 Sensitivity

Variation in $\delta^{15}N_{AA}$ values with tissue mass was observed in all of the trophic amino acids studied, however the pattern was not consistent (Figure 4-6). For valine, the $\delta^{15}N$ values increased slightly from 25.30 ± 0.77‰ to 27.10 ± 0.04‰ with increasing tissue mass. Alanine $\delta^{15}N$ values were relatively constant with values varying by 0.77‰ between tissue masses. The greatest variation recorded for a trophic amino acid was in glutamic acid, with $\delta^{15}N$ values ranging from 24.38 ± 0.44‰ (3.0 mg) to 26.99 ± 0.94‰ (7.5 mg). The average $\delta^{15}N$ values for the source amino acid, phenylalanine ranged from 17.19 ± 0.38‰ (1.0mg) to 18.37 ± 0.65‰ (4.0mg). As for glutamic acid, there was no consistent increase or decrease in $\delta^{15}N$ with increasing tissue mass.

Duplicate $\delta^{15}N$ values of leucine, glycine and aspartic acid were also measured with increasing tissue mass (Figure 4-7). The $\delta^{15}N$ values of leucine and aspartic were relatively constant over tissue weights of 0.5 to 5.0 mg, but with a noticeable increase at 7.5 mg to 24.90 ± 0.11‰ and 29.37 ± 0.42‰ respectively. Glycine had the greatest standard deviations between duplicate measurements and although the $\delta^{15}N$ values for 0.5 and 7.5 mg were similar, 15.12 ± 0.78‰ and 15.62 ±0.25‰ respectively, values decreased to 12.87 ± 1.14‰ at 3 mg of tissue. Despite some variation in the $\delta^{15}N$ values of some amino acids, there was no significant relationship

between the tissue weights and $\delta^{15}N_{AA}$ values (Spearman's Rank correlation; Table 4-6).

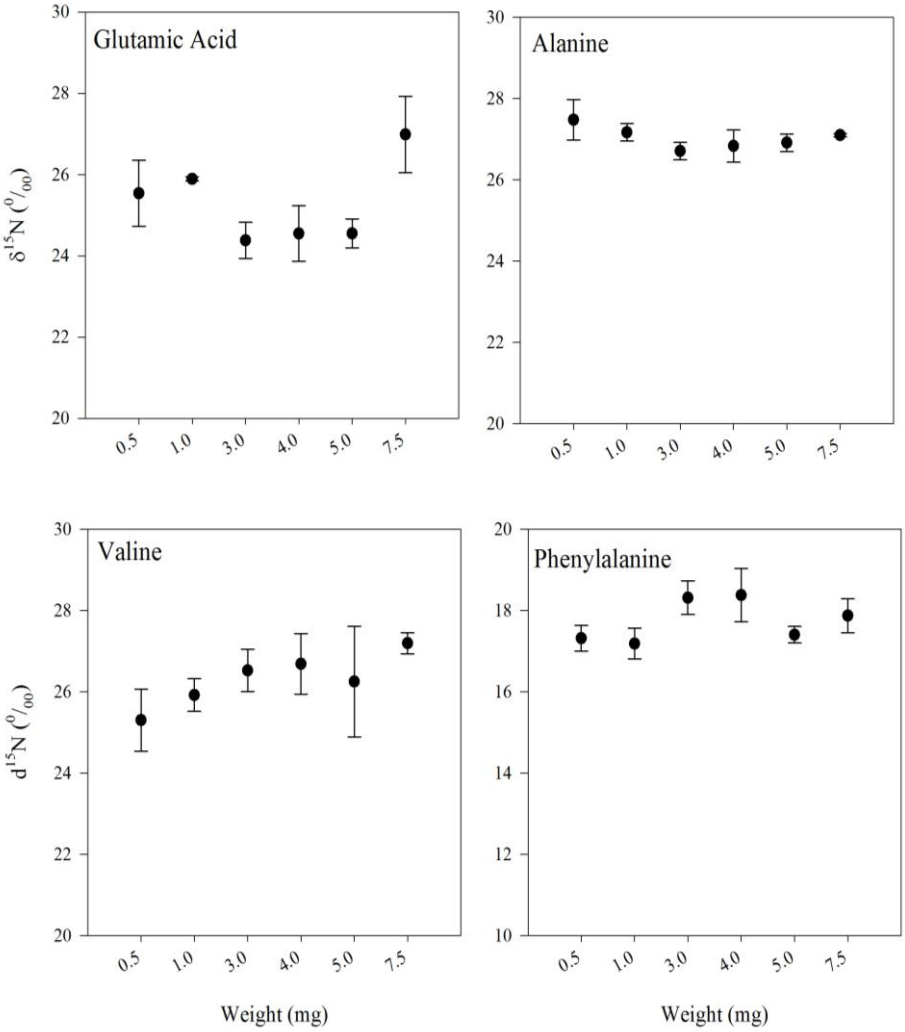

Figure 4-6 Mean $\delta^{15}N$ values obtained from duplicate analyses of different tissue masses of *Arenicola marina* for trophic amino acids; glutamic acid, alanine and valine and the source amino acid; phenylalanine.

	Spearman's Rank Correlation
Alanine	R_s = -0.429, d.f. = 4, P = 0.419
Valine	R_s = 0.829, d.f. = 4, P = 0.058
Leucine	R_s = 0.086, d.f. = 4, P = 0.919
Glycine	R_s = 0.257, d.f. = 4, P = 0.658
Aspartic Acid	R_s = 0.029, d.f. = 4, P = 1.000
Glutamic Acid	R_s = 0.116, d.f. = 4, P = 0.805
Phenylalanine	R_s = 0.486, d.f. = 4, P = 0.356

Table 4-6 Spearman's Rank correlation results between the $\delta^{15}N$ values of the trophic; glutamic acid, alanine, valine, and, source amino acids; phenylalanine, as well as leucine, glycine and aspartic acid and tissue mass of *Arenicola marina.*

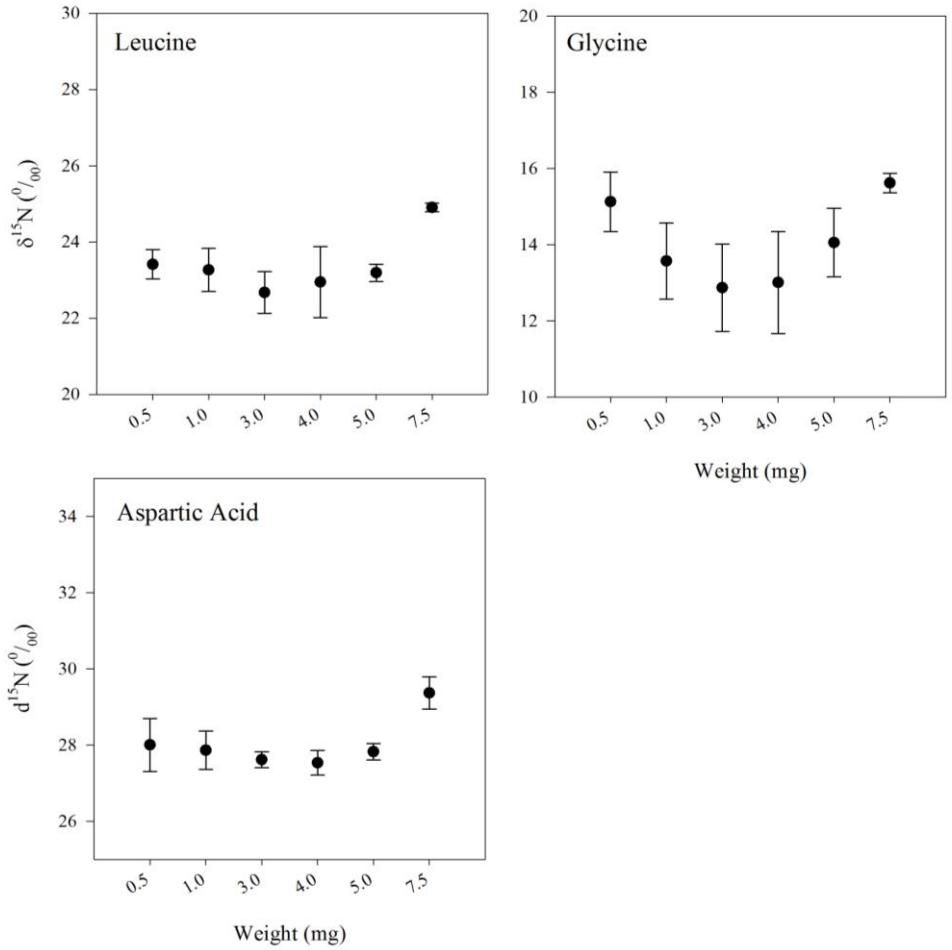

Figure 4-7 The mean $\delta^{15}N$ values obtained from duplicate analyses of different tissue masses of *Arenicola marina* for the amino acids; leucine, glycine and aspartic acid.

4.3.2.2 Preservation

The $\delta^{15}N$ values for the three trophic amino acids:- glutamic acid, alanine and valine, and single source amino acid, phenylalanine, used to determine trophic level, are shown in Figure 4-8. No significant trend was observed within any of the trophic and source amino acids over time or with preservation method. Of the trophic amino acids, glutamic acid was isotopically lightest ranging from 23.8 ±0.5‰ (1W_E) to 26.0 ±0.2‰ (1Y_E), and valine the heaviest ranging from 25.6 ± 0.4‰ (6M_Fr) to 27.9 ± 0.7‰ (1W_E). As expected the source amino acid Phenylalanine was depleted in ^{15}N relative to the trophic amino acids ranging from 14.3 ± 2.3‰ (1Y_E) to 18.1 ± 4.7‰ (6M_Fr). A single outlier was removed from statistical analysis (Figure 4-8); this specimen from the 6 month frozen treatment had a phenylalanine $\delta^{15}N$ value of 24.6 ‰.

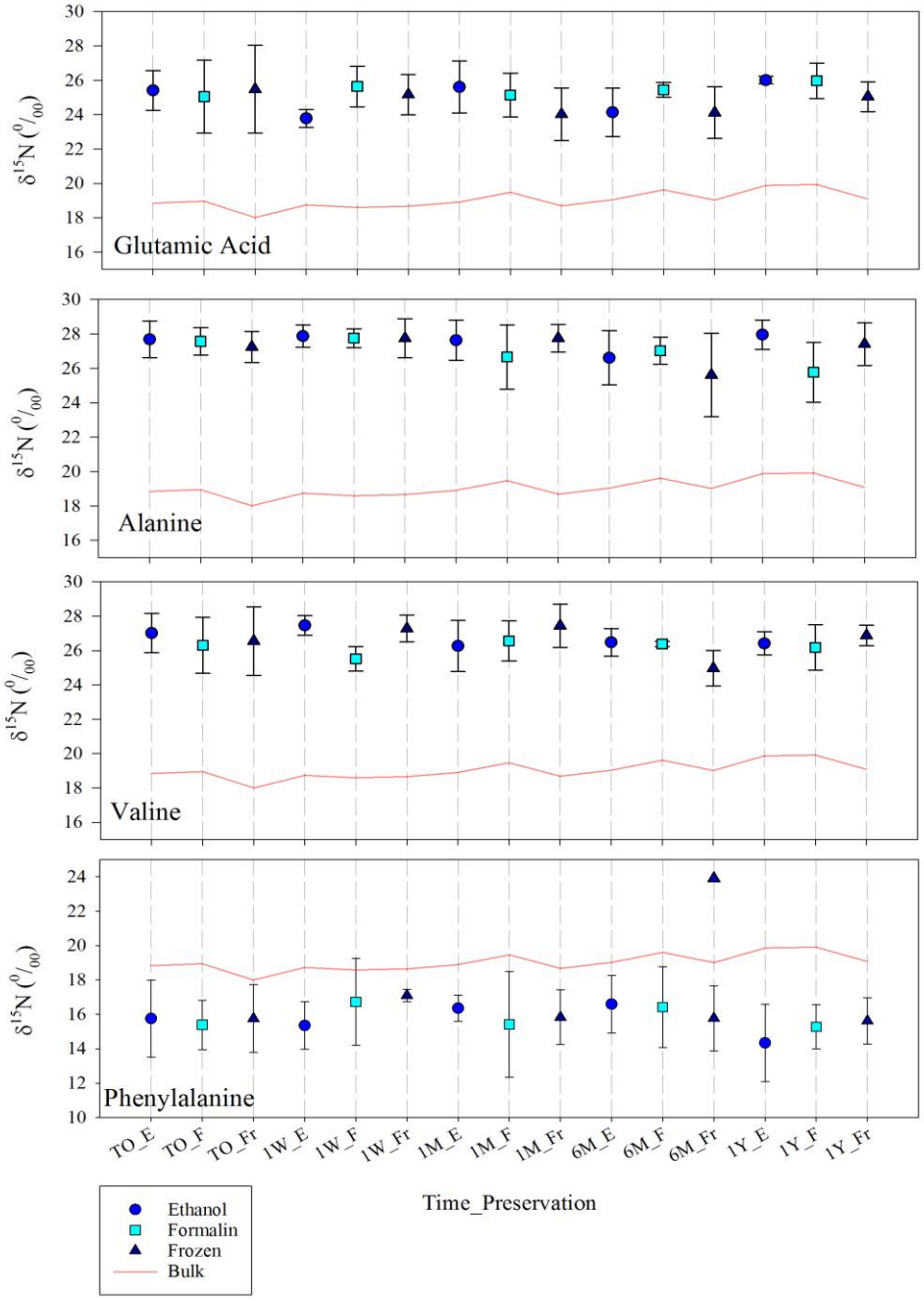

Figure 4-8 Average $\delta^{15}N$ values for amino acids; glutamic acid, alanine, valine and phyenylalanine, with a running mean of $\delta^{15}N_{Bulk}$ for each time-preservation treatment. Where T_0 = time zero, 1W = 1 week, 1M = 1 month, 6M = 6 months and 1Y = 1 year and E = ethanol, F = formalin, Fr = frozen. Note the additional outlying data point from the 6MF treatment, which was excluded from the calculations and statistical analysis.

The $\delta^{15}N_{AA}$ values analysed including leucine, glycine and aspartic acid also were not significantly different between preservation methods and over time (Figure 4-9, Table 4-3). Both leucine and aspartic acid had higher $\delta^{15}N$ values than bulk material, with leucine, ranging from 22.9 ± 1.0‰ (6M_Fr) to 24.8 ± 0.7‰ (6M_F) and aspartic acid from 26.5 ± 1.0‰ (T0_F) to 28.4 ± 0.6‰ (1Y_E). Glycine had lower $\delta^{15}N$ values than bulk tissue, ranging from 12.5 ± 0.6‰ (1Y_Fr) to 14.3 ± 0.6‰ (1Y_F).

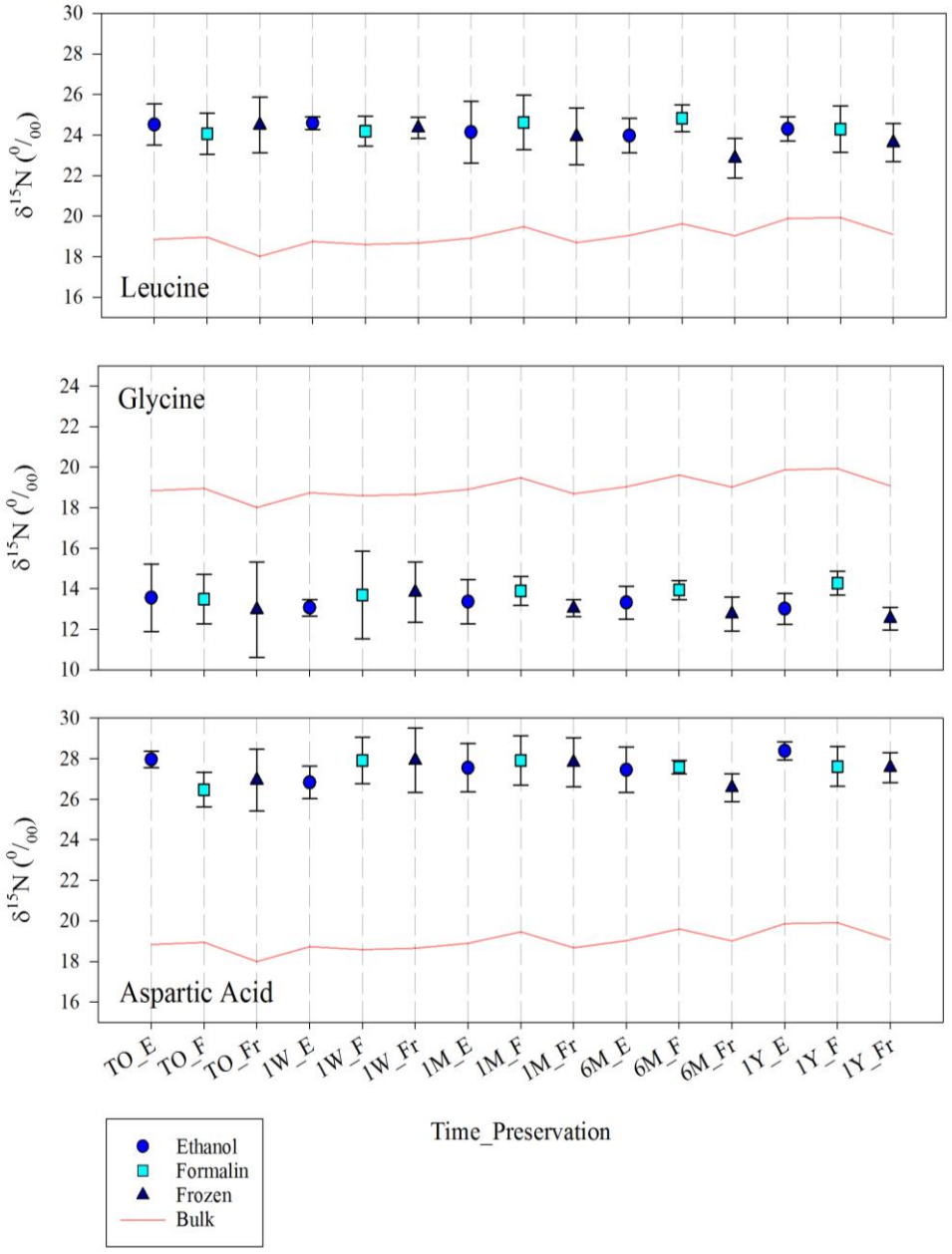

Figure 4-9 Average $\delta^{15}N$ values for amino acids; leucine, glycine and aspartic acid, with a running mean of $\delta^{15}N_{Bulk}$ for each time-preservation treatment. Where T_0 = time zero, 1W = 1 week, 1M = 1 month, 6M = 6 months and 1Y = 1 year and E = ethanol, F = formalin, Fr = frozen.

4.3.3 Trophic level

4.3.3.1 Sensitivity

Variation in the calculated trophic levels reflected that of the source and trophic amino acids (Figure 4-10). Thus, no consistent increase or decrease in trophic level was observed with increasing tissue mass. The range of values recorded for different tissue masses was greatest for $TL_{Glu/Phe}$ with estimates from 1.4 to 1.8, reflecting the greater variation in its trophic amino acid isotopic composition (Figure 4-6). Variations in the $TL_{Val/Phe}$ and $TL_{Ala/Phe}$ trophic levels were similar, at 0.2 and 0.3 respectively. In comparison to trophic levels calculated using $TL_{Glu/Phe}$, they were much higher at 1.8 to 2.0 ($TL_{Val/Phe}$) and 1.9 to 2.2 ($TL_{Ala/Phe}$). As could be expected using the source and trophic amino acid data to calculate trophic level estimates, statistical analysis reflected the results observed between $\delta^{15}N_{AA}$ values and tissue match. No significant relationship was found between tissue mass and trophic level for any of the three equations (Table 4-7).

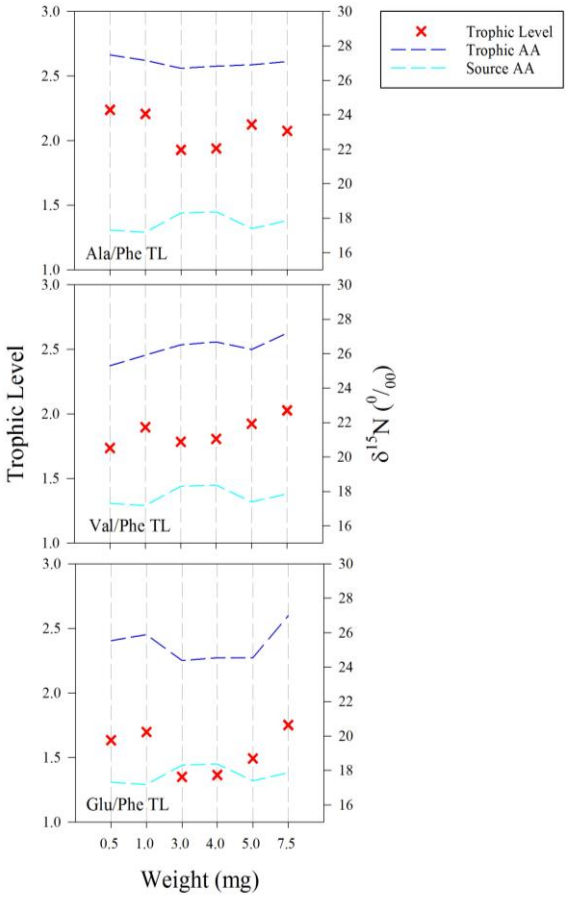

Figure 4-10 Mean trophic level obtained from different tissue masses of *Arenicola marina* using the Val/Phe (top), Ala/Phe (middle) and Glu/Phe (bottom) (Chikaraishi et al., 2009). The mean $\delta^{15}N$ of the relevant trophic amino acid and the source amino acid (phenylalanine) also shown.

	Spearman's Rank Correlation
Val/Phe TL	R_s = 0.829, d.f. = 4, P = 0.058
Ala/Phe TL	R_s = -0.486, d.f. = 4, P = 0.356
Glu/Phe TL	R_s = 0.143, d.f. = 4, P = 0.803

Table 4-7 Spearman's Rank correlation results between the trophic level calculated using the Val/Phe, Ala/Phe and Glu/Phe equations 4.1 to 4.3 (Chikaraishi et al., 2009) and tissue mass of *Arenicola marina.*

4.3.3.2 Preservation method

There was no significant effect of time, preservation or interaction on the trophic level recorded using any of Equations 4.1 to 4.3 (Table 4-8). The $TL_{Glu/Phe}$ estimates produced the lowest calculated trophic levels ranging from 1.6 ± 0.4 (6 months ethanol) to 2.1 ± 0.3 (1 year ethanol), followed by $TL_{Ala/Phe}$ from 2.1 ± 0.5 (6 months frozen) to 2.6 ± 0.3 (1 week ethanol) and the highest values were recorded for $TL_{Val/Phe}$ from 2.1 ± 1.1 (6 month frozen) to 3.0 ± 0.5 (1 year ethanol) (Figure 4-11). The $TL_{Glu/Phe}$ trophic level had the lowest variability with each preservation time treatment (SD < ± 0.8 within each sample), whilst $TL_{Val/Phe}$ had the highest standard deviation of ± 1.2.

	Time	Preservation	Time*Preservation
Val/Phe TL	$F_{(4,44)}$ = 1.345, P = 0.269	$F_{(2,44)}$ = 0.767, P = 470	$F_{(8,44)}$ = 0.628, P = 0.750
Ala/Phe TL	$F_{(4,44)}$ = 1.197, P = 0.326	$F_{(2,44)}$ = 0.654, 0.525	$F_{(8,44)}$ = 0.871, P = 0.548
Glu/Phe TL	$F_{(4,44)}$ = 2.367, P = 0.067	$F_{(2,44)}$ = 0.504, P = 0.604	$F_{(8,44)}$ = 0.463, P = 0.876

Table 4-8 Two-way ANOVA results for each trophic level equation in Chikaraishi et al. (2009). All data passed both normality (Shapiro-Wilk) and equal variance (Brown-Forsythe) tests.

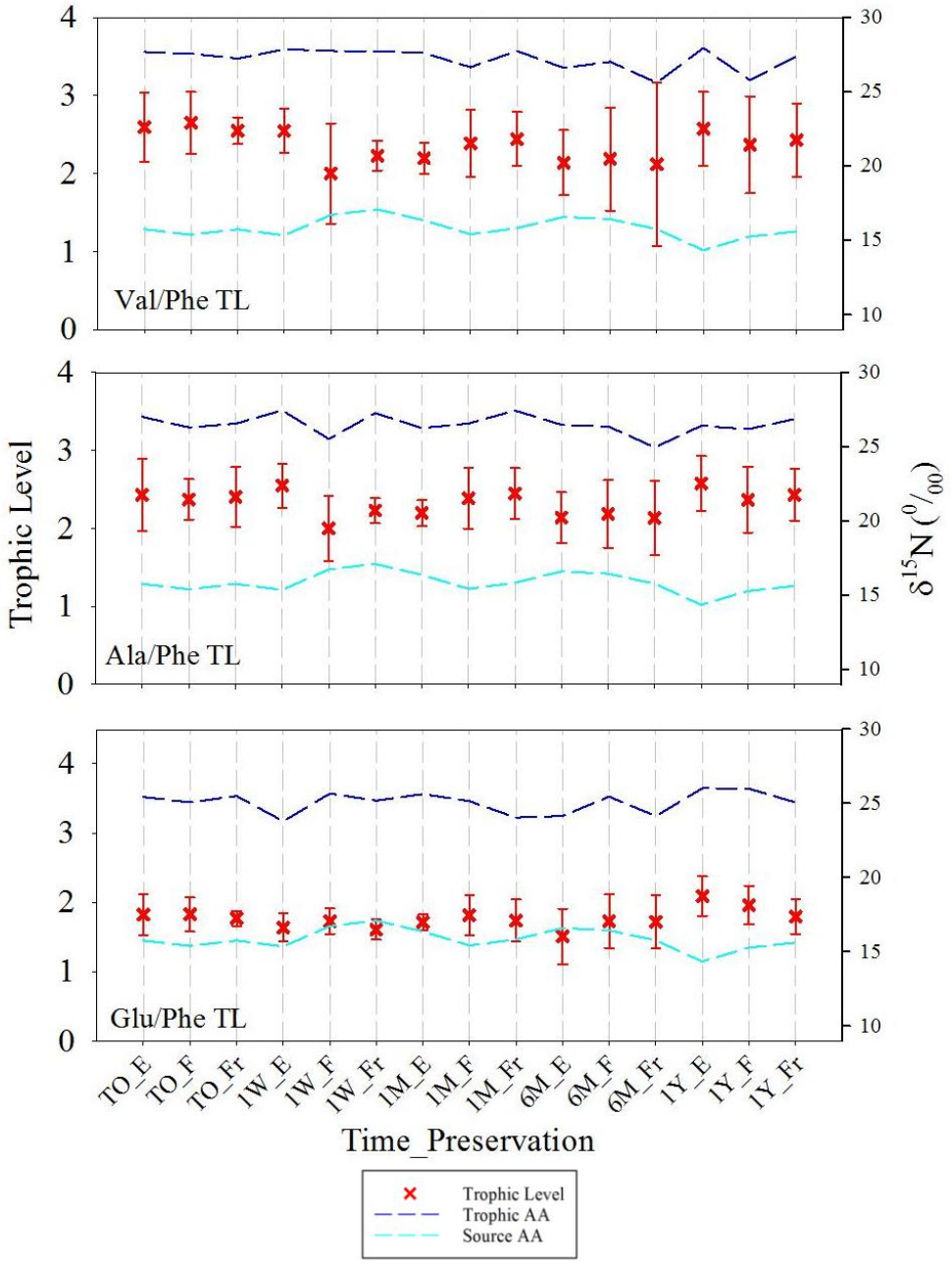

Figure 4-11 Mean trophic level of *Arenicola marina* for each time-preservation treatment using the Val/Phe (top), Ala/Phe (middle) and Glu/Phe (bottom) equations in Chikaraishi et al. (2009). The mean $\delta^{15}N$ of the relevant trophic amino acid and the source amino acid (phenylalanine) also shown. Where T_0 = time zero, 1W = 1 week, 1M = 1 month, 6M = 6 months and 1Y = 1 year and E = ethanol, F = formalin, Fr = frozen. Note that the edited value for the 6M_Fr treatment has the outlier from Figure 4-8 removed.

4.4 Discussion

4.4.1 Total nitrogen

The total nitrogen content of marine organisms is related to food supply and demand which, varies within and between species. The interspecific variation may reflect feeding methods and physiology, but may also vary temporally and spatially (Hawkins and Bayne, 1985, Fourqurean et al., 1997, Hillebrand and Sommer, 1999). *Arenicola marina* is a deposit feeder that lives in burrows in muddy sediments and obtains its nitrogen from organic matter, a heterogeneous, highly variable food source which could explain some of the variability observed in this study (Jacobsen, 1967).

There was considerable variation in the total nitrogen values from replicate individuals within certain preservation method and time scenarios. This could be a result of intra-species trait variations not considered within this study, such as body size, which can affect nutrient content (Cross et al., 2003). Overall, preservation time did not affect the total nitrogen content recorded for *A. marina,* however preservation method did have a significant effect. Williams and Robins (1982) recorded a loss in dry weight and total nitrogen content in preserved specimens of the copepod *Calanus helgolandicus*, which was greater in frozen material than in specimens preserved in formaldehyde. This could be the case in the *A. marina* data, as the only constant pattern was that the total nitrogen values of the specimens preserved in formalin were always greater than those preserved in ethanol or frozen. The loss in nitrogen with preservation could be assigned to the degradation of proteinaceous material that, again varies with preservation method (Hopkins, 1968).

4.4.2 $\delta^{15}N_{Bulk}$

With the exception of two outliers, the $\delta^{15}N_{bulk}$ values of tissue masses of $\geq$ 0.5 mg were relatively constant; therefore, for reliable $\delta^{15}N_{bulk}$ data, samples of $\geq$ 0.5 mg were used for all preservation tests.

Significant differences in $\delta^{15}N_{bulk}$ values were observed over time, with enriched $\delta^{15}N_{bulk}$ values between T0 to 1Y. How formalin preservation affects isotopic signatures has been discussed in the literature (e.g. Fanelli et al., 2010), however many of these refer to significant differences in $\delta^{13}C$ composition. With regard to nitrogen, potential effects of preservation that may alter $\delta^{15}N_{Bulk}$ values include the exchange of ^{14}N for ^{15}N (Hobson et al., 1997, Edwards

et al., 2002) and the hydrolysis of proteins (Arrington and Winemiller, 2002). As discussed in Fanelli et al. (2010) and Barrow et al. (2008) who reviewed multiple preservation studies, the impacts of preservatives on stable isotopic signatures are highly variable and taxon specific. Based on the 16 studies reviewed in Barrow et al. (2008) for the majority of species $\delta^{15}N_{Bulk}$ values obtained were not significantly affected by preservation frozen (>90%), in ethanol (~70%) or formalin (~60%). For those species that were affected, in the majority of cases and as recorded for *A. marina*, $\delta^{15}N$ values were enriched over time for both ethanol and formalin.

4.4.3 $\delta^{15}N_{AA}$

Although there was some variation in $\delta^{15}N_{AA}$ values with tissue mass, there was no consistent or significant relationship between the two variables observed here. However, it would still be advised to use the same tissue mass where possible for $\delta^{15}N_{AA}$ analysis. No significant variation in the $\delta^{15}N_{AA}$ values over time, or with preservation method was recorded. The only significant results for amino acids was a significant effect of time and preservation on aspartic acid signatures. This reflected the relatively lower and more variable $\delta^{15}N$ values at T_0 for the formalin and frozen specimens and at 1 week for ethanol samples.

Given the significant effects of preservation method and time on $\delta^{15}N_{bulk}$ values, it might have been expected that some of the $\delta^{15}N_{AA}$ values would reflect this. It is possible that the significant variation in the $\delta^{15}N_{Bulk}$ values were related to changes in other amino acids not measured here. As shown in Figure 4-2, amino acids including isoleucine, threonine, serine and proline were present within the derivatized specimens. However these amino acids were often absent from the GC/C/IRMS chromatograms or were at low amplitudes and thus deemed unreliable for analysis.

4.4.4 Trophic level

Tissue mass did not have a significant effect on trophic level estimates. This is useful information for future studies as preserved specimens may be smaller or less tissue is available. However where possible, using a constant tissue mass is still advised to limit any additional variation.

The trophic levels calculated varied depending on which trophic amino acid was used. The highest and most variable values were for $TL_{Val/Phe}$, followed by $TL_{Ala/Phe}$ and the lower and more consistent trophic level was determined using $TL_{Glu/Phe}$. The differences in variability can be assigned to the variation in the trophic amino acid data. Chikaraishi et al. (2009) considered

that the trophic level calculated from $TL_{Glu/Phe}$ has the lowest error. Later research by Chikaraishi et al. (2010) investigated the mean isotopic differences between the trophic amino acid and phenylalanine and the level of $\delta^{15}N$ enrichment in more detail and confirmed that Equation 4.1 is most suitable for aquatic food webs.

The $TL_{Glu/Phe}$ estimates ranged from 1.55 to 2.09, suggesting that *Arenicola marina* is an omnivorous feeder. This result reflects the uncertainty surrounding the diet of *A. marina* within the literature. Given the nature of its feeding behaviour, a subsurface deposit feeder, its diet will reflect the local environment i.e. if there are small organisms within the sediment $\delta^{15}N$ signatures and trophic level estimates may be higher suggesting more predator or scavenger traits. Alternatively if there is a highly available quality of organic matter, signatures may be more indicative of herbivorous feeding. The recorded omnivory suggests that perhaps the species is a more flexible or selective feeder than some studies suggest (e.g. Riisgard and Banta, 1998).

4.5 Conclusions

The SIA and CSIA results presented here for experiments assessing the influence of preservation method and time on the $\delta^{15}N$ composition of *Arenicola marina* were essential to this study as the main subject of this thesis, namely Antarctic polychaetes (Chapter 5 and 6) were all preserved in ethanol. The main findings include:

- A minimum of 0.5 mg of tissue should be used for $\delta^{15}N_{Bulk}$ analysis of polychaetes.
- An increase in $\delta^{15}N_{Bulk}$ values with increasing preservation time was recorded for tissues preserved by freezing, or in formaldehyde or ethanol.
- The significant variation in $\delta^{15}N_{Bulk}$ values could not be explained by variation in any of the seven amino acids examined here.
- Variation in $\delta^{15}N_{AA}$ values with tissue mass were not consistent across the different amino acids analysed. Where possible at least 5 mg of tissue should be used for $\delta^{15}N_{AA}$ analysis.
- Estimates of trophic levels vary dependent upon the trophic amino acid and equation used. The $TL_{Glu/Phe}$ was considered to be most reflective of the 'true' trophic level of *A. marina*.

4.6 References

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5 Trophic traits of Antarctic polychaetes

The $\delta^{15}N_{AA}$ data presented in this chapter will be submitted for publication with data collected from symbiotic polynoids in Chapter 6.

5.1 Introduction

5.1.1 Diversity, traits and ecosystem function

Despite the increase in trait based research in the last decade, commonly used terms such as 'biological' or 'functional' trait as well as 'functional diversity' and 'ecosystem function' remain unclear or often undefined (Hooper et al., 2005; Petchey and Gaston, 2006; Bremner, 2008). In this project biological traits are viewed as well-defined measureable properties of an organism that are related to their behavioural, reproductive and morphological characteristics (McGill et al., 2006, Tyler et al., 2012, Faulwetter et al., 2014). Some biological traits may also be referred to as functional traits. These are considered to be fundamentally important to ecosystem function e.g. traits that affect resource use, feeding interactions, or habitat structure (Bremner, 2008). 'Ecosystem function' is generally considered an umbrella term for the processing and operation of an ecosystem encompassing various physical, chemical and biological factors (Loreau, 2008). For example, Bremner et al. (2006a) defined ecosystem function in three ways: as a process (e.g. nutrient cycling), as a particular property of an ecosystem (e.g. stability), or the flow of energy and materials through the abiotic and biotic components of the ecosystem. Measures of 'functional diversity' use trait data to understand species contributions to ecosystem function (Petchey and Gaston, 2006). Measures of functional diversity can include the number, type and distribution of functions performed by organisms within an ecosystem that subsequently contribute to ecosystem function (Díaz and Cabido, 2001). Functional diversity is often, but not always, related to species diversity and thus not all species contribute equally to ecosystem function (Hooper et al., 2005).

The increased number of functional studies in the last 10 years reflects a shift in research interest from understanding the diversity of ecosystems to understanding how they work and, the services they provide. Functional research approaches range from experimental studies to macro-ecological models. This progression could be related to the increased awareness of the importance of ecosystem services and a need for ecosystem-based management (Bremner, 2008). Additionally, there is increasing concern about the potential impacts of biodiversity loss

on ecosystem function (Solan et al., 2004; Bulling et al., 2010). Furthermore, there are an increased number of publicly-available trait databases such as the Biological Traits Information Catalogue (BIOTIC) for marine organisms (MarLIN, 2006) and taxa specific databases inlcuding Polytraits for polychaetes (Faulwetter et al., 2014) that are facilitating more trait investigations.

The assignment of species-level traits is laborious and time-consuming (Faulwetter et al., 2014), so there are still large gaps in our trait knowledge (Tyler et al., 2012). Often, if species level trait data are not readily available, traits from higher taxomonic groupings are used. This works on the basis that closely-related species might have evolved similar environmental and ecological adaptations and so are functionally similar (Usseglio-Polatera et al., 2000). However, more recent investigations have found that even closely related species and cryptic species may be functionally different (Davidson and Haygood, 1999, McGovern and Hellberg, 2003) and thus more accurate information on the evolutionary relationships between species is required (Tyler et al., 2012).

5.1.2 Stable isotope analysis (SIA)

Stable isotopic ratios have been used in ecosystem research to examine trophic relationships since the late 1970s (Peterson and Fry, 1987). The two most common elements used for SIA in food web studies are carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$). In both cases the ratio of the heavier to the lighter isotope is determined relative to a standard (Equation 5-1). Thus the lower the δ value relative to the standard, the more depleted in the heavier isotope the sample is. Differences in the ratio of heavy to light isotopes due to their source or fractionation (described below) is typically quite small; in order to accurately detect these small differences isotopic ratios are measured using a mass spectrometer in gaseous samples of CO_2 and N_2 generated from the sample of interest.

$$\delta X = [(R_{sample}/R_{standard})\text{-}1) \times 10^3$$

Equation 5-1 Calculation of isotopic composition relative to a standard expressed in parts per thousand (‰), where X is ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$ (Peterson and Fry, 1987). Where standards are, calcium carbonate for C and atmospheric N_2 for N (Craig, 1957, Mariotti, 1983, Carter and Barwick, 2011).

Linking consumers to food sources using bulk stable isotopes ratios is based on the assumption that the isotopic composition of a consumer reflects the weighted mean isotopic composition of its food source (Gannes et al., 1997, Fry, 2006). Controlled studies have shown through

comparison of $\delta^{13}C$ values from food sources and consumers it is possible to identify the energy sources at the base of food webs (Deniro and Epstein, 1978). Trophic enrichment in $\delta^{13}C$ is generally considered to be small (0.4 to 1.0‰), as indicated by both controlled laboratory experiments and field analyses (see Post, 2002, Michener et al., 2007). The minor enrichment in $\delta^{13}C$ could be associated with several factors including, loss of ^{12}C during respiration or preferential uptake of ^{13}C (Deniro and Epstein, 1978, Rau et al., 1983, Fry et al., 1984). $\delta^{13}C$ values can also be dependent on the types of tissues sampled. For example, McCutchan et al. (2003) found the mean trophic enrichment factor for carbon in muscle tissue was 1.0‰ higher than whole body samples.

In comparison to carbon, trophic enrichment for $\delta^{15}N$ is generally much larger between food sources and consumers. This is a result of the increased fractionation of nitrogen, which affects the relative abundance of ^{14}N and ^{15}N. When nitrogen is consumed, the lighter isotope, ^{14}N, is more readily used in metabolic processes such as deamination and lost by excretion than the heavier isotope, ^{15}N. Subsequently, the consumer becomes 'enriched' in the heavier isotope relative to its food source. As a result, the $\delta^{15}N$ values of animal consumers are 3 to 5 ‰ greater than their dietary nitrogen (Peterson and Fry, 1987). The increased fractionation of nitrogen makes it a useful as an indicator of trophic level rather than food source (Deniro and Epstein, 1981, Minagawa and Wada, 1984). The average trophic enrichment is ~3.4 ± 1.0 ‰ for $\delta^{15}N$ (Post, 2002), and this has been used to calculate trophic level in a number of studies (e.g Jennings et al. (2002a), Jennings et al. (2002b) and Jennings et al. (2002c)).

Trophic enrichment factors can vary; this results from multiple interacting factors. For example, McCutchan et al. (2003) found that trophic enrichment was lowest in consumers raised on invertebrate diets (1.4 ± 0.2‰), whereas those raised on high protein animal diets where significantly higher (3.3 ± 0.3‰). Another source of variation is the means by which consumers obtain nitrogen, i.e. in the pelagic food web phytoplankton are the primary food sources, while in littoral or benthic food webs they are dominated by detrital material (Post, 2002). The form of nitrogen excreted by the organism e.g. urea, uric acid, ammonia, guanine or amino acids also influence $\delta^{15}N$ signatures (Vanderklift and Ponsard, 2003). Overall the insight into trophic relationships based on SIA has several advantages over conventional methods such as gut content analysis. Most importantly perhaps, is that SIA values provide a medium to long-term average of an organism's feeding habits as isotopic ratios are related to

food assimilated into tissue, rather than just recently ingested material (Hobson and Welch, 1992).

5.1.3 Compound specific stable isotope analysis (CSIA)

Stable isotope analyses of whole organisms and tissues samples have improved our understanding of food web relationships and the flow of different elements through ecosystems. However, the trophic level defined from 'bulk methods' described above can be misleading (Chikaraishi et al., 2009). The degree of fractionation and enrichment of $\delta^{15}N$ with each trophic step can vary with species and physiology (Bearhop et al., 2004). This may be associated with differences in metabolic activity between individuals (Hobson and Clark, 1992, Bearhop et al., 2002) as well as their nutritional condition (Hobson et al., 1993). Furthermore, the $\delta^{15}N$ of some primary producers such as phytoplankton varies temporally and spatially. Such variation can result from the different sources of nitrogen available (nitrate, ammonium and N_2), the incomplete utilisation of nitrogenous nutrients of the uptake of partially denitrified nitrate (Popp et al., 2007). Although knowledge of the $\delta^{15}N$ of food sources at the time of sampling are useful in the determination of trophic end-members, they are only provide a snapshot of the assimilated food consumed by other organisms. This increases the potential for error when estimating the trophic level of different organisms. An alternative to bulk analysis is to analyse the stable isotope ratios of specific compounds, e.g. amino acids. The advantage of this is that analysis of determine single tissue sample yields information on the base of the food web and trophic level (Macko et al., 1997, McClelland and Montoya, 2002).

Using gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS), McClelland and Montoya (2002) demonstrated that changes in bulk N-isotope ratios with trophic level reflected the relative abundance and the degree of ^{15}N enrichment of amino acids in the consumer. The authors observed that some amino acids were enriched in consumers by ~7‰, e.g. alanine, valine, isoleucine, proline and glutamic acid, whereas others remained virtually unchanged, e.g. glycine, serine and phenylalanine. These two groups of amino acids became known as 'trophic' and 'source' amino acids, respectively (Popp et al., 2007). The difference in ^{15}N enrichment between trophic and source amino acids is associated with the process of transamination, the transfer of an amino group. For trophic amino acids there is signification isotopic fractionation during this process as a result of the cleavage of the carbon-nitrogen bond; however in source amino acids carbon-nitrogen bonds are neither formed nor broken, so their $\delta^{15}N$ is conserved (Chikaraishi et al., 2007). As a result, the $\delta^{15}N$ values of

source amino acids provide information on the nitrogen sources at the base of the food web. Using controlled feeding experiments and comparison with previous data, Chikaraishi et al. (2009) estimated the isotopic differences among the amino acids in aquatic primary producers and the ^{15}N enrichment factor at each trophic level. These data were used to calculate trophic level using phenylalanine and glutamic acid as the source and trophic amino acids (Equation 5-2). This amino acid combination was chosen over other trophic and source amino acids as it had the lowest associated error.

$$\text{Trophic level} = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - 3.4)/7.6 + 1$$

Equation 5-2 Calculating trophic level using the isotopic values of the trophic and source amino acids glutamic acid ($\delta^{15}N_{Glu}$) and phenylalanine ($\delta^{15}N_{Phe}$), respectively. Where 3.4‰ is the mean isotopic difference between glutamic acid and phenylalanine and 7.6‰ is the ^{15}N trophic enrichment factor at each trophic level.

It has now been shown that amino acids provide a sensitive indicator of trophic level in zooplankton (McClelland and Montoya, 2002, McCarthy et al., 2007, Hannides et al., 2009), invertebrates (Pakhomov et al., 2004, Chikaraishi et al., 2007, Ohkouchi et al., 2013) and fish (Popp et al., 2007, Chikaraishi et al., 2009). The use of amino acids provides the potential to accurately measure the trophic level of an individual without the need for source material. This is of great benefit as source materials may be multiple and it is often difficult to collect or is unavailable, especially when using preserved archive material.

5.1.4 Trophic ecology of the Southern Ocean

The ecology of the Southern Ocean is tightly linked to the seasonal cycle of primary production, which includes: phytoplankton, ice algae and benthic macroalgae. The latter are less likely to influence the trophic ecology of the organisms studied here given the distance from land and reduced impact of glacial sedimentation (Anderson et al., 1979). Primary production in the Southern Ocean is highly seasonal, limited and controlled by light, nutrients and temperature (Dayton et al., 1994). Light, which is controlled by solar irradiance and sea ice cover, is considered to be the key driver of seasonal primary production in Antarctic waters (El-Sayed, 1985; Clarke, 1988; Clarke and Leakey, 1996). An eight to ten week summer phytoplankton bloom occurs consistently after the retreat of winter sea ice, normally from November to January, when stable conditions in the water column are established. An abrupt decline in chlorophyll biomass and so phytoplankton, is normally observed in February indicating the end of the bloom. During and after the bloom period there is a seasonal disposition of particulate organic carbon to the seafloor (Clarke, 1988). The amount of phytodetritus reaching the

seafloor is variable dependent on depth, the productivity of overlying surface waters and benthic currents. Thus near shore shelf communities may experience very different phytodetrial food inputs compared to slope and abyssal communities (Ramirez-Llodra et al., 2010).

Applications of SIA to aid our understanding of Antarctic food webs started to appear in the 1980s. Many studies target large charismatic fauna, including marine species such as seals and seabirds (Rau et al., 1991a, Rau et al., 1991b, Rau et al., 1992, Burns et al., 1998). However, SIA has been applied to benthic invetebrates from various sites around Antarctica including the Scotia Arc (Kaehler and Pakhomov, 2001, Corbisier et al., 2004), the Western Antarctic Peninsula (Conlan et al., 2006, Mincks et al., 2008), the Ross Sea (Norkko et al., 2007) and the Weddell Sea (Nyssen et al., 2002, Mintenbeck et al., 2007).

The current changes in the extent and seasonal retreat of Antarctic sea ice has led to an increased research effort to document Antarctic primary productivity and the implications of any changes on marine ecosystems. Mincks et al. (2008) determined $\delta^{13}C$ values of -22.9 to -25.7‰ and $\delta^{15}N$ values of 5.7 to 7.9‰ for phytodetritus derived from the summer phytoplankton bloom collected in March 2001 on the West Antarctic Peninsula (WAP) continental shelf. The collection of $\delta^{13}C$ and $\delta^{15}N$ values of four potential food sources allowed the determination of feeding preferences of benthic organisms, namely deposit feeders on fresh detritus or reworked organic material, or predatory feeding utilizing epibenthic or pelagic food sources. Differences in $\delta^{15}N$ values of presumed deposit feeders compared to predator/scavengers was also noted, for example, the predatory polychaete *Laetomonice producta* had a recorded $\delta^{15}N$ value of 11.4 ‰ while that of the deposit feeder *Aurospio* sp. was 7.9 ‰. Additionally, Mincks et al. (2008) and Norkko et al. (2007) recorded a lack of seasonal feeding traits in organisms feeding on detrital matter. They concluded that seasonal primary production is integrated into the sediment, providing a food bank for benthic feeders.

Temporal and spatial variability in the $\delta^{15}N$ values of Antarctic organisms has also been recorded. The $\delta^{15}N$ values of bivalve and urchin species indicated a shift in consumption from detrital to fresh algal material between areas of permanent sea ice to those of ice-free water (Norkko et al., 2007, Mincks et al., 2008). Spatial variation in isotopic composition has also been recorded in the Southern Ocean, for example there is a correlation between latitude and $\delta^{13}C$ values of the blood of penguin chicks (Cherel and Hobson, 2007). In the same study, the variation in $\delta^{13}C$ values could also be used to identify inshore and offshore foraging sites between different populations. Such studies could inform the prediction of climate change

impacts on higher trophic levels via changing sea ice conditions and the onset, duration and intensity of phytoplankton blooms.

$\delta^{15}N$ signatures also vary with depth as recorded within the Weddell Sea by Mintenbeck et al. (2007). The observed changes varied with feeding guild, because of the particle-size preferences between feeding guilds and the biogeochemical reworking of particulate organic matter (Mintenbeck et al., 2007). In contrast, no change was observed in the $\delta^{15}N$ signatures of predators between 1000 and 5600 m depths in the Arctic, although an enrichment in the $\delta^{15}N$ signatures of suspension feeders and a depletion in the $\delta^{15}N$ signatures of deposit feeders was recorded within increasing depth (Bergmann et al., 2009). These differences were related to bacterial action on particulate organic matter with aging (or depth) as well as the sources consumed by different feeding groups. For carbon, changes in bulk $\delta^{13}C$ values with depth have also been recorded in bottom dwelling shrimp (Pakhomov et al., 2004). It is essential to consider both potential temporal and spatial variation when analysing specimens from different regions and depths to avoid any bias and misinterpretation of stable-isotope-based trophic information.

5.1.5 Benthic feeding guilds

In this chapter, the $\delta^{15}N$ amino acid ($\delta^{15}N_{AA}$) signatures of 16 polychaete species from the Southern Ocean are used to determine their trophic traits. To date, the trophic traits of these species have been defined based on morphological features, gut content and some bulk isotope analyses. Feeding guilds or diet types are often defined in different ways. Here, I describe various feeding guilds associated with the Southern Ocean benthos as well as those included in many polychaete studies and in the Polytraits database (Knox, 1994, Fauchald and Jumars, 1979, Faulwetter et al., 2014). It is possible for species to exhibit a variety of feeding guilds; this may reflect opportunistic behaviour or non-selective feeding.

5.1.5.1 Herbivores

Herbivores only consume plant material; they are generally found in shallow water grazing on macroalgal films on the seafloor by deposit feeding, but may also capture phytoplankton from the water column by suspension feeding. These feeding types are not exclusively herbivorous as organisms may graze on, or capture other animal material. Thus the terms suspension or deposit feeder are used more frequently.

- Suspension feeders

Suspension feeders extend specialised feeding structures into bottom currents to collect organic particles from the water column. Organic particles could include, but are not limited, to detrital material, such as phytodetritus and faecal pellets, living plankton, larvae and eggs. Gut content analyses have shown that species may ingest a range of food sources, for example Tatian et al. (2005) observed that ingested material in the guts of invertebrate suspension feeders off King George Island mostly consisted of macroalgal detritus and faecal pellets, whilst microalgae appeared to be a minor dietary component. No suspension feeders were targeted in this study, however many suspension-feeding polychaetes are known to occur in the Southern Ocean (Beylev and Uschakov, 1957). In some texts, suspension feeding is referred to as filter feeding, as organisms filter material for consumption from the surrounding seawater.

- Deposit feeders

Polychaetes are amongst the most prominent deposit feeders in the Southern Ocean benthos (Knox, 1994). Deposit feeders consume material from the surface of the seafloor hence termed surface deposit feeders, or they may burrow ingesting sediment below the surface. Many deposit feeders are motile, using palps and tentacles to collect and ingest organic matter or sediment microalgae (Fauchald and Jumars, 1979).

5.1.5.2 Omnivores

Omnivores have a broad diet and can be classed as both herbivores and carnivores. For example, gut content analysis of the giant amphipod *Glyptonotus antarctica* contained ophuroids, gastropods, echinoids, pycnogonids, sponges, crinoids, brachiopods, algae and amphipods (Dearborn, 1967). “Omnivore” is often used as a catch-all term to describe feeding behaviour and often creates confusion between true omnivores, which are opportunistic and temporalyl omnivorous species, for which limited data are available.

5.1.5.3 Carnivores

Carnivorous species feed on other organisms, they include species with predatory or scavenging behaviours. Often authors are unsure whether organisms actively hunt live prey or feed on dead material and so may describe species as predator/scavenger but the differences between the two are discussed below.

- Necrophage/scavenger

Necrophagy within Antarctic invertebrates was first described by Arnaud (1970). Organisms adopting this feeding method can also be described as scavengers and they consume dead material on the seafloor. Necrophagy may be more common in winter when primary food material is not readily available, or species may be opportunistic necrophages when food falls create discrete organic enrichment at the seafloor. This trait is considered to be widespread among amphipods, gastropods, ophiuroids, echinoids and nemerteans and may be more common in Antarctica than other oceans (Presler, 1986). Early theories suggested that this was a result of lower water temperatures and that necrophagy provided a more energy-efficient foraging strategy (Arnaud, 1970). However it is now considered to be related to limited food availability and any decline in necrophagy in Antarctic waters may be associated with the increase in energy-rich prey rather than increasing temperature (McClintock, 1994).

- Predators

Predators feed by preying on other animals. This could be by active hunting or luring of individuals. Predators have evolved specialist predatory mechanisms, for example many infaunal polychaetes often possess a muscular eversible pharynx, large jaws, and prey-detecting sensory devices. Their prey include: amphipod crustaceans, bivalves, and other polychaetes (Fauchald and Jumars, 1979). In soft bottom communities, infaunal predatory polychaetes can significantly reduce the abundance of other species. For example the Nereids, *Nereis virens* controls the abundance of the amphipod *Corophium volutator* while *N. diversicolor* influences the abundance of nematode, turbellarian and juvenile cockle species (Reise, 1979, Commito, 1982).

5.1.6 Current knowledge of the trophic traits of polychaetes

Polychaetes are often the numerically dominant taxa within the benthos. As a group they are considered to have key roles in the food web, acting as prey for larger predators and contributing to the cycling of organic matter (Gambi et al., 1997). Despite this there is a lack of species specific data and feeding guilds are often assigned by family level morphological characteristics. Gaston (1987) was one of the first to attempt to describe the diet of polychaetes. The gut contents of polychaetes from 20 different families to their previously considered categorical feeding guilds were analysed and compared, although the majority of specimens examined had extruded guts. Later, Gambi et al. (1997) determined the categorical feeding

guilds of 77 polychaete species from the Ross Sea, where the majority of species were considered to be deposit feeders with some carnivores. In contrast, Saiz-Salinas et al. (1998) described Southern Ocean polychaetes to be mostly omnivorous. This inconsistency highlights a need for better species level trait analysis.

There are a few examples of studies that use biochemical analysis to define the trophic level and dietary components of different Antarctic polychaete species. For example polychaete species have been included in SIA studies of macrofauna on the Antarctic Peninsula (Mincks et al., 2008), King George Island (Kaehler and Pakhomov, 2001, Corbisier et al., 2004) and the Weddell Sea (Nyssen et al., 2002). In a more comprehensive study of polychaetes Würzberg et al. (2011) identified the feeding preferences of 79 polychaetes belonging to 18 different families from the Weddell Sea between 600 m and 5337 m depth using fatty acids as trophic biomarkers. Certain fatty acids were indicative of dietary components and were used to define feeding guilds and selective preferences; those relevant to this study are included below. Feeding patterns were relatively consistent within families at deeper sites but varied between individuals collected from the shallower shelf stations. Our current knowledge of trophic traits of families including species analysed in this study are described below.

5.1.6.1 Acrocirridae

Acrocirridae are generally considered to be surface deposit or filter feeders. Würzberg et al. (2011), used fatty acid analysis to demonstrate that an unidentified species of Acrocirridae was primarily feeding on freshly deposited diatoms. The fatty acid distributions of Acrocirridae were comparable to those of Spionidae species examined in the same study, and may be related to their taxomonic position in the same superfamily, Spionida.

5.1.6.2 Glyceridae

Glyceridae have large eversible pharynges tipped with four jaws, which has often led to the assumption that they are carnivorous. Gaston et al. (1987) considered *Glycera capitata*, *G. robusta* and *G. dibranchiata* to be mobile carnivores based on previous studies. Ingested material documented during gut content analysis of *G. dibranchiata* included an amphipod and a polychaete. Other studies have listed Glyceridae as detritivores feeding on faecal pellets; this feeding behaviour combined with the absorption of dissolved organic matter may fulfill part of their energy requirements (see references in Fauchald and Jumars, 1979).

5.1.6.3 Nephtyidae

Species of the family Nepthyidae have large eversible pharynges, within which there is a small pair of jaws (Fauchald and Jumars, 1979). As a result, Nephtyidae are generally perceived to be carnivorous; they are free-living, highly mobile and are often found in burrows. The gut contents of the Nephtyidae species *Aglaophamus circinata* collected by Gaston et al. (1987), contained coarse sand, foraminifera and polychaetes. Other studies have found similar dietary components in the guts of nephtyids. Rauschenplat (1901) observed large quantities of sand in the guts of *Nephtys* spp. as well as the remnants of other marine invertebrates. Variation in the diet of *N. incisa* from different localities has been recorded. Sanders (1956, 1960) found no evidence of carnivorous behaviour from specimens in New England and suggested the species was a motile deposit feeder; this was in contrast to individuals collected in Europe (Southward, 1957, Clark, 1962). *Aglaophamus* species have also been studied in the Antarctic, for example, Würzberg et al. (2011) found evidence of consumption of foraminifera in the fatty acid composition of polychaete tissue. Corbisier et al. (2004) recorded enriched $\delta^{13}C$ values for *Aglaophamus ornatus* compared to both primary food sources and the $\delta^{13}C$ signatures of the primary consumers i.e. deposit feeders, suggesting *A. ornatus* feeds carnivorously.

5.1.6.4 Paraonidae

Paraonidae have short, eversible sac-like pharynges with a soft proboscis used for feeding. Fauchald and Jumars (1979) described the feeding biology of a single species, *Paraonis fulgens,* and considered it to be a non-selective, burrowing deposit feeder or surface feeder (Day, 1967, Pearson, 1971, Rasmussen, 1973). These statements have generally been applied to the family level, classifying all 50 known species of Paraonidae with the same feeding traits (Fauchald and Jumars, 1979). As discussed earlier for *Nephtys incise,* there is variability in the documented diets of *P. fulgens*. Mortensen (1922) suggests that the species feeds on plant debris and dead animals, whilst Röder (1971) suggested that its diet consists of pennate diatoms as well as foraminifera and small crustaceans. Since then there has been an increase in the number of *Paraonidae* species studied and Gaston et al. (1987) examined 28 individuals from the genus *Aricidea*. These included representatives from the three species *A. catherinae, A. cerrutii* and *A. simplex,* all of which contained phytodetritus in their guts and were considered to be surface deposit feeders.

5.1.6.5 Polynoidae

All polynoids have a muscular eversible pharynx armed with jaws and are considered to be carnivores. Polynoid prey items include small crustaceans, echinoderms, polychaetes, gastropods, sponges and hydroids (for references see Fauchald and Jumars, 1979). Algal fragments may also be an important component of the diet of some species including *Harmothoe imbricata,* which can comprise 18% of an individual's body weight, however the most common prey type are amphipods, comprising up to 66% of an individual's body weight (Streltzov, 1966). Fatty acid analyses of unidentified Antarctic polynoids also provide evidence for carnivorous behaviour (Würzberg et al., 2011). The widespread Antarctic Polynoidae species *Laetmonice producta* had stable isotopic signatures indicative of predatory feeding (Mincks et al. (2008). Variation in the feeding guilds of polynoids has been observed, and is most often associated with commensal relationships with host species (Fauchald and Jumars, 1979). None of the polynoid species investigated in this study have associated host species and will be considered as omnivores, predators and scavengers.

5.1.6.6 Scalibregmatidae

Scalibregmatidae have sac-like eversible pharynges, and actively burrow in soft sediments being buried below the sediment surface, at up to 30-60cm deep (Ashworth, 1901, Hertweck and Reineck, 1966). They are generally considered to feed on detritus found in the sediment, for example, Mare (1942) suggested that *Scalibregma inflatum* feeds non-selectively on surface sediments. Although there is little evidence for selectivity within this family their burrowing behaviour can be affected by sediment composition and therefore the diet of different populations of the same species can be depend on regional productivity (Fauchald and Jumars, 1979). In contrast, fatty acids associated with copepods and deep-sea zooplankton have been recorded in Southern Ocean Scalibregmatidae (Würzberg et al., 2011)). This suggests either active hunting or ingestion of detrital matter at the seafloor (Würzberg et al., 2011).

5.1.6.7 Spionidae

The majority of the Spionidae polychaetes live in mud tubes, although most species are capable of leaving their tubes. They are generally considered to be surface deposit feeders, using ciliated palps to select food particles from the surrounding sediment. Evidence suggests that they are able to select particles based on both size and content (Fauchald and Jumars, 1979). Southward (1957) considered the ciliary feeding spionid *Laonice cirrata* to have an active

mode of life, resulting from its lack of permanent burrow and the presence of large eye spots. Selectivity within the Spionidae has been suggested from previous studies, including feeding experiments when individuals were offered natural and organic enriched sediments (Fauchald and Jumars, 1979, Kishlinger and Woodin, 2000). In the Southern Ocean, Spionidae have been observed to preferentially feed on freshly deposited organic matter high in nutritional value (Würzberg et al., 2011). SIA indicates that the Antarctic Spionidae, *Auriospio* sp., is a surface deposit feeder (Mincks et al., 2008), however, the isotope values did not vary seasonally with phytodetrial deposition and so did not support preferential or selective feeding. Other feeding guilds documented within this family, for example *Polydora* species (not investigated here), are considered wholly or partial filter feeders; rock, shell and coral drilling forms also exist, but again are not included in this study (Fauchald and Jumars, 1979).

5.1.7 Aims

In this chapter I aim to investigate the trophic traits of Antarctic polychaetes, using CSIA to determine the $\delta^{15}N$ of source and trophic amino acids in order to determine trophic level quantitatively.

The species to be examined include a range of morphologically different and cryptic species, all having assigned family level categorical feeding guilds. The sample set also contains representatives from different locations and depth combinations. The data presented are first $\delta^{15}N_{AA}$ values for Antarctic benthic polychaetes and provide descriptive information about the trophic traits and trophic variability of the taxa in the West Antarctic.

5.2 Methods

5.2.1 Antarctic species selection and preparation for CSIA

As described in Chapter 4, 5.0 mg of freeze-dried tissue is needed for reliable $\delta^{15}N_{AA}$ isotope signatures. Dry weight for the majority of species was determined on freeze-drying multiple unbarcoded specimens (Table 5-1). Hence, multiple individuals were pooled to obtain enough tissue for CSIA; this reduced the biogeographic coverage if only one specimen was barcoded from specific depth/site localities.

Species	Dry weight per individual (mg)
Flabelligena sp.	0.94
Glycera sp.	0.12
Aglaophamus sp.	1.83
Aricidea sp.	1.60
Harmothoe fuligineum	3.02
Macellicephala sp.	8.57
Macelicephaloides sp. B	4.57
Scalibregma sp.	1.67
Laonice sp	3.82

Table 5-1 The average individual dry weight obtained each morphospecies to be used for CSIA.

The sample set listed in Table 5-2 was chosen for CSIA based on the number of individuals available to ensure enough tissue mass for reliable data. Ideally, the CSIA data set should allow investigation of trophic variation across species at the same locality and, within the same species from different depths and locations. It also includes examples of cryptic species as well as different morphospecies within the same family. Sites within the same location (e.g. inner Amundsen Sea) and specimens from depths of less than 500 m were pooled to obtain enough tissue for CSIA. The barcoded *Aglaophamus* specimens were generally larger and so single individuals could be used for CSIA. Cryptic species of *Aglaophamus trissophyllus* were identified from COI sequences (Chapter 1) and only those with CO1 barcodes were used for CSIA. The biogeographic and depth distribution of these species is listed in Table 5-3.

5.2.2 CSIA

Samples were prepared for GC/C/IRMS following esterification and acylation of amino acids released by hydrolysis of tissues according to Chapter 4. Each sample was weighed before and after freeze drying, as the amount of tissue derivitised may be important when interpreting results and to ensure that the correct amount of the internal standard, L-Norleucine (L-Nle), was added to each sample. For each mg of freeze dried tissue mass 4µl of L-Nle (5000 ng μL^{-1} in 0.1M HCl; Sigma-Aldrich, suitable for amino acid analysis) was added.

		Amundsen Sea				Scotia Arc		Weddell Sea	
	Feeding	Inner		Outer		SG	ST		
Species (Family)	guild	500	1000	500	1000	500	500	500	1000
Flabelliena sp. A (MB) (Acrocirridae)	SDF	10 (2mg)							
Flabelliena sp. B (MB) (Acrocirridae)	SDF	8 (2mg)							
Glycera sp. (MB1)* (Glyceridae)	O/P/S			2 (11mg)		4 (4mg)		4 (15mg)	
Glycera sp. (MB2)* (Glyceridae)	O/P/S	4 (4mg)	2 (1mg)	2 (1mg)	2 (7mg)				
Aricidea cf. *belgicae* (MB1)* (Paraonidae)	SSDF	3 (3mg)	4 (3mg)	3 (1mg)					
Aricidea simplex (Paraoindae)	SSDF							4 (3mg)	
Harmothoe fuligineum (Polynoidae)	O/P/S	8 (9mg)		4 (3mg)		3 (7mg)			
Macellicephaloides sp. (MB1) (Polynoidae)	O/P/S	9 (7mg)		2 (3mg)	3 (2mg)				
Scalbregma sp. (MB1)* Scalibregmatidae	SSDF	9 (5mg)							
Scalbregma sp. (MB3)* Scalibregmatidae	SSDF						3 (7mg)		
Laonice weddellia (Spionidae)	SDF	1 (9mg)		3 (1mg)	2 (10mg)		3 (8mg)	2 (10mg)	2 (3mg)
Laonice cf. *vieitezi* (MB) (Spionidae)	SDF	3 (11mg)							
Laonice cf. a*ntarctica* (MB) (Spionidae)	SDF							3 (4mg)	

Table 5-2 The categorical feeding guild of each genetically identified species to be analysed, * indicates cryptic species, with the number of individuals from each location that were combined for derivatisation and the total dry weight used (mg). For trophic traits SDF = surface deposit feeder, SSDF = subsurface deposit feeder and O/P/S = omnivore, predator, scavenger. Location and depth (500 and 1000 m) are also noted where SG = South Georgia and ST = Southern Thule.

Species	Feeding guild	Amundsen Sea Inner	Scotia Arc EI	LI	SG	ST
Aglaophamus cf. *trissophyllus* (MB1)	O/P/S		1000 m (11mg)	200 m (x2) (12mg)	300 m (x2) (10mg)	200 m (12mg)
Aglaophamus sp. (MB2)	O/P/S	500 m (10mg) 1000 m (10mg)				
Aglaophamus sp. (MB3)	O/P/S				500m (10mg)	

Table 5-3 The geographic and depth distribution of genetically identified cryptic species of the omnivore/predator (O/P) *Aglaophamus trissophyllus,* where multiple individuals available as indicated (x#) and dried tissue mass derivatised (mg). Where EI = Elephant Island, LI = Livingston Island, SG = South Georgia and ST = Southern Thule.

5.2.3 $\delta^{15}N_{AA}$ data correction and analysis

All $\delta^{15}N_{AA}$ duplicate values were corrected using a two-step calibration procedure:

1. $\delta^{15}N_{AA}$ measurements of each amino acid in the sample were adjusted to the known $\delta^{15}N_{AA}$ value of the internal standard L-Norleucine following Yarnes and Herszage (2017). This allows for any isotopic fractionation of the target analytes during hydrolysis, derivatisation and combustion in the reactor.

Nle correction = $\delta^{15}N_{AA}$ + (L-Nle Known – L-Nle Sample) (Equation 5-3)

Where L-Nle = L-Norleucine, with a known $\delta^{15}N$ of 14.8‰.

2. The adjusted values were then scale corrected. $\delta^{15}N_{AA}$ measurements of each amino acid in the standard mixture were normalized to the international reference scale using a linear regression of the known $\delta^{15}N_{AA}$ values and the measured $\delta^{15}N_{AAi}$ values following Chikaraishi et al. (2007):

$\delta^{15}N_{AA}$ Scale correction = $m\delta^{15}N_{AAi}+b$, (Equation 5-4)

Where $\delta^{15}N_{AAi}$ = measured $\delta^{15}N$ in the individual amino acids in the standard mixture, m =slope and b=intercept.

A mean value of each $\delta^{15}N_{AA}$ from the duplicate analyses was calculated. If the standard deviation of the duplicate measurements was greater than 1.0‰, samples were rerun. Averages for each morphotype by site and depth were calculated with standard deviation. Given the absence of replicate samples at the same location, statistical analyses of the amino acid and trophic level data were not possible. However, patterns in the data are described with regard to feeding guild, depth and location.

5.2.4 Trophic level

There has been some debate as to which source and trophic amino acids should be used to estimate trophic level. For this reason, three methods were used to determine the trophic level of the polychaetes. Phenylalanine was the targeted source amino acid in this project and was used to calculate trophic level (Equation 5-5, Chikaraishi et al. (2009)). However, as a result of an ephemeral peak co-eluting with phenylalanine, the reliability of the data was questionable (see section 5.4). So other trophic level calculations were used for comparison, using glycine (Equation 5-6), as well a source average (SAA = $\delta^{15}N$ phenylalanine and glycine) and trophic average (TAA = $\delta^{15}N$ alanine, aspartic and glutamic acid) amino acids (Equation 5-7) both from Popp et al. (2007) and used in Hannides et al. (2009).

$TL_{Glu/Phe}$: (δ^{15}N Glu - δ^{15}N Phe – 3.4)/7.6 +1 (Equation 5-5)

$TL_{Glu/Gly}$: (δ^{15}N Glu - δ^{15}N Gly/7 +1 (Equation 5-6)

$TL_{TAA/SAA}$: (δ^{15}N T_{AA} - δ^{15}N S_{AA})/7 +1 (Equation 5-7)

As individuals were combined for CSIA, there were no replicate samples of the same species at any location. Thus, the isotopic differences between sites and depths within species were not examined statistically. Linear regression was used to test the fit of each trophic level estimate relative to both the source and trophic amino acids used. Any relationship between trophic level and 'source' amino acid could indicate fractionation of the conserved 'source' amino acids. The δ^{15}N of the trophic and source amino acids, as well as estimated trophic level of each species is discussed with regard to its feeding guild. The reliability of δ^{15}N source amino acid values from benthic polychaetes is also discussed.

5.3 Results

All trophic and source amino acid values as well as calculated trophic level for each species by depth and location are shown in Appendix 3. Note that duplicate values were not obtained for all species. Despite re-running samples, in some cases only a single analysis was deemed reliable. Threonine data are not included in this results section but are listed in Appendix 2. With the exception of *Laonice vietezi* all threonine values were negative ranging from -17.86 to -5.10‰. Originally classed as a source amino acid (McClelland and Montoya, 2002) however due to the unique isotopic fractionation of threonine leading to depletion in ^{15}N during metabolism, it is now referred to as a 'metabolic' amino acid (McMahon and McCarthy, 2016, Wallace and Hedges, 2016). This fractionation may result from an inverse isotope effect, whereby enzymes select for the heavier isotope leaving the residual threonine depleted in ^{15}N (Hare et al., 1991). For this reason it has been excluded from this section.

5.3.1 Trophic Amino Acids

Of five trophic amino acids measured, alanine and valine had the most enriched δ^{15}N values for each species. Glutamic acid, which is often the trophic amino acid of choice in trophic level estimates, usually had lighter δ^{15}N values than those of alanine and valine. Valine had the most variable with standard deviations up to ±2.8‰. Aspartic acid had the lowest δ^{15}N values of trophic amino acids and, was the least variable across species.

5.3.1.1 Glutamic Acid

Glutamic acid was the target trophic amino acid in this study. $\delta^{15}N$ values ranged from 12.2 to 24.5‰ for *Flabelligena* sp. A (MB) and *Glycera* sp. (MB2), respectively (Figure 5-1). There were differences between feeding guilds, where O/P/S were more enriched in ^{15}N glutamic acid than SDF ranging from 17.8 to 24.5‰ and 12.2 to 17.2‰, respectively. With the exception of *Aricidea simplex* (15.9‰), SSDF $\delta^{15}N$ glutamic acid values ranged from 19.4 to 21.9‰ and were more enriched in ^{15}N than SDF, and more comparable to O/P/S. There was no obvious trend between sites or with depth. However, relatively consistent values were recorded from species at 500 m in the Scotia Arc, ranging from 17.7 to 19.4‰, irrespective of feeding guild.

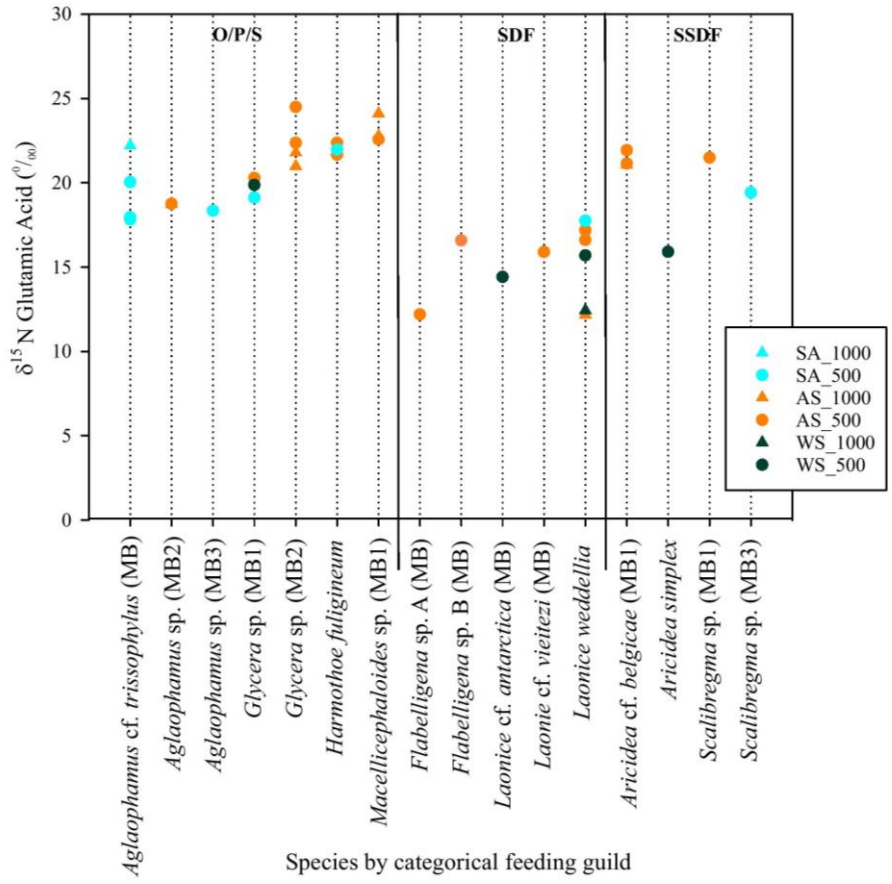

Figure 5-1 $\delta^{15}N$ values of glutamic acid (means of duplicate analyses) for each species by site and depth. Where SA = Scotia Arc, AS = Amundsen Sea and WS = Weddell Sea, 1000 = 1000 m and 500 = 500 m depth. Species listed by feeding guild where O/P/S = Omnivore, predator, scavenger, SDF = surface deposit feeder and SSDF = subsurface deposit feeder.

5.3.1.2 Alanine

The $\delta^{15}N$ values of alanine reflected those of glutamic acid, but were more variable within species (Figure 5-2). *Aglaphamus trissophyllus* displayed the largest range in $\delta^{15}N$ alanine, from 16.3 to 24.8‰ within the Scotia Arc at depths of 500 and 1000 m, respectively. This covered most of the overall $\delta^{15}N$ alanine range from 13.8 to 27.1‰ measured from *Flabelligena* sp. A (MB) and *Macellicephaloides* sp. (MB1), respectively. Within O/P/S species, the polynoid *Harmothoe fuligineum* had consistently higher $\delta^{15}N$ alanine values than other species. Again the variation between sites and depths were not consistent across species or feeding guilds. However, *Aricidea simplex* had more depleted $\delta^{15}N$ alanine values than other SSDFs and it signature was more comparable to SDF from the same location.

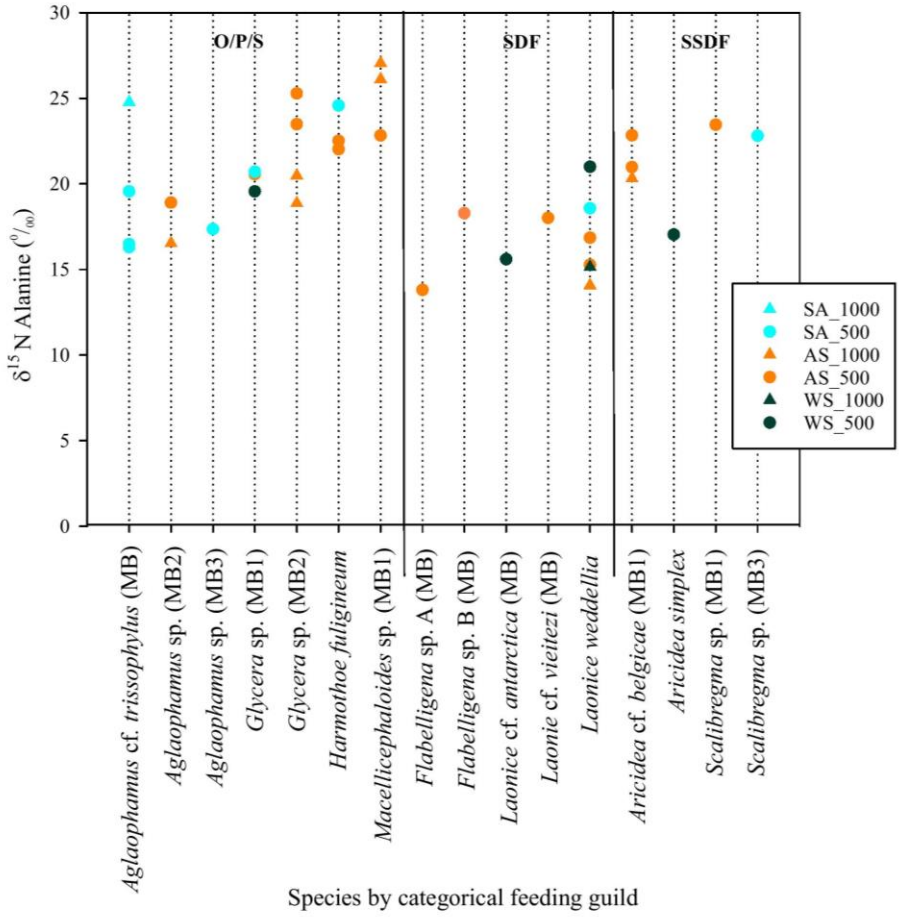

Figure 5-2 $\delta^{15}N$ values of alanine (means of duplicate analyses) for each species by site and depth. Where SA = Scotia Arc, AS = Amundsen Sea and WS = Weddell Sea, 1000 = 1000 m and 500 = 500 m depth. Species listed by feeding guild where O/P/S = Omnivore, predator, scavenger, SDF = surface deposit feeder and SSDF = subsurface deposit feeder.

5.3.1.3 Valine

$\delta^{15}N$ values of valine were generally the most enriched. In comparison $\delta^{15}N$ alanine, they were less variable within species. The most enriched $\delta^{15}N$ valine values were recorded in O/P/S species being comparable between individuals of *Aglaophamus* cf. *trissophyllus* (MB) at 26.7‰, *Glycera* sp. (MB2) at 27.0‰ and *Macellicephaloides* sp. (MB1) at 27.1‰, (Figure 5-3). $\delta^{15}N$ valine values for SDF and SSDF were more variable but in comparison to $\delta^{15}N$ of glutamic acid the difference between the two guilds was much smaller. Overall $\delta^{15}N$ valine values for SDF ranged from 12.9 to 22.5‰, for *Flabelligena* sp. A (MB) and *Laonice* cf. *vieitezi* (MB), respectively and in SSDF from 17.8 to 23.2‰ for *Aricidea simplex* and *Scalibregma* sp. (MB1), respectively (Figure 5-3). As recorded in the $\delta^{15}N$ glutamic acid, the values for the Scotia Arc at 500 m were least variable across feeding guilds.

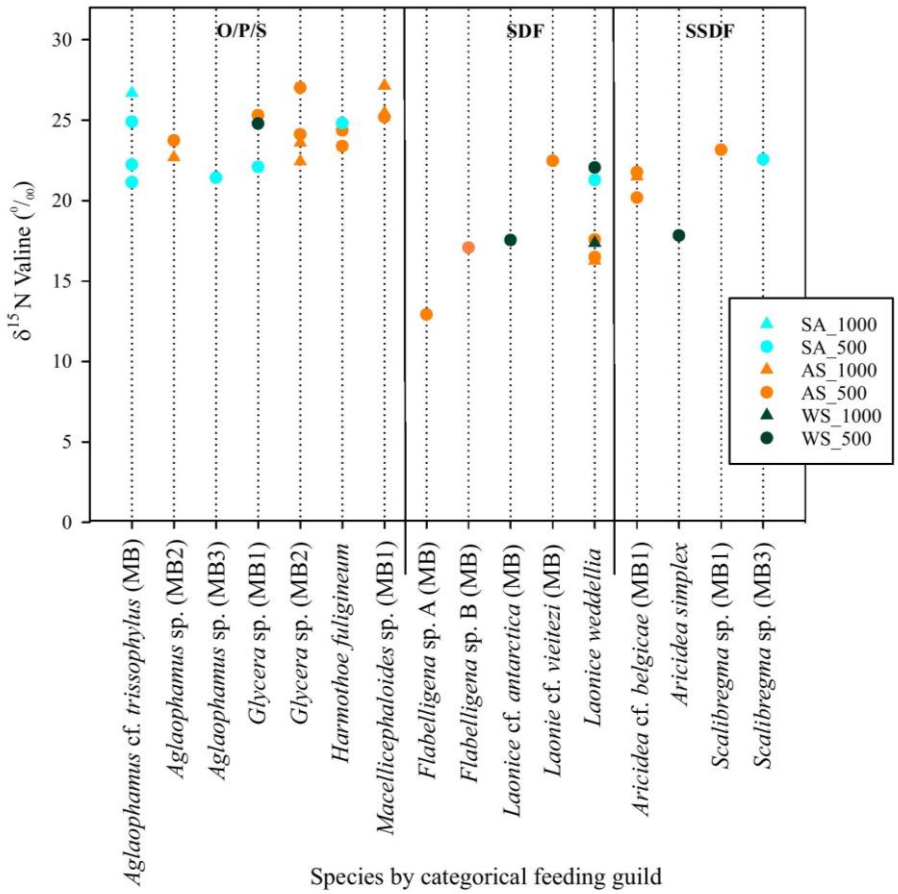

Figure 5-3 $\delta^{15}N$ values of valine (means of duplicate analyses) for each species by site and depth. Where SA = Scotia Arc, AS = Amundsen Sea and WS = Weddell Sea, 1000 = 1000 m and 500 = 500 m depth. Species listed by feeding guild where O/P/S = Omnivore, predator, scavenger, SDF = surface deposit feeder and SSDF = subsurface deposit feeder.

5.3.1.4 Leucine

$\delta^{15}N$ values of leucine reflected those of glutamic acid, but were generally heavier (Figure 5-4). For O/P/S/, $\delta^{15}N$ leucine ranged from 18.8 to 25.4‰, for *Aglaophamus* cf. *trissophyllus* (MB) and *Macellicephaloides* sp. (MB1), respectively. In the SDFs, $\delta^{15}N$ leucine ranged from 12.9 to 19.4‰, *Flabelligena* sp. A (MB) and *Laonice weddellia*, respectively. SSDFs signatures were again more enriched with $\delta^{15}N$ leucine ranging from 16.8 to 22.0‰ for *Aricidea simplex* and *Scalibregma* sp. (MB1), respectively.

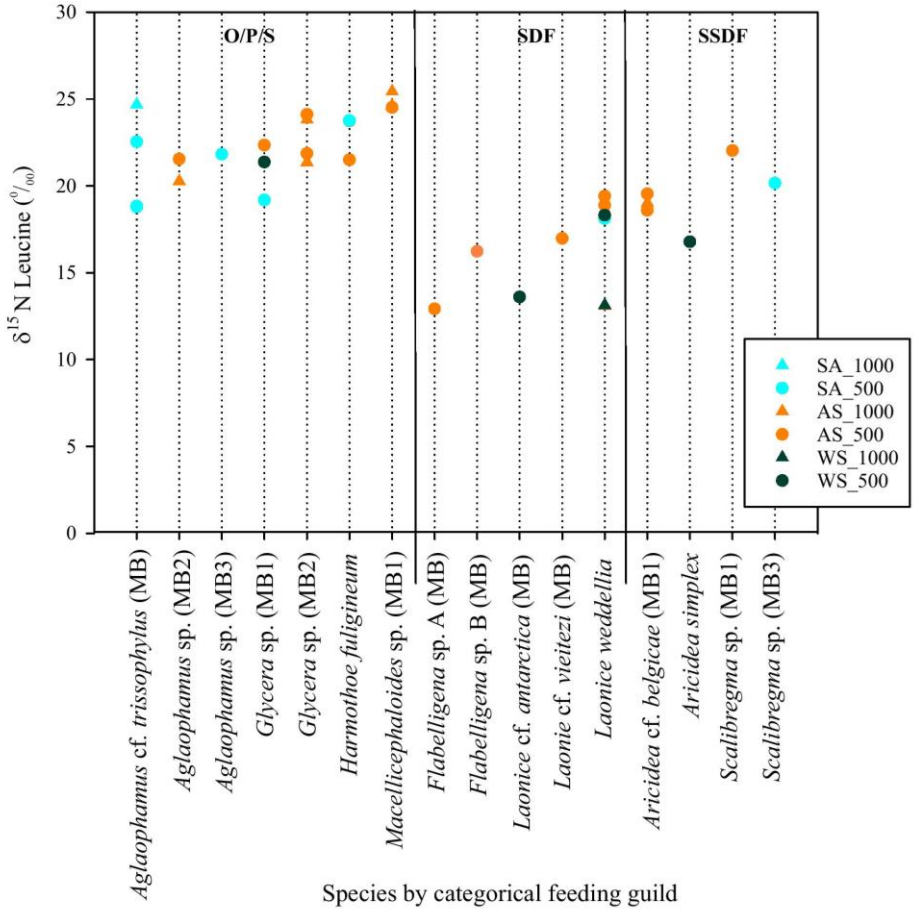

Figure 5-4 $\delta^{15}N$ values of luecine (means of duplicate analyses) for each species by site and depth. Where SA = Scotia Arc, AS = Amundsen Sea and WS = Weddell Sea, 1000 = 1000 m and 500 = 500 m depth. Species listed by feeding guild where O/P/S = Omnivore, predator, scavenger, SDF = surface deposit feeder and SSDF = subsurface deposit feeder.

5.3.1.5 Aspartic Acid

Aspartic acid was the least enriched in ^{15}N of all trophic amino acids. $\delta^{15}N$ aspartic acid values reflected a similar trend as the other trophic amino acids across polychaete feeding guilds (Figure 5-5). In O/P/S $\delta^{15}N$ aspartic acid values ranged from 16.1 to 20.6‰ for *Aglaophamus* cf. *trissophyllus* (MB) and *Glycera* sp. (MB2), respectively. For SDFs, $\delta^{15}N$ aspartic acid values ranged from 10.4 to 16.9‰ for *Flabelligena* sp. A (MB) and *Laonice weddellia*, respectively. Within SSDF, $\delta^{15}N$ aspartic acid values ranged from 14.2 to 19.6‰ for *Aricidea simplex* and *Scalibregma* sp. (MB1), respectively.

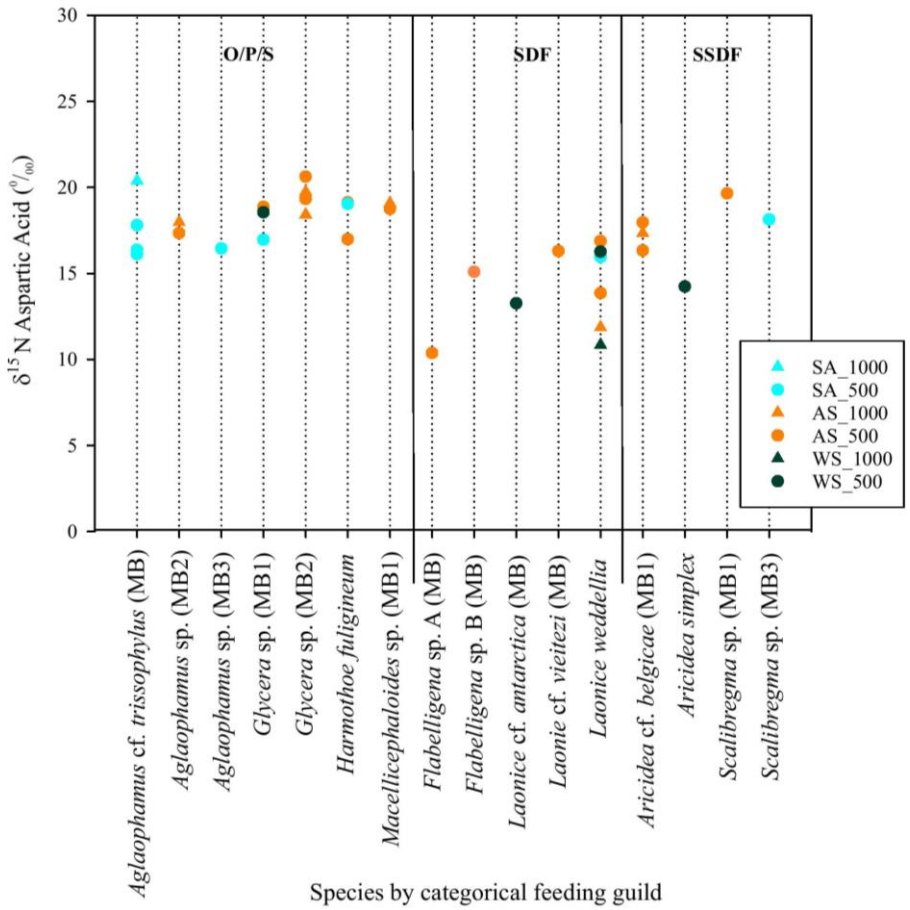

Figure 5-5 $\delta^{15}N$ values of aspartic acid (means of duplicate analyses) for each species by site and depth. Where SA = Scotia Arc, AS = Amundsen Sea and WS = Weddell Sea, 1000 = 1000 m and 500 = 500 m depth. Species listed by feeding guild where O/P/S = Omnivore, predator, scavenger, SDF = surface deposit feeder and SSDF = subsurface deposit feeder.

5.3.2 Source Amino Acids

As expected the source amino acids values were lower than those of the trophic amino acids. Fractionation in source amino acids across trophic levels should be minimal (McMahon and McCarthy, 2016). Thus there should be similar $\delta^{15}N$ source amino acid signatures across

feeding guilds. Phenylalanine often considered to be the most conserved source amino acid had the lowest range of $\delta^{15}N$ values ranged at 6.7‰, followed by glycine at 15.9‰ and serine at 17.6‰ across all species.

5.3.2.1 Phenylalanine

The $\delta^{15}N$ values for phenylalanine were variable compared to data collected for *Arenicola marina* (Chapter 4). However, the data presented here are from multiple sites and species thus greater variation might be expected. Overall values ranged from 3.8 to 10.5‰ for *Laonice* cf. *vieitezi* (MB) and *L. wedellia*, respectively (Figure 5-6). No consistent pattern was recorded in the $\delta^{15}N$ phenylalanine values between locations or depths (Figure 5-7).

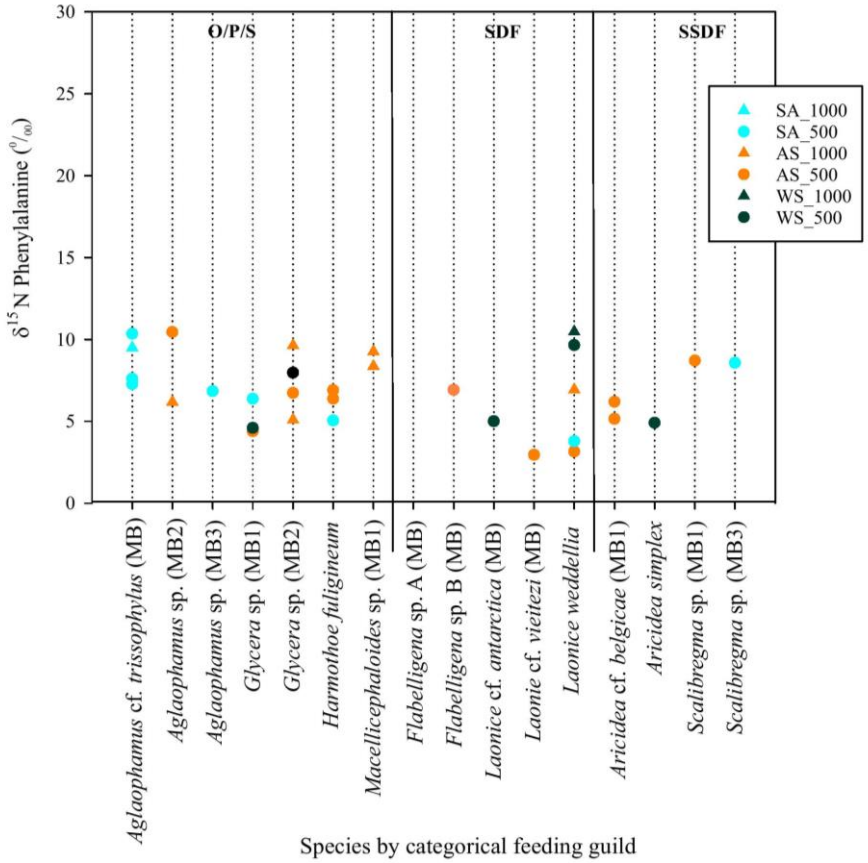

Figure 5-6 $\delta^{15}N$ values of phenylalanine (means of duplicate analyses) for each species by site and depth. Where SA = Scotia Arc, AS = Amundsen Sea and WS = Weddell Sea, 1000 = 1000 m and 500 = 500 m depth. Species listed by feeding guild where O/P/S = Omnivore, predator, scavenger, SDF = surface deposit feeder and SSDF = subsurface deposit feeder.

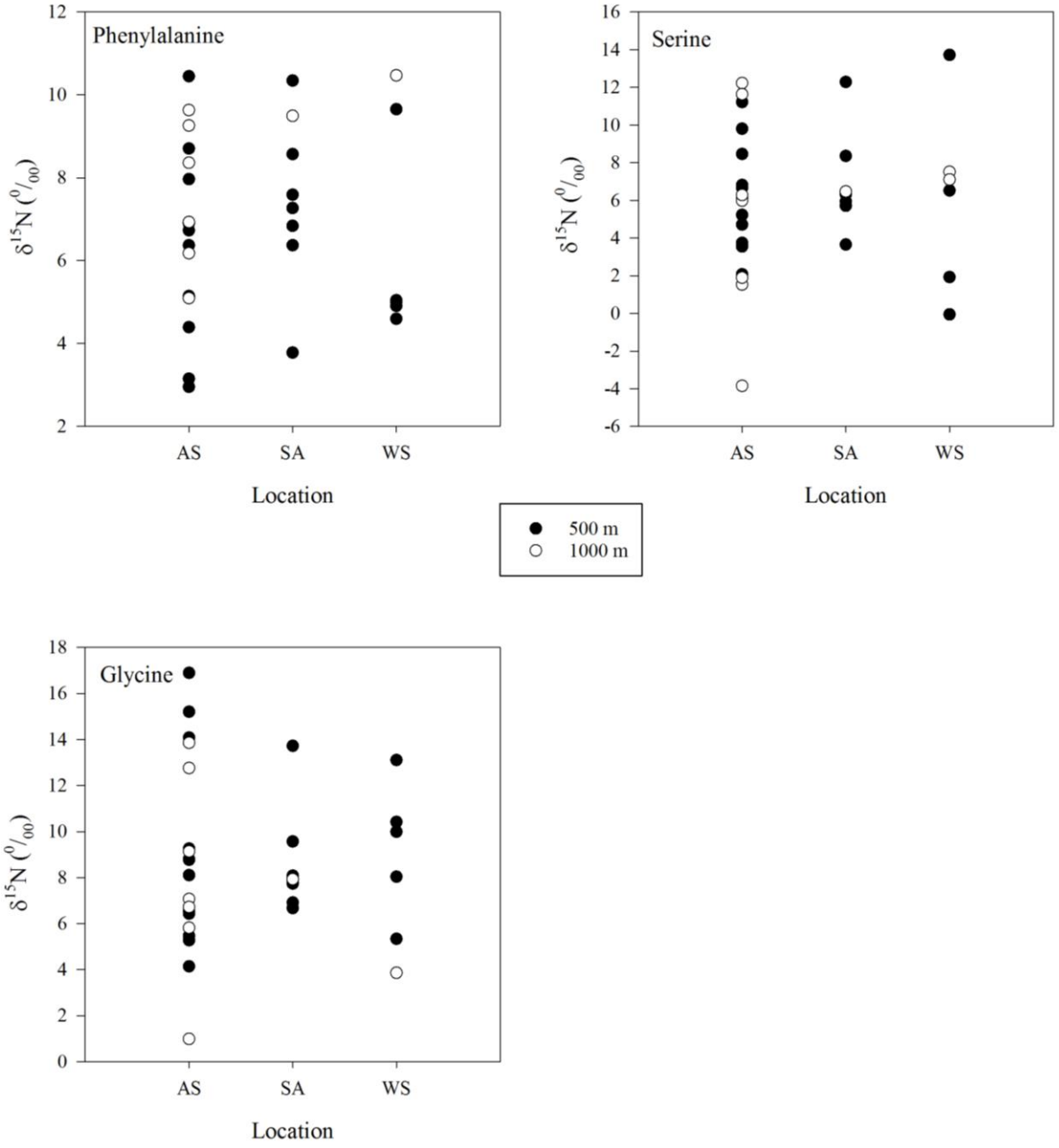

Figure 5-7 The $\delta^{15}N$ values for phenylalanine, glycine and serine by location and depth, where AS = Amundsen Sea, SA = Scotia Arc and WS = Weddell Sea.

5.3.2.2 Glycine

δ^{15}N glycine values for both *Glycera* species were noticeably higher than all other species, with a maximum value of 16.9‰ for the Amundsen Sea at 1000 m (Figure 5-8). To put this in context, these values are heavier than some of the δ^{15}N glutamic acid signatures of SDF species. In *Laonice weddellia* δ^{15}N glycine values ranged from 1.0 to 8.1‰ in the Amundsen Sea (1000 m) and in the Scotia Arc (500 m), respectively. Excluding these species, the overall range was 5.3 to 10.4‰, for *L.* cf. *vieitezi* and *Harmothoe fuligineum*, respectively. δ^{15}N glycine values were more enriched at 500 m within the Scotia Arc and Weddell Sea compared to the Amundsen Sea values. However, no obvious trends were recorded in the δ^{15}N glycine values between locations or depths (Figure 5-7).

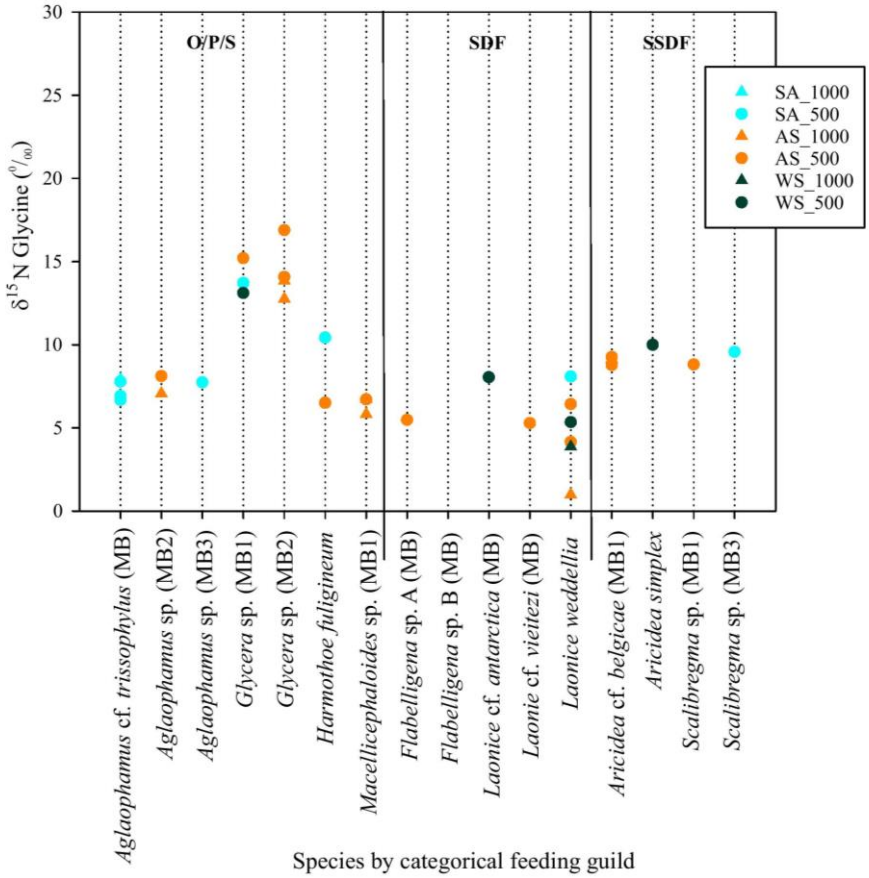

Figure 5-8 δ^{15}N values of glycine (means of duplicate analyses) for each species by site and depth. Where SA = Scotia Arc, AS = Amundsen Sea and WS = Weddell Sea, 1000 = 1000 m and 500 = 500 m depth. Species listed by feeding guild where O/P/S = Omnivore, predator, scavenger, SDF = surface deposit feeder and SSDF = subsurface deposit feeder.

5.3.2.3 Serine

δ^{15}N serine values mirrored those of δ^{15}N glycine. The most enriched δ^{15}N serine values were recorded in *Glycera* spp. at 13.7‰. The largest range of δ^{15}N serine was recorded in *L. weddellia* (-3.9 to 7.1‰). Excluding these species, δ^{15}N serine values ranged from 1.5 to 8.5‰ for *Macellicephaloides* sp. (MB1) and *Scalibregma* sp. (MB1), respectively. Again there was no trend in ^{15}N serine values across different depths and locations (Figure 5-7).

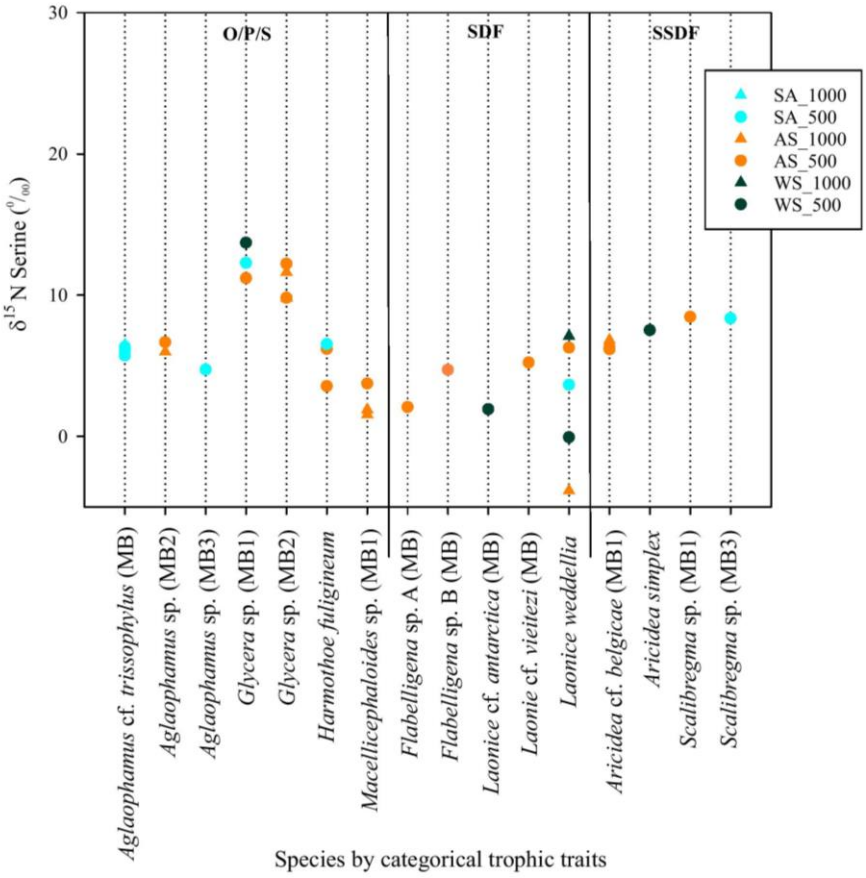

Figure 5-9 δ^{15}N values of serine (means of duplicate analyses) for each species by site and depth. Where SA = Scotia Arc, AS = Amundsen Sea and WS = Weddell Sea, 1000 = 1000 m and 500 = 500 m depth. Species listed by feeding guild where O/P/S = Omnivore, predator, scavenger, SDF = surface deposit feeder and SSDF = subsurface deposit feeder.

5.3.3 Trophic Level

The estimated trophic levels of the polychaetes studied here ranged from 1.2 to 3.1 for $TL_{Glu/Phe}$, to 1.7 to 3.6 for $TL_{Glu/Gly}$, and 1.8 to 3.3 for $TL_{TAA/SAA}$ (Figure 5-10 and Appendix 2). As there was no obvious trend in trophic amino acids with location or depth, the trophic level data have been analysed to determine variability in estimated trophic level within species and to ascertain the reliability of the trophic level calculations based on the amino acid data.

The variation within and between species mirrors that of the source and trophic amino acids used. For this reason differences in estimated trophic level between species did not follow the same pattern. For example, *Aglaophamus trissophylus* (MB1), *Aglaophamus* sp. (MB2) and *Laonice weddellia* had low $\delta^{15}N$ phenylalanine signatures; this is reflected in the $TL_{Glu/Phe}$ estimates in comparison to $TL_{Glu/Gly}$ and $TL_{TAA/SAA}$ (Figure 5-10 and Table 5-4). Additionally, *Glycera* sp. (MB1) had noticeably higher $\delta^{15}N$ glycine values than other species and its $TL_{Glu/Gly}$ is on average 0.7 – 0.8 lower when compared to the other trophic level estimates.

	Feeding guild	**$TL_{Glu/Phe}$**	**$TL_{Glu/Gly}$**	**$TL_{TAA/SAA}$**
Aglaophamus trissophylus (MB1)	O/P/S	2.1 ± 0.7	2.6 ± 0.2	2.5 ± 0.4
Aglaophamus sp. (MB2)	O/P/S	1.4 ± 0.2	2.6 ± 0.1	2.4 ± 0.1
Glycera sp. (MB1)	O/P/S	2.5 ± 0.1	1.8 ± 0.1	2.4 ± 0.1
Glycera sp. (MB2)	O/P/S	2.5 ± 0.5	2.1 ± 0.1	2.5 ± 0.2
Harmothoe fuligineum	O/P/S	2.8 ± 0.3	3.0 ± 0.3	3.0 ± 0.1
Macellicephaloides sp. (MB1)	O/P/S	2.9 ± 0.0	3.4 ± 0.2	3.2 ± 0.1
Flabelligena sp. (MB)	SDF	2.4	2.0	1.9
Flabelligena sp. (MB)	SDF	2.0		2.4
Laonice cf. *antarctica* (MB)	SDF	1.9	1.9	2.1
Laonice cf. *vietezi* (MB)	SDF	2.5	2.5	2.8
Laonice weddellia	SDF	1.9 ± 0.5	2.5 ± 0.2	2.4 ± 0.3
Aricidea cf. *belgicae* (MB1)	SDF	2.7 ± 0.3	2.8 ± 0.1	2.7 ± 0.3
Aricidea simplex	SSDF	2.1	1.8	2.2
Scalibregma sp. (MB1)	SSDF	2.5	2.8	2.8
Scalibregma sp. (MB3)	SSDF	2.4	2.4	2.6

Table 5-4 The average (and standard deviation) trophic level for each species using equations 5-5 ($TL_{Glu/Phe}$), 5-6 ($TL_{Glu/Gly}$) and 5-7 ($TL_{TAA/SAA}$). Where no standard deviation is given only a single specimen was processed.

As there was no trend in source or trophic amino acid $\delta^{15}N$ signatures with location and depth, all the trophic level data were combined and linear regression analyses used to investigate which trophic level equation provides the most reliable estimates. If the source amino acid values truly represent the base of the food web and are conserved then, the trophic level estimates should be related to the trophic amino acids (Figure 5-11 and Table 5-5).

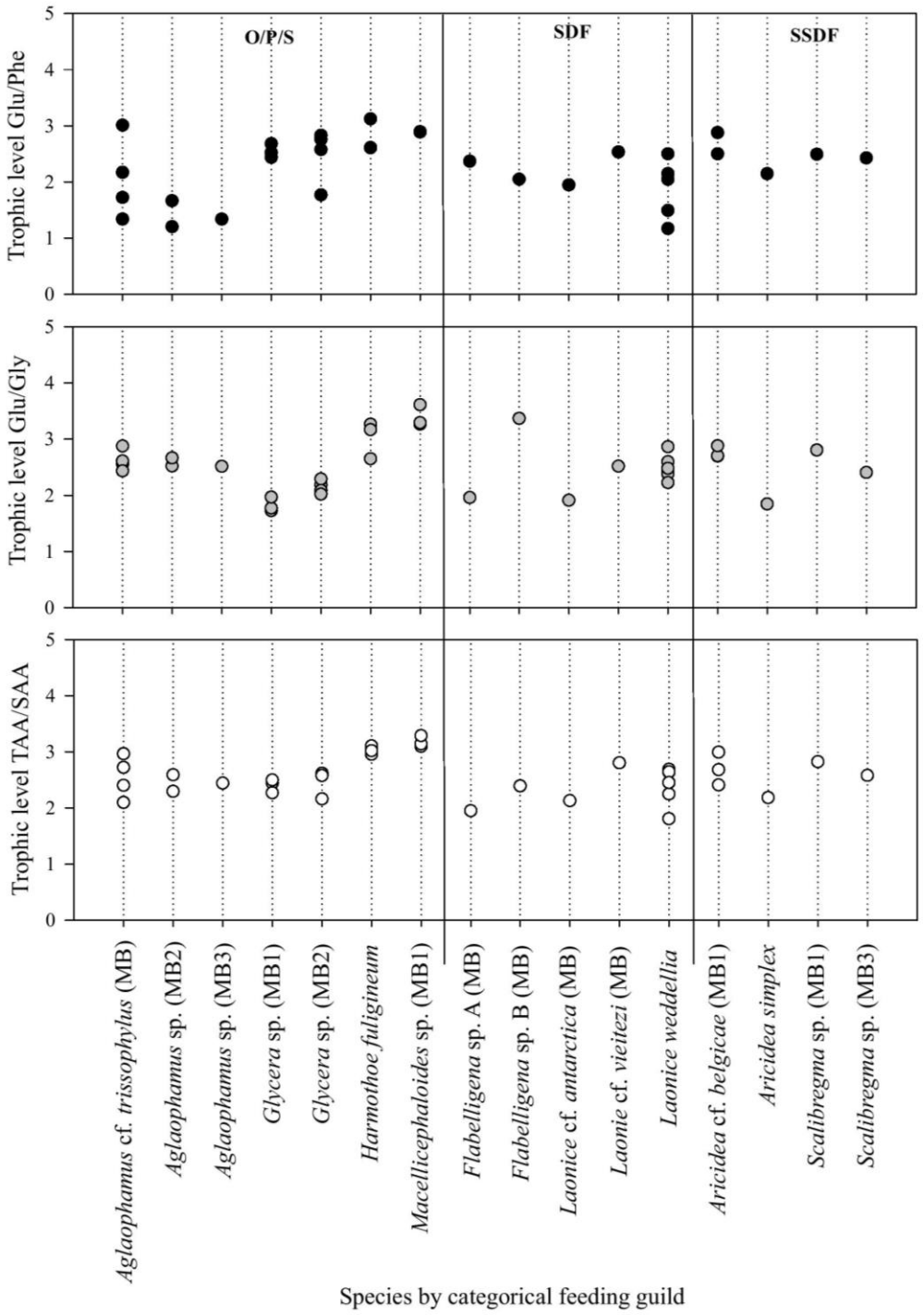

Figure 5-10 Calculated trophic level for each species by site and depth. Where SA = Scotia Arc, AS = Amundsen Sea and WS = Weddell Sea, 1000 = 1000 m and 500 = 500 m depth. Species listed by feeding guild where O/P/S = Omnivore, predator, scavenger, SDF = surface deposit feeder and SSDF = subsurface deposit feeder. Using three trophic level equations including Glu/Phe (Equation 5-5), Glu/Gly (Equation 5-6) and TAA/SAA (Equation 5-7).

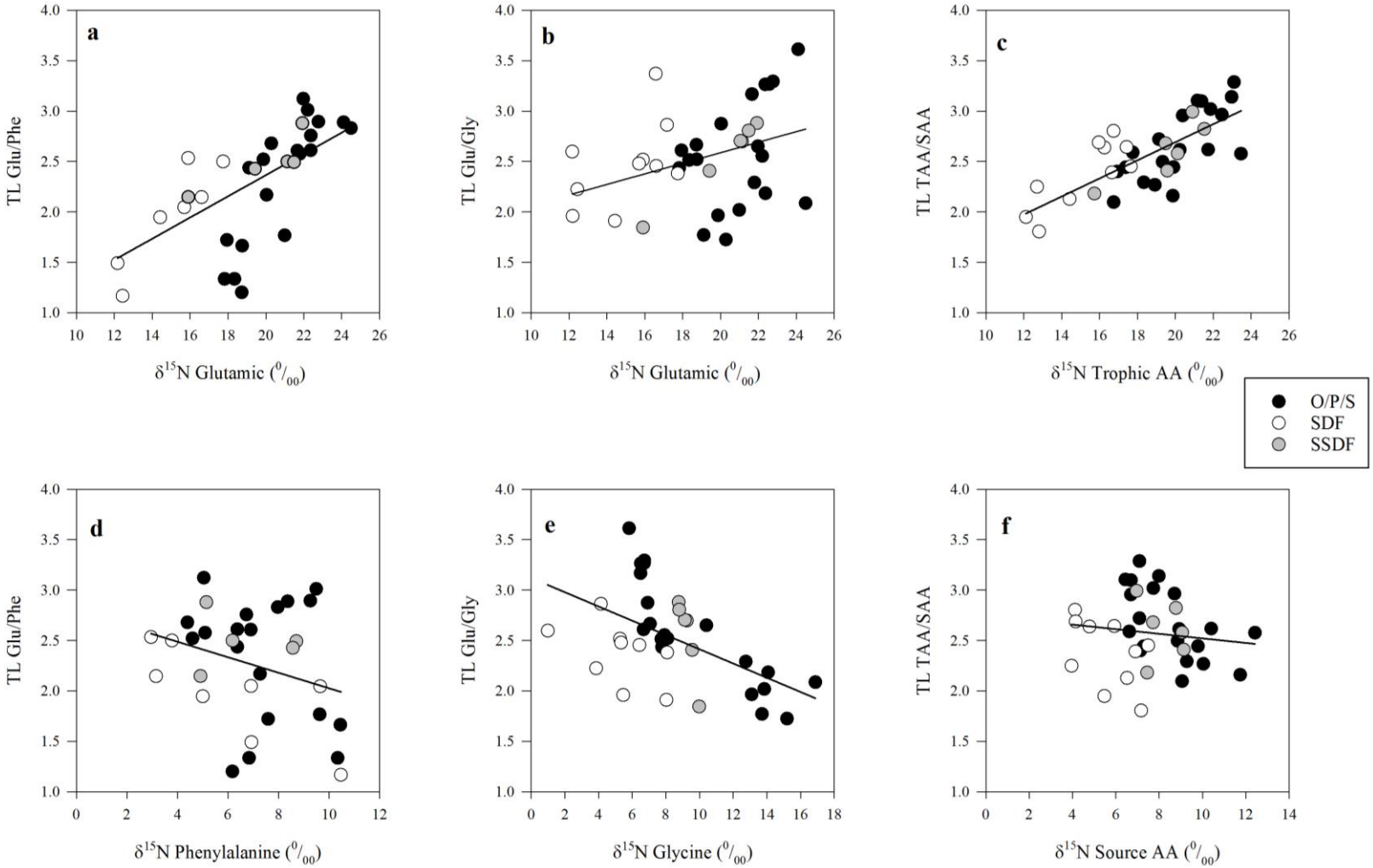

Figure 5-11 The relationship between trophic amino acids and corresponding TL equations (a-c) and source amino acids and corresponding equations (d-f), with regression lines.

Regression	R	Df	P
Glu *vs*. $TL_{Glu/Phe}$	**0.64**	**31**	**0.001**
Glu *vs*. $TL_{Glu/Gly}$	**0.36**	**34**	**0.031**
Trophic AA *vs*. TAA/SAA	**0.75**	**34**	**0.001**
Phe *vs*. $TL_{Glu/Phe}$	0.30	30	0.098
Gly *vs*. $TL_{Glu/Gly}$	**0.53**	**33**	**0.001**
Source AA *vs*. $TL_{TAA/SAA}$	0.12	34	0.475

Table 5-5 Linear regression results for the comparison of trophic amino and source acids with their corresponding trophic level equation estimates. Where Glu = glutamic acid, Phe = phenylalanine, $TL_{Glu/Phe}$ = trophic level estimates using equation 5-3, Gly = glycine, $TL_{Glu/Gly}$ = trophic level estimates using equation 5-4, Trophic AA = averaged trophic amino acids, Source AA = averaged source amino acids, $TL_{TAA/SAA}$ = trophic level estimates using equation 5-5.

The estimated trophic levels from each equation were significantly related to the trophic amino acid $\delta^{15}N$ values (Table 5-5). The strongest relationship was observed between the averaged yrophic amino acids and $TL_{TAA/SAA}$. It was also the corresponding source amino acid and $TL_{TAA/SAA}$ that had the weakest relationship. Additionally, the $TL_{Glu/Gly}$ estimates had the weakest relationship with the trophic amino acid (Glutamic acid) and was the only trophic level estimate to have a significant relationship with its source amino acid (Glycine).

5.4 Discussion

5.4.1 Trophic traits of benthic Antarctic polychaetes

The $\delta^{15}N_{AA}$ data presented show variation both within and between species, cryptic species, feeding guilds, and, with depth and location. The observed variation in each feeding guild for any amino acid, ranged by up to 10.8‰ in the case of $\delta^{15}N$ alanine. The greatest $\delta^{15}N_{AA}$ ranges were generally observed in *Aglaophamus cf. trissophyllus* (MB) and *Laonice weddellia*; these species also contained some of the largest numbers of individuals. The lack of variation in other species could therefore be a result of limited sample numbers.

There was a notable difference in the isotopic composition of trophic amino acids between feeding guilds (Table 5-6). This was most evident between the O/P/S and SDF feeders, which would have been expected given that the diet of O/P/S (secondary consumers) is more enriched in $\delta^{15}N$ than the SDF that were likely feeding on organic material at the seafloor. In general the $\delta^{15}N$ signatures of the SSDF were in the same range as both the O/P/S and SDF, so without knowledge of the organism's life style or habitat, it would be difficult to determine trophic traits based on $\delta^{15}N_{AA}$ signatures alone. The enriched signatures of SSDF to SDF could be associated with bacterial decomposition of organic matter or animal tissue within the sediment (Calleja et al., 2013, Carstens et al., 2013, Bui and Lee, 2015), which could be consumed by the SSDF species. Additionally, subsurface polychaetes secrete mucus which lines their

burrows (Steward et al., 1996, Papaspyrou et al., 2006); this alters the bacterial and microbial communities surrounding the burrow and, if re-ingested, could have different $\delta^{15}N_{AA}$ signatures to the overlying phytodetritus.

	O/P/S	SDF	SSDF
Glutamic Acid	20.9 ± 2.0	15.1 ± 2.1	20.2 ± 2.2
Alanine	21.2 ± 3.3	16.7 ± 2.3	21.2 ± 2.4
Valine	24.1 ± 1.8	18.1 ± 3.0	21.2 ± 1.9
Leucine	22.3 ± 2.0	16.1 ± 2.6	19.4 ± 1.7
Aspartic Acid	18.3 ± 1.3	14.1 ± 2.4	17.3 ± 1.8
Phenylalanine	7.3 ± 1.9	6.1 ± 2.9	6.7 ± 1.8
Glycine	9.7 ± 3.6	5.3 ± 2.2	9.3 ± 0.5

Table 5-6 The average trophic and source amino acid values (‰) by categorical feeding guilds where O/P/S = omnivore, predator, scavenger, SDF = surface deposit feeder and SSDF = subsurface deposit feeder.

The $\delta^{15}N$ trophic amino acid values for *Aricidea simplex* were noticeably lower than the other species considered to be SSDF. *Aricidea simplex* was the only SSDF collected in the Weddell Sea and its $\delta^{15}N$ trophic amino acid values were closer to those of SDF from the same location. It is possible that either this species feeds on organic material on the surface of the sediment, or that the factors influencing the $\delta^{15}N$ vary between locations. The lack of replicates across sites and depths makes it difficult to determine whether the difference in $\delta^{15}N$ trophic amino acid values are site specific. The influence of spatial variation is discussed further with regard to source amino acids, however the same processes could influence the $\delta^{15}N$ signatures of trophic amino acids.

The overlapping trophic amino acids values across the proposed feeding guilds could also be associated with the increased omnivory amongst both deep-sea and polar species. Given the seasonal supply of organic matter and food-limited environment, it may be ecologically advantageous to be a less selective and more flexible feeder (Sweetman and Witte, 2008, Mincks et al., 2008, McMeans et al., 2015). The term omnivore is often used when there is uncertainty regarding the trophic traits; however the trophic variability within species and within feeding guilds shown here demonstrates that omnivory is a valid categorisation.

5.4.2 Determining source amino acids

Source amino acids are conserved in food webs (McMahon and McCarthy, 2016), which enables them to be used as indicators of the base of the food web without the need to sample

primary food sources (McClelland and Montoya, 2002, Chikaraishi et al., 2007). Phenylalanine has been the primary choice of source amino acid and has been utilised frequently in food web studies to calculate trophic levels (see references within Table 5-7). Glycine has been used as the primary source amino acid in a limited number of studies (e.g. Popp et al., 2007) (Table 5-7). More recently the use of averaged source and trophic amino acid isotopic composition has been proposed and has gained traction (e.g. Popp et al., 2007, Hannides et al., 2009, Chikaraishi et al., 2015, Choy et al., 2015). Additional source amino acids averaged for trophic level estimates include: glycine, lysine, tyrosine and serine. Lysine and tyrosine were excluded from my work as the analytical method chosen (Chapter 4) does not allow their detection. Since phenylalanine was successfully used to determine the trophic level of *Arenicola marina* (Chapter 4), it was chosen as the target source amino acid. The variability observed in the Antarctic data was greater than expected and so glycine and serine were also analysed for comparison.

Without source material for comparison, the interpretation of variability within the data is challenging. As source amino acids reflect the nitrogen signature base of primary producers, these values can vary both spatially and temporally as a result of local and regional differences in biogeochemical cycling that affects the $\delta^{15}N$ values of nitrogenous nutrients (Vizzini and Mazzola, 2003, Cherel and Hobson, 2007, Ménard et al., 2007, Stowasser et al., 2012). Spatial variation within the source amino acids has been noted in CSIA trophic investigations of different species and habitats (e.g. Popp et al., 2007, Choy et al., 2014, Ruiz-Cooley et al., 2014, Lorrain et al., 2015). In the Southern Ocean, phenylalanine values have been shown to vary with location in amphipods and salps (Kruse et al., 2015) and penguins (Lorrain et al., 2009). Kruse et al. (2015) suggested that variation in source amino acid signatures with location may be associated with differences in nitrate concentration. Such differences could propagate through the food web as observed by Lorrain et al. (2009), where the source amino acid values in carnivorous penguin species showed regional variation. The limited data set accrued in this study did not allow the relationship between location and depth to be investigated.

Study	Organism	Location	Source AA					
			Glycine	Lysine	Phenylalanine	Serine	Threonine	Tyrosine
Bradley et al. (2014)	Bluefin Tuna	Hawaii, Pacific Ocean	✗	✗	✗	✗	✗	✗
Bradley et al. (2015)	Teleost Fish	Hawaii, Pacific Ocean	✗	✗	✓	✗	✗	
Chikaraishi et al. (2007)	Algae, gastropods	Laboratory	✗		✓	✗		
Chikaraishi et al. (2009)	Algae, zooplankton, fish	Laboratory	✗		✓	✗		
Chikaraishi et al. (2010)	Algae, zooplankton, gastropods, sharks	Aquaria and Japan, Pacific Ocean	✗		✓	✗		
Chikaraishi et al. (2015)	Algae. Mollusc, Crustacea, fish	Aquaria and Japan, Pacific Ocean	✓		✓	✓		
Choy et al. (2012)	Mesopelagic fish	Atlantic and Pacific Ocean			✓			
Choy et al. (2015)	Fish and micronekton	Pacific Ocean	✓	✓	✓			
Dale et al. (2011)	Stingray	California, Pacific Ocean			✓			
Germain et al. (2013)	Harbour seal	Aquaria	✗	✗	✓	✗	✗	
Gerringer et al. (2017)	Fish spp.	Kermadec Trench, Pacific Ocean		✓	✓			
Hannides et al. (2009)	Zooplankton spp.	Hawaii, Pacific Ocean	✓		✓			
Hoen et al. (2014)	Cartilaginous fish and diet species	Laboratory/aquaria	✗	✗	✓	✗	✗	
Kruse et al. (2015)	Amphipods and salps	Southern Ocean	✗		✓			
Lorrain et al. (2009)	Penguin spp.	Southern Ocean	✗		✓			
Lorrain et al. (2015)	Particulate organic matter, barnacles and Yellowfin Tuna	Indian and Pacific Ocean	✗		✓			
McClelland and Montoya (2002)	Algae and zooplankton	Laboratory/Atlantic Ocean	✗	✗	✓	✗	✗	✗
McMahon et al. (2015a)	Gentoo penguins	Southern Ocean	✗		✓	✗	✗	
McMahon et al. (2015b)	Teleost fish	Aquaria	✗	✗	✓	✗	✗	
Miller et al. (2013)	Eel Larvae	Aquaria and Japan, Pacific Ocean			✓			
Mompeán et al. (2016)	Zooplankton	Atlantic Ocean	✗	✗	✓	✗	✗	
Popp et al. (2007)	Yellowfin Tuna	Eastern Equatorial Pacific	✓		✓			
Ruiz-Cooley et al. (2014)	Sperm Whale	Pacific Ocean	✗	✗	✗	✗		✗
Vokhshoori and McCarthy (2014)	Mussels	California, Pacific Ocean	✗	✗	✓	✗	✗	✗

Table 5-7 Marine trophic studies using compound specific stable isotope analysis to collected $\delta^{15}N$ of amino acids. This included the organisms used, collection location and the source amino acids for which $\delta^{15}N$ was recorded (✗) and used to calculate trophic level/position (✓). Note that some studies regard threonine as a metabolic amino acid including Germain et al. (2013), Ruiz-Cooley et al. (2014), Ruiz-Cooley et al. (2014), McMahon et al. (2015a) and (2015b). In some studies trophic level was not calculated, however trophic enrichment factors were, Bradley et al. (2014) and Ruiz-Cooley et al. (2014). With the exception of Popp et al. (2007) most studies using source amino acids other than phenylalanine to calculate trophic level were using an average of the source amino acids measured; Hannides et al. (2009), Chikaraishi et al. (2015) and Choy et al. (2015).

Glycine and serine were chosen as source amino acids based on their reliable analysis on the GC/C/IRMS. They were originally classified as source amino acids as there was minimal enrichment between consumers and food sources in controlled feeding experiments (McClelland and Montoya, 2002). Further field research has confrimed that glycine is conserved reflecting the base of the food web in different marine organisms (McClelland et al., 2003, Schmidt et al., 2003). Glycine has been used to estimate trophic level in yellow fin tuna, where phenylalanine was not suitable because it co-eluted with glutamic acid rendering the measurement of $\delta^{15}N$ difficult (Popp et al., 2007). However meta-analysis by McMahon and McCarthy (2016) of a broad range of consumers found fractionation in both glycine and serine of up to 14.2 and 9.7‰ respectively. Glycine has also been excluded from studies due to co-elution (Gerringer et al., 2017), its variability in comparison to other source amino acids (Kruse et al., 2015) and potential fractionation between consumers and their food sources (McMahon and McCarthy, 2016).

In the present study, there is clear fractionation in glycine and serine in both *Glycera* species, which has been ascribed to ammonia and uric acid production (Hoskin et al., 2001, Matthews et al., 1981). Polychaetes however are known to be ammontelic, i.e. ammonia is their primary product of waste nitrogen (Rastogi, 2001). Ammonia production can vary between polychaete species (Nithart et al., 1999) and it is possible that differences in the metabolism of the *Glycera* species led to the isotopic enrichment of glycine and serine. Glycine may also be affected by microbial degradation, so if the consumer is feeding directly on bacteria directly or on another bacterial consumer, e.g. foraminifera, this could influence its $\delta^{15}N$ signature (McCarthy et al., 2007, Calleja et al., 2013). Additionally, glycine can be produced via the transformation of other amino acids in the polychaete tissues and digestive track; this has been observed in other benthic species (Woulds et al., 2012). For these reasons, the glycine-derived estimates of trophic level for *Glycera* should be treated with caution (Nielsen et al., 2015).

Phenylalanine, which is often considered the more reliable choice of trophic amino acid has also been found to fractionate with trophic level. Although the changes are 'small' compared to those of trophic amino acids, they can propagate through the food web (Ruiz-Cooley et al., 2014). The variability in phenylalanine values recorded here are perhaps greater than expected, which may be associated with site-specific differences in ^{15}N of food source, feeding selectivity, the quality of organic matter consumed, bacterial action and analytical error.

Furthermore, phenylalanine could be altered in the water column via microbial resynthesis of sinking particulate organic matter (McCarthy et al., 2007).

During data collection, 'shoulders' on the GC/C/IRMS phenylalanine peaks were also noted. The extent to which these may have influenced the data is yet to be determined. The shoulders may indicate co-elution with another amino acid, which could lead to analytical error in the isotopic measurement. The $\delta^{15}N$ values of phenylalanine measured in the polychaetes in the present study are high when compared to those obtained from other Southern Ocean species. For example in pelagic species, reported $\delta^{15}N$ phenylalanine ranged from -1.0 to 0.9‰ in salps; 0.6 to 2.3‰ in amphipods and from -0.2 to 5.1‰ in penguins (Lorrain et al., 2009, Kruse et al., 2015).

5.4.3 CSIA-based trophic levels

This is the first time that the trophic levels of Antarctic polychaete species have been determined. The $\delta^{15}N$ values of trophic amino acids reported here do reflect the suggested feeding guilds and findings from previous trophic analyses using bulk stable isotopes and fatty acid composition (Mincks et al., 2008, Würzberg et al., 2011). However, when considering the trophic traits of each feeding guild, the average trophic level estimates only differed by 0.5 at most (Table 5-8). Concomitantly, both the $TL_{Glu/Gly}$ and $TL_{TAA/SAA}$ estimates for SSDF were greater than those for O/P/S, which could be related to the consumption of degraded or dead material within the sediment with enriched $\delta^{15}N$ values. Regression analysis identified that $TL_{TAA/SAA}$ had the strongest relationship with the trophic amino acids and the weakest relationship with source amino acids. Thus this method could be regarded as the most realistic estimate of trophic level used. $TL_{Glu/Gly}$ had the weakest correlation with its trophic amino acid, which is likely to be result of the high variability in its source amino acid isotopic composition (glycine) between species and perhaps isotopic fractionation in *Glycera* spp.

	OPS	SDF	SSDF
$TL_{Glu/Phe}$	2.3 ± 0.6	2.0 ± 0.5	2.5 ± 0.3
$TL_{Glu/Gly}$	2.6 ± 0.5	2.4 ± 0.3	2.6 ± 0.4
$TL_{TAA/SAA}$	2.7 ± 0.4	2.4 ± 0.3	2.6 ± 0.3

Table 5-8 Average trophic level estimate for each categorical feeding guilds using the equations 5-1 ($TL_{Glu/Phe}$), 5-2 ($TL_{Glu/Gly}$) and 5-3 ($TL_{TAA/SAA}$).

The trophic level estimates depend both on the accuracy of the source amino acids and the trophic enrichment factors in the equation (Nielsen et al., 2015, McMahon and McCarthy, 2016). In Chapter 4, trophic level estimates for *Arenicola marina* reflected its biology with the best estimates being from the $TL_{Glu\text{-}Phe}$ equation, which was designed for marine organisms by Chikaraishi et al. (2009). However, for the Antarctic samples, greater variability is recorded between species and individuals which could imply that trophic level equations are not universal.

Linked to diet, physiology and mode of excretion, trophic enrichment factors vary between species and with trophic level (McMahon and McCarthy, 2016). Laboratory and field studies have been used to determine species or taxon-specific trophic enrichment factors for molluscs, fish, marine mammals and penguins (Lorrain et al., 2009, Dale et al., 2011, Ruiz-Cooley et al., 2014, Bradley et al., 2015, Choy et al., 2015). In many cases the calculated enrichment factors were less than the 7.6‰ suggested by Chikaraishi et al. (2009). Most studies have investigated a group of species from a single taxon, however, meta-analyses of 359 marine species of different feeding traits by Nielsen et al. (2015) found a mean trophic enrichment factor of 6.6 ± 1.7‰ between glutamic acid and phenylalanine. Furthermore enrichment factor estimates can vary within taxa where biological traits, such as body size, vary between individuals (Dale et al., 2011). In McMahon and McCarthy (2016) evidence shows that the variation in enrichment factors is not simply 'noise' but mechanically linked to animal physiology and biochemistry via diet quality and nitrogen excretion. Across 88 consumer-diet combinations McMahon and McCarthy (2016) recorded a range in the trophic enrichment factor between glutamic acid and phenylalanine of greater than 10. The observed variation was associated with the quality of diet. The greatest amino acid imbalances (and highest trophic enrichment factor) between food source and consumer were associated with 'lower' quality diets. In the same study trophic enrichment was also found to vary between urea/uric acid-producing consumers and ammonia-producing consumers.

These causes of variation may explain some of the trophic level similarities between the O/P/S and SSDF feeders; based on feeding guilds and that in the deep sea benthic, SSDFs are more likely to be feeding on lower quality organic matter. Using a more appropriate trophic enrichment factor could provide a more realistic trophic level estimate for these organisms, however source materials would be needed to determine this.

5.5 Summary

To the best of my knowledge, the data presented here provide the first $\delta^{15}N_{AA}$ data of Southern Ocean benthic polychaetes. The results highlight that the benthic system is subject to trophic variability, which may be linked to regional differences in nitrate chemistry as well as biological and physiological differences within and between species. Despite these uncertainties there are general patterns in the $\delta^{15}N$ values of the trophic amino acids that can be used to interpret the trophic traits of the polychaetes studied. Variation in the $\delta^{15}N$ values of the source amino acids is somewhat harder to interpret and further analyses and method development may be needed to determine the variability in nitrogen sources at the base of the food web. This variability in $\delta^{15}N$ values of source amino acids and the use of universal trophic enrichment factors indicate that the trophic level estimates should be treated with caution. These caveats also lead me to question the value in attempting to define a linear trophic level for a species in a highly complex interactive benthic system and so trophic variability needs to be investigated further.

5.6 References

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6 Classifying symbiotic relationships: Corals and Polynoidae of the South Orkney Islands Southern Shelf MPA

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6.1 Introduction

The South Orkney Islands form a small archipelago located 375 miles from the Antarctic Peninsula. In 2009 the commission for the Conservation of Antarctic Marine Living Resources (CCAMLR) designated a 94,000km^2 marine protected area (MPA) within this region, known as the South Orkney Islands Southern Shelf MPA (SOISS MPA). This is an area of exceptionally high biodiversity (Dickens et al., 2014), and the aim of this MPA is to protect both benthic and pelagic habitats in this region. In addition to the MPA, CCAMLR affords special protection to benthic habitats that are particularly vulnerable to the effects of bottom fishing. Such habitats are identified, based on the presence of indicator taxa, which are known as Vulnerable Marine Ecosystem (VME) taxa, and were assigned by CCAMLR (2009a) to include species that significantly contribute to the creation of a complex three-dimensional structures, create a complex surface by clustering in high densities, change the structure of the substratum, provide substrata for other organisms, or are rare or unique. At present twenty seven taxonomic groups classified as 'VME Indicator Organisms' are listed in the VME Taxa Classification Guide CCAMLR (2009b) including species of cnidarians, poriferans, pterobranchia, ascidians, bryozoans, stalked crinoids, and euryalida cideroida.

The State of the Antarctic Ecosystem (SO-AntEco) research cruise led by British Antarctic Survey (Austral summer 2016) was the first comprehensive research expedition to document and describe the fauna of the SOISS MPA. Owing to its geology and benthic substrata, the South Orkney slope provides a suitable habitat for many coral species (Davies and Guinotte, 2011). Overall 762 individual coral colonies were collected during SO-AntEco. The majority (84%), belonged to the class Octocorallia (soft corals) with the remainder including various Scleractinia (hard corals) and Stylasteridae (lace corals). The octocorals were also the most diverse with twenty-seven species from primary identifications. In recent years deep-water coral habitats and ecosystems have received much global research interest as they provide refuge and nursery grounds for a large number of fish and invertebrate species, thereby creating

and contributing to diversity hotspots (Jensen and Frederiksen, 1992, Rogers, 1999). As shallow-water fish stocks are exploited and depleted, fisheries are moving into deeper waters and deep-water corals have become an attractive area for fishing (Fosså et al., 2002, Roberts, 2002, Roberts et al., 2006). Deep-water corals are thought to be particularly at risk from these activities because of their arborescent morphology and assumed slow growth rates (Roberts, 2002). It is for these reasons that they are included in CCALMR's VME taxa. The stations sampled during SO-AntEco targeted deeper areas within the fishable depth range for long-lining (500 to 2000 m).

During SO-AntEco nearly 100 individual polynoids were found to be living on various coral species. As described by de Bary (1879), we define symbiosis as the relationship between two species living together where the symbiont (polychaete) is found in association with a host species (coral). The type of symbiotic relationship between the two species is categorized based on the effect of the symbiont on the host which may be any one of the following: - 1) mutualism: both species benefit from the relationship, 2) commensalism: the symbiont profits staying with the host, while the host is unaffected and 3) parasitism: the symbiont benefits, but the host suffers from the relationship. Furthermore, symbioses can be facultative or obligate; facultative species are able to survive without their host whilst obligate species cannot. Associated taxa may also be monoxenous, by which they have specificity to one host species or polyxenous, having several potential host species.

Symbiotic relationships between polychaetes and other marine invertebrates are well documented across different marine habitats, depths and latitudes. Britayev et al. (2014) recorded around 550 polychaete associations, 200 of which were scaleworm species including many polynoid species. Within the deep sea, many polychaete taxa have been collected from different host species from several locations including the Atlantic, Pacific, Indian and Southern Oceans (Martin and Britayev, 1998, Buhl-Mortensen and Mortensen, 2004, Serpetti et al., 2016). The abundance of symbiotic associations within the polar benthos has been noted within the Weddell and Ross Sea (see Alvaro and Barco, 2013, Schiaparelli, 2014). The most common symbioses observed within the Southern Ocean benthos appear to be shifted towards parasitism with polychaetes and mollsucs being the most common symbionts (Schiaparelli et al., 2007, Schiaparelli, 2014). Very few symbiotic relationships documented in the Southern Ocean have been studied in detail, exceptions include inquilistic commensalism and parasitic species on echinoderm hosts (Schiapelli et al. 2010; 2011).

Defining symbiotic relationships between host species and their associated fauna is challenging because of the lack of direct observations and damaged samples associated with studying and sampling deep-sea environments (Buhl-Mortensen and Mortensen, 2004). However, some symbiotic relationships e.g. the Eucinidae polychaete *Eunice norvegica* and its coral host is now relatively well researched through aquaria studies (Roberts, 2005, Mueller et al., 2013). Ecological benefits of these symbioses included increased calcification around the polychaete tubes by corals promoting growth, strengthening the reef framework, a decreased risk of predation compared to free-living species for the polynoid and a constant food supply as the polyps concentrate organic matter or by predating on coral grazers (Roberts, 2005). Although the polychaete is considered to 'steal' organic food from the host this behvaiour also cleans the host and decreases risk of predation (Buhl-Mortensen et al., 2001). Further studies using isotopic tracers demonstrated that food assimilation by *E. norvegica* was 2 to 4 times higher in the presence of the *L. pertusa* highlighting the energetic benefits of the symbiotic traits within an often food limited system (Mueller et al., 2013).

Stable isotope analysis can be used to determine the trophic traits of marine organisms (Peterson and Fry, 1987). More specifically the nitrogen composition, $\delta^{15}N$ (the ratio of ^{14}N to ^{15}N relative to a standard), of organisms can be used to define their trophic level (Deniro and Epstein, 1981, Minagawa and Wada, 1984). This is due to the loss of isotopically light nitrogen (^{14}N) with each trophic transfer and thus the consumer becomes 'enriched' in isotopically heavier nitrogen (^{15}N). The degree of ^{15}N enrichment has been estimated to be ~3.4‰ per trophic level. This value has been used to estimate the trophic status of different taxa in many marine studies (Peterson and Fry, 1987, Post, 2002, Fry, 2006). However ^{15}N enrichment between each trophic level can vary between species associated with species-specific physiology and trophic ecology (Hobson and Clark, 1992, Hobson et al., 1993, Bearhop et al., 2002, Bearhop et al., 2004). Thus if the isotopic signatures of primary and secondary food sources are not available, the trophic level calculated for a given species may not be a true reflection of its ecology. More recently compound specific stable isotope analysis (CSIA) has enabled the determination of $\delta^{15}N$ values of different amino acids (Hare et al., 1991, Gaebler et al., 1966, Macko et al., 1997). During cultured feeding experiments using green algae and zooplankton, McClelland and Montoya (2002) noted that some amino acids are highly conserved (e.g. phenylalanine) whilst others were enriched by ~7‰ with each trophic level (e.g. glutamic acid). These amino acids have since been referred to as 'source' and 'trophic' amino acids, the $\delta^{15}N$ of which can be compared to obtain a more realistic measure of trophic

position than the previously described 'bulk methods' (Popp et al., 2007). Determining the trophic level of marine species using the 'amino acid method' has now been documented in zooplankton (McClelland and Montoya, 2002, McCarthy et al., 2007, Hannides et al., 2009), invertebrates (Pakhomov et al., 2004, Chikaraishi et al., 2007, Ohkouchi et al., 2013) and fish (Popp et al., 2007, Chikaraishi et al., 2009). The method is especially useful when examining the trophic ecology of preserved collection material where primary food sources are unavailable.

The research described in this chapter uses a molecular and biochemical approach to define the symbiotic relationship between the polynoids and corals hosts collected within and around the SOISS MPA. DNA sequencing was used to identify the polynoid symbionts to species level and to assess the level of genetic diversity and connectivity within the SOISS MPA region. The use of DNA to describe the behavioural strategies and the potential role of hosts in the speciation process of symbiotic polynoids was recently applied by Serpetti et al. (2016) in the South West Indian Ocean. Sequence comparison also allows the detection of cryptic species, i.e. those that are morphologically identical but genetically distinct, which appear to be a common feature within the Antarctic benthos (see review by Grant et al., 2011). The determination of $\delta^{15}N$ of the polynoids allowed the investigation of the influence of genetic diversity on trophic and functional diversity. Finally, by comparison to $\delta^{15}N$ signatures from free-living Antarctic polychaetes (Chapter 5), and trophic level estimates from the literature, these data are used to improve our understanding of the symbiotic relationships between deep-water corals and polynoid species of the SOISS MPA region.

6.2 Methods

6.2.1 Sample collection

All polynoid specimens used in this study were collected during the SO-AntEco cruise led by British Antarctic Survey onboard the *RRS James Clarke Ross*. Six localities were sampled including two sites within and four sites outside of the SOISS MPA. Larger macro- and megafaunal species were collected using an Agassiz trawl with a 1 cm mesh size and a 2 m wide mouth which was towed along the seafloor at 1 knot for 10 minutes. At each location the trawl was deployed three times to discrete depth horizons of approximately 500, 750, 1000 and 1500 m. Once aboard, samples were separated into taxonomic group and any associated polynoids were removed from their host species and preserved in 90% ethanol. A total of 89

polynoid individuals were collected from at least 9 host coral species and 2 provisional morphospecies identified (Table 6-1). Not all specimens were removed directly from corals, nine individuals were considered to have 'fallen off' during sampling and sorting. The majority of individuals (81%) were collected from Octocorallia species and all but one of the purple specimens were collected from *Acanthogorgia* species (Table 6-1).

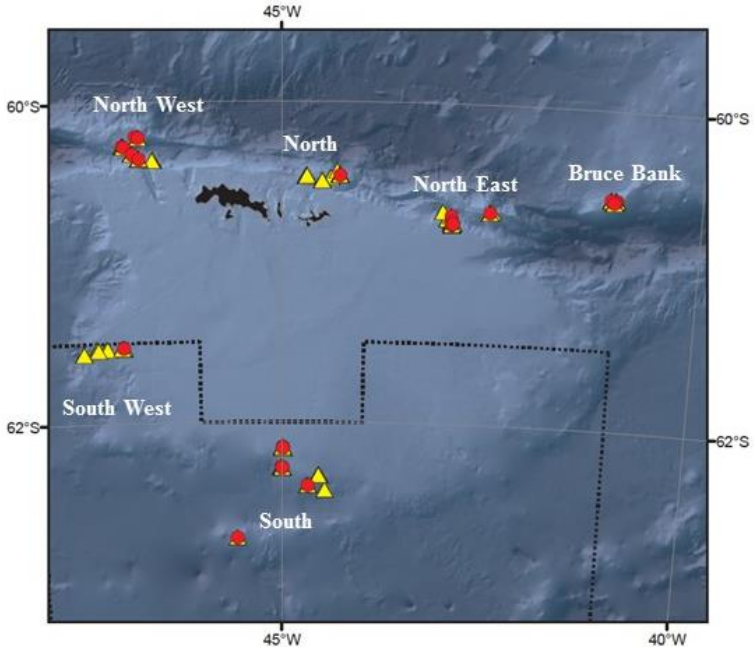


Figure 6-1 Location of the 6 sites sampled during the SOAntEco expedition. Red circle indicate sites where Polynoidae specimens were collected and yellow triangles, host coral species. The dotted line indicates the northern limit of the South Orkney Island Southern Shelf Marine Protected Area. West and South sites were within the MPA and all Northern sites and Bruce Bank were outside the MPA area.

				Striped			
Host	**Site**	**Bruce Bank**	**North**	**North West**	**South**	**West**	**Total per host**
Unidentified Anthozoa				1			1
Octocorallaria	*Acanthogorgia*	3					3
Octocorallaria	*Anthomastus*				1		1
Octocorallaria	Bayergorgia vermidoma			2	1		3
Octocorallaria	Dasystenella acanthina	19		9	2	1	31
Octocorallaria	Isididae	2					2
Octocorallaria	*Onogorgia*		1				1
Octocorallaria	*Thouarella* spp.	14		1	4		19
Hydrozoa	Stylasteridae	1			1		2
Unknown		8		1	4		13
				Purple			
Octocorallaria	*Acanthogorgia*	12					12
Unknown				1			1
	Total per site	59	1	15	13	1	89

Table 6-1 The host species in which the symbionts were collected from by location and morphotype. Unknown includes unidentified hosts and individuals that had fallen off corals.

6.2.2 Morphological species identification

All 88 polynoid specimens were examined under a stereomicroscope and photographed, being assigned to two different morphotypes including 'striped' and 'purple' varieties (Figure 6-2). Following phylogenetic sequence analysis specimens were re-examined to ensure that morphological variation between potential cryptic species had not been overlooked.

Figure 6-2 The two polynoid morphotypes collected from corals on JCR15005 including 'striped' (left) and 'purple' (right). Scale bar = 5 mm.

6.2.3 DNA barcoding and sequence analysis

The spatial distribution and number of specimens of each morphotype from which DNA was extracted is shown in Table 6-2. DNA was extracted from dissected parapodia, from whole specimens, segments and or incomplete specimens, using a Hamilton Microlab STAR Robotic Workstation at the Natural History Museum Sequencing Facility. The primary gene targeted for species identification was the so-called 'Folmer fragment', a 660 base pair region of the mitochondrial protein-coding COI gene (Folmer et al., 1994). An internal transcribed species (ITS) gene was also sequenced from a reduced number of specimens chosen from preliminary COI phylogenetic analyses. This rRNA gene can be used to check genetic differences based on both paternal and maternal inheritance and has been used previously for polynoid species discrimination (Nygren et al., 2011).

	Striped			Purple
Depth (m)	Bruce Bank	North West	South	Bruce Bank
500	4	7	3	6
750	30	2	3	5

Table 6-2 The number of individuals from which DNA sequenced for each polynoid morphotype, by depth and location. See Figure 6.1 for site locations.

DNA extractions were amplified using a PCR mix of 21 µL Red Taq DNA Polymerase 1.1X MasterMix (VWR), 1 µL of each primer (10 µM) (Table 6-3), and 2-5 µL of DNA extract. The

PCR temperature profile consisted of an initial 5-minute denaturation stage at 95ºC, followed by 35 cycles of 95ºC denaturation for 1 minute, 55ºC annealing for 1 minute, 74ºC extension for 2 minutes with an additional 5 minutes extension phase after the last cycle. For primer sequences and references see Table 1. PCR products were purified using a Millipore Multiscreen 96-well PCR purification system and sequenced on an ABI 3730XL DNA Analyser (Applied Biosystems) at the Natural History Museum Sequencing Facility using the same primers as in the PCR.

Primer name	Sequence (5-3')	Reference
LCO	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)
HCO	TAAACTTCAGGGTGACCAAAAA ATCA	Folmer et al. (1994)
CO1-E	TATACTTCTGGGTGTCCGAAGAATCA	Carr et al. (2011)
ITS18SFPOLY	GAGGAAGTAAAAGTCGTAACA	Nygren et al. (2009)
ITS5.8SFPOLY	GAATTGCAGGACACATTGAAC	Nygren et al. (2009)
ITS5.8SRPOLY	GTTCAATGTGTCCTGCAATTC	Nygren et al. (2009)
TS28SRPOLY	ATGCTTAAATTCAGCGGGT	Nygren et al. (2009)

Table 6-3 COI and ITS primers used for PCR of the polynoid DNA

Overlapping sequences (from forward and reverse primers) were assembled into consensus sequences and aligned in Geneious 7.1.4 (Kearse et al., 2012). For phylogenetic analysis, additional sequences of the polynoidae family were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). COI sequences were aligned using MUSCLE (Edgar, 2004) and ITS using MAFTT (Katoh et al., 2002) both using the default settings provided as plug-ins in Geneious.

Bayesian phylogenetic analyses of the COI and ITS dataset were conducted separately. For each dataset the best nucelotide substitution model was chosen using the jModelTest Akaike and Bayesian information criterion (Posada, 2008). Either GTR+I+G or GTR+G models were chosen as the best-fit model for each alignment dependant on the jModelTest results. All analyses were run three times for 10,000,000 generations using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) with 2,500,000 generations discarded as burn-in. The polynoid species *Harmothoe bathydomus* was used as an outgroup. This was chosen based on its position in a polynoidae tree constructed in preliminary DNA analyses. All phylogenetic trees were edited in FigTree 1.4 (Rambaut, 2007) and Adobe Illustrator CS5.1.

The inclusion of publicly available sequences allowed the comparison of genetic distances between polynoid clades or potential species. Thus, if the genetic distances were greater than or comparable to the genetic distances between known species, this could be used to

discriminate between potential species. For this the Kimura's two-parameter substitution model (K2P) (Kimura, 1980) was calculated using Mesquite for pairwise comparisons of sequence divergence within and between species based on the number of nucleotide substitutions.

6.2.4 Total nitrogen, bulk and compound specific stable isotope analysis

These analyses targeted a subsample of polynoids chosen based on their site of collection and genetic results obtained from DNA analyses. This ensured spatial cover throughout the sampled region whilst maintaining replicated individuals within the same species for comparison within and between sites and depths. Those chosen are displayed in Table 6-4.

	Striped			Purple
Depth (m)	Bruce Bank	North West	South	Bruce Bank
500	4	4	3	3
750	4	2	3	3

Table 6-4 The number of individuals from which CSIA was conducted for each polynoid species identified from DNA analysis by depth and location. See Figure 6.1 for site locations.

From each polynoid, 1mg of freeze dried tissue was used for total nitrogen (TN) analyses, 1 mg of freeze dried tissue was used for bulk $\delta^{15}N$ analysis and 0.5mg for CSIA. All analyses were run in duplicate on the 'full' and 'Phe' methods outlined in Chapter 4.

6.2.5 Data correction

All samples were analysed in duplicate for total nitrogen, bulk $\delta^{15}N$ ($\delta^{15}N_{Bulk)}$ and amino acid $\delta^{15}N$ ($\delta^{15}N_{AA}$). Total nitrogen and $\delta^{15}N_{Bulk}$ data were corrected as described in Chapter 4. All $\delta^{15}N_{AA}$ values, excluding phenylalanine, were corrected using the two-step calibration procedure described in Chapter 5. Phenylalanine values were corrected to an offset, the difference between the recorded phenylalanine of nearest standard and its known $\delta^{15}N$ value.

Phenylalanine correction: $\delta^{15}N_{Phe}$ Sample – ($\delta^{15}N_{Phe}$ Known - $\delta^{15}N_{Phe}$ Standard)

(Equation 6-1)

Where $\delta^{15}N_{Phe}$ Known = 1.7‰.

A mean value of each $\delta^{15}N_{AA}$ from the duplicate analyses for each individual polynoid was then calculated. Averages for each morphotype by site and depth were calculated with the standard deviation.

6.2.6 Estimation of trophic level

Trophic level was calculated for each individual (Equations 6-2 to 6-5) and data averages calculated for each location by depth. This approach was chosen due to differences within the literature regarding which equation best represents the 'true' trophic level. Both the enrichment and abundance of different amino acids varies between species and habitats thus this approach will allow multiple scenarios when incorporating biochemical and trophic variation (McMahon and McCarthy, 2016).

$$TL_{Bulk:}\ (\delta^{15}N\text{Polynoid} - 1.8)/3.4 + 1 \qquad \text{(Equation 6-2)}$$

$$TL_{Glu/Phe}:\ (\delta^{15}N\ \text{Glu} - \delta^{15}N\ \text{Phe} - 3.4)/7.6 + 1 \qquad \text{(Equation 6-3)}$$

$$TL_{Glu/Gly}:\ (\delta^{15}N\ \text{Glu} - \delta^{15}N\ \text{Gly})/7 + 1 \qquad \text{(Equation 6-4)}$$

$$TL_{TAA/SAA}:\ (\delta^{15}N\ T_{AA} - \delta^{15}N\ S_{AA})/7 + 1 \qquad \text{(Equation 6-5)}$$

Equations 6-2 to 6-5 Calculations used to estimate trophic level. Where TL = trophic level, Bulk = bulk $\delta^{15}N$, Glu = Glutamic acid, Phe = Phenylalanine, Gly = Glycine, T_{AA} = averaged trophic amino acids (alanine, aspartic and glutamic acid) and SAA = averaged source amino acids (phenylalanine and glycine).

These equations were chosen on the basis of data available from the SIA and CSIA analyses and relevant literature. Equation 6-2 included the average $\delta^{15}N$ particulate organic matter values (1.8‰) collected in the Scotia Sea near the South Orkneys within the Antarctic Summer (Stowasser et al., 2012). Equation 6-3 is from Chikaraishi et al. (2009), where 3.4 is the isotope difference between amino acids (glutamic acid and phenylalanine) in primary producers and 7.6 is the ^{15}N enrichment factor. Likewise in Equations 6-4 and 6-5, 7 is the ^{15}N enrichment factor for glycine and the average of trophic amino acids (TAA: alanine, aspartic and glutamic acid) respectively. Equation 6-7 uses an average value of the source amino acids (SAA) including phenylalanine and glycine (Popp et al., 2007). Note that phenylalanine data was only available for 18 of the 26 specimens, so the average value for each morphotype at each site and depth was taken and used as the phenylalanine value in Equation 6-3.

6.2.7 Data analysis

One-way ANOVA was used to test for significant differences in total nitrogen for $\delta^{15}N_{Bulk}$ and individual $\delta^{15}N_{AA}$ and trophic level estimates between the 'purple' and 'striped' morphotypes from the same location (Bruce Bank). Data obtained from the striped morphotype were used to investigate potential biogeographic patterns in trophic traits. Two-way ANOVA was used

to test for differences in the mean values between site, depth, as well as any interaction effect, i.e. depth was only a significant factor at certain sites. All statistical analyses were conducted in R after passing normality and equal variance tests.

6.3 Results

6.3.1 Genetic diversity based on DNA barcoding

Phylogenetic analyses of the COI data from 60 symbiotic polynoid specimens formed two main clades, one of which contained the purple morphotype and the other the striped individuals (Figure 6-3). A single purple individual (#59) did not fall within either clade. Two individuals, one from each morphotype (purple #81 and striped #11) formed an intermediate clade. Within the striped clade no genetic structuring with locality or depth was found, nor was there genetic structuring with depth within the purple clade. The polynoids sequenced here appear to be related to *Polyeunoa laevis* McIntosh, 1885 specimens collected from deep-sea corals in the South West Indian Ocean (Serpetti et al., 2016) and the striped clade matched larval sequences from the Ross Sea (Gallego et al., 2014).

Figure 6-3 Phylogenetic tree of the symbiotic polynoids from Bayesian analysis using COI (mtDNA) only. Including sequences from JR15005 specimens labelled by morphotype 'purple' and 'stripe', and GenBank sequences of *Polyeunoa laevis*. Outgroup: *Harmothoe bathymodus* from the NHM database, * indicates significant node values (>95%) for Bayesian posterior probabilities.

The K2P distance was used to investigate the presence of the 'barcoding gap' used in the determination of species (Figure 6-4). The intra morphotype variation was comparable to that of the inter morphotype pairwise comparisons, ranging from 0 to 3.05% within and, 1.69 to 3.81% between the purple and the striped South Orkney morphotypes, respectively. For this reason we refer to purple and striped morphotypes from here on. Pairwise distances between the purple and striped morphotypes of *P. laevis* from the Southern West Indian Ocean were greater, ranging from 3.68 to 5.15%.

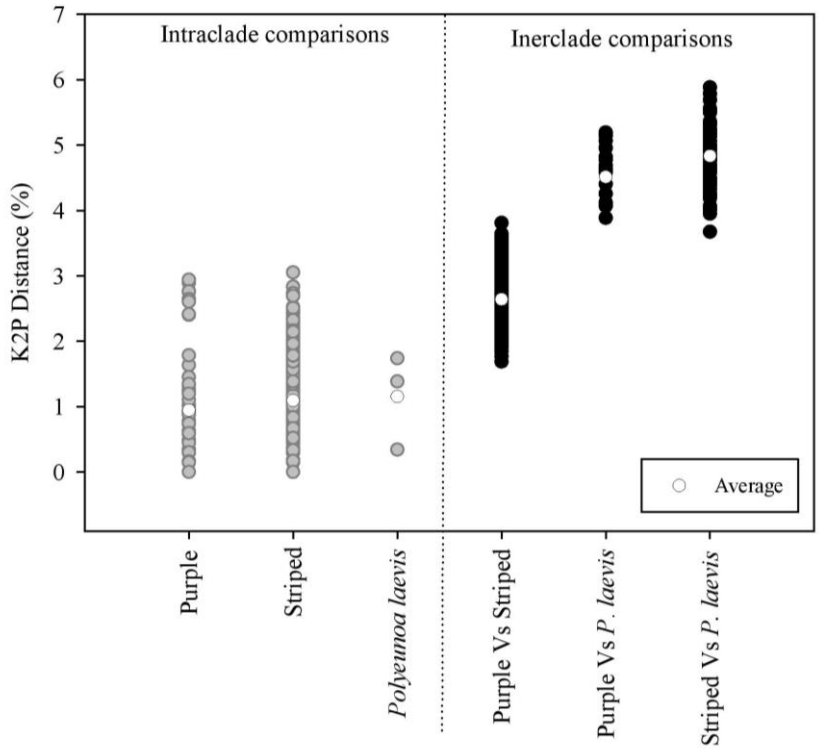


Figure 6-4 Average and pairwise K2P distances (%) from pairwise comparisons of COI sequences within and between morphotypes purple and striped and *Polyeunoa laevis*. Note that the larval sequence KF713377 was excluded from the analysis and unsure of adult morphotype.

6.3.2 Nitrogen isotopic signatures between symbiotic polynoid morphotypes

The average total nitrogen, $\delta^{15}N_{Bulk}$ and $\delta^{15}N_{AA}$ data for both morphotypes by location and depth is shown in Table 6-5. As a result of limited phenylalanine data an average was taken by region and for each species. The purple morphotype was only collected from the Bruce Bank locality and thus for the investigation of trophic differences between morphotypes these data were only compared to the striped individuals from the same locality (Figure 6-5).

	Total N (%)	Bulk $\delta^{15}N$ (‰)	Amino Acid $\delta^{15}N$ (‰)								
			Glutamic acid	Alanine	Valine	Leucine	Threonine	Aspartic acid	Glycine	Serine	Phenylalanine
Purple **Bruce Bank**											
500m (n=3)	13.6 ± 0.1	10.2 ± 0.2	19.4 ± 0.6	21.4 ± 0.9	23.5 ± 1.2	21.7 ± 0.9	-16.1 ± 3.2	16.6 ± 0.3	1.1 ± 0.5	2.5 ± 0.6	7.1 ± 0.9
750m (n=3)	14.1 ± 0.2	11.6 ± 0.5	20.6 ± 0.3	22.6 ± 1.1	25.6 ± 0.2	23.7 ± 0.7	-14.8 ± 3.3	17.8 ± 0.4	2.4 ± 2.0	2.5 ± 0.8	8.8 ± 1.3
Striped **Bruce Bank**											
500m (n=4)	13.2 ± 0.4	9.3 ± 0.7	19.1 ± 0.6	18.1 ± 3.2	20.3 ± 3.2	20.7 ± 1.1	-15.1 ± 3.9	16.5 ± 1.7	2.2 ± 1.8	1.6 ± 2.2	8.8 ± 1.0**
750m (n=4)	13.4 ± 0.1	10.1 ± 0.7	19.0 ± 0.9	20.7 ± 1.4	24.6 ± 0.7	23.0 ± 0.9	-13.3 ± 2.6	17.1 ± 0.6	3.4 ± 1.6	4.7 ± 1.7	8.9 ± 2.0**
North West											
500m (n=3)	12.3 ± 1.2	10.2 ± 0.5	19.2 ± 2.2	20.6 ± 0.9	24.2 ± 1.6	23.1 ± 1.7	-15.1 ± 4.0	17.0 ± 2.4	1.4 ± 3.6	17.0 ± 2.5	10.2 ± 0.2**
750m (n=3)	12.8 ± 0.0	8.6 ± 0.8	17.2 ± 0.1	17.3 ± 1.2	22.0 ± 0.1	20.1 ± 0.4	-16.2 ± 2.4	15.2 ± 0.4	1.6 ± 2.2	15.2 ± 0.4	9.3*
South											
500m (n=3)	13.6 ± 0.2**	9.3 ± 0.4**	19.3 ± 1.0	18.4 ± 2.1	21.3 ± 4.2	23.0 ± 1.2	-18.8 ± 4.7	16.4 ± 1.3	0.5 ± 1.0	16.4 ± 1.3	8.5 ± 0.5**
750m (n=3)	13.2 ± 0.1**	9.1 ± 0.6	17.4 ± 0.5	16.8 ± 2.9	20.2 ± 2.5	19.8 ± 0.6	-15.9 ± 2.4	14.9 ± 0.6	3.1 ± 3.6	14.9 ± 0.6	9.0 ± 1.3

Table 6-5 Average total nitrogen with $\delta^{15}N$ values of bulk and isolated amino acids of the striped and purple polychaete morphotypes from three locations within the South Orkney region at 500 and 750m depth. Number of individuals (n) varied between sample groups where ** = 2 individuals and * = 1 individual, due to insufficient material for all analyses or technical difficulties with the GC/C/CIRMS.

Significant differences in the nitrogen signatures between morphotypes were recorded in total nitrogen, $\delta^{15}N_{Bulk}$, and $\delta^{15}N$ values of glutamic acid, alanine (Table 6-6). Where significant differences were found the total nitrogen, $\delta^{15}N_{Bulk}$, and $\delta^{15}N_{AA}$ values were higher or heavier in the purple morphotype than in the striped individuals. This pattern was reflected in all other trophic amino acids (valine, leucine and aspartic), but was not statistically significant. For the source amino acids, glycine was enriched in ^{15}N in the striped morphotype, but highly variable in both morphotypes (Figure 6-5). The average $\delta^{15}N$ of glycine at Bruce Bank was similar between the two species being 2.4‰ and 2.6‰, for the purple and striped morphotypes, respectively (Table 6-5).

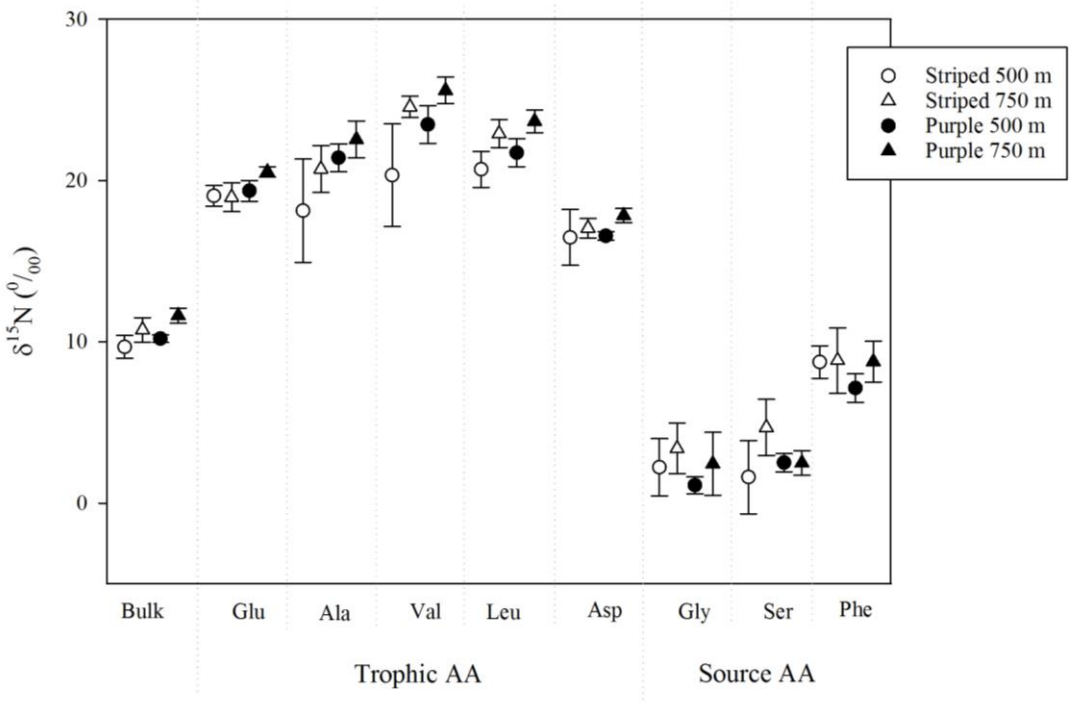


Figure 6-5 Average bulk $\delta^{15}N$ and amino acid $\delta^{15}N$ data (‰) for both the purple and striped morphotype at Bruce Bank by depth. Threonine not included but shown in Table 6-5. Where Glu = glutamic acid, Ala = alanine, Val = valine, Leu = Leucine, Asp = aspatic, Gly = glycine, Ser = serine, Phe = phenylalanine.

	Purple vs Stripe
Total Nitrogen	**$F_{(1,12)} = 12.12, P = 0.005$**
Bulk	**$F_{(1,12)} = 7.60, P = 0.017$**
Glutamic Acid	**$F_{(1,12)} = 5.28, P = 0.040$**
Alanine	**$F_{(1,12)} = 4.73, P = 0.050$**
Valine	$F_{(1,12)} = 2.21, P = 0.162$
Leucine	$F_{(1,12)} = 0.69, P = 0.422$
Threonine	$F_{(1,12)} = 0.58, P = 0.463$
Aspartic Acid	$F_{(1,12)} = 0.61, P = 0.450$
Glycine	$F_{(1,12)} = 1.48, P = 0.246$
Serine	$F_{(1,12)} = 0.37, P = 0.553$
Phenylalanine	$F_{(1,8)} = 0.10, P = 0.347$
Weight	$F_{(1,12)} = 0.13, P = 0.722$

Table 6-6 One-way ANOVA results of total nitrogen (%), bulk $\delta^{15}N$ and $\delta^{15}NAA$ values (‰) between the purple and striped moprhotypes collected from Bruce Bank. Significant values in bold type.

6.3.3 Nitrogen isotopic signatures of Polynoidae within the South Orkney Region

The nitrogen data from the striped morphotype was collected at three localities within the South Orkney region (Bruce Bank, North West and South) at depths of 500 and 750 m. Total nitrogen values were relatively constant between sites and depths although the variation at 500 m in the North West was more than three times greater than the other sites (Table 6-7 and Figure 6-6). A significant interactive effect of depth and site on $\delta^{15}N_{Bulk}$ signatures was recorded with a depletion in $\delta^{15}N$ with depth at the South and North West sites and enrichment with increasing depth at Bruce Bank (Figure 6-6).

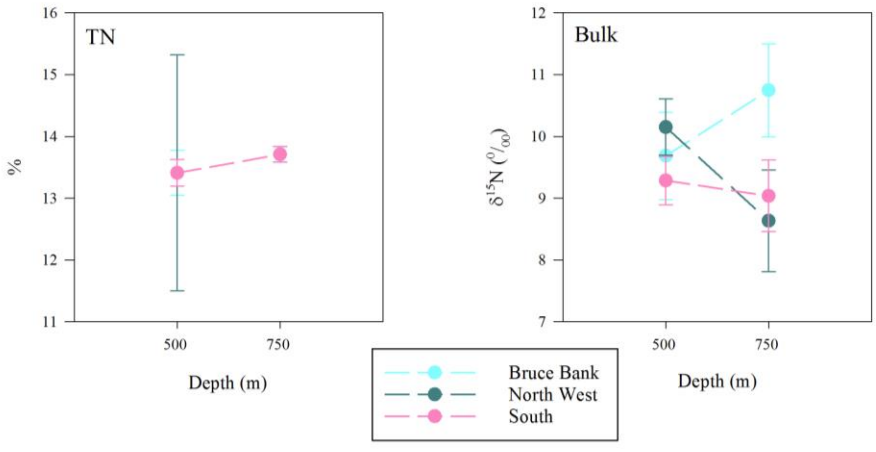
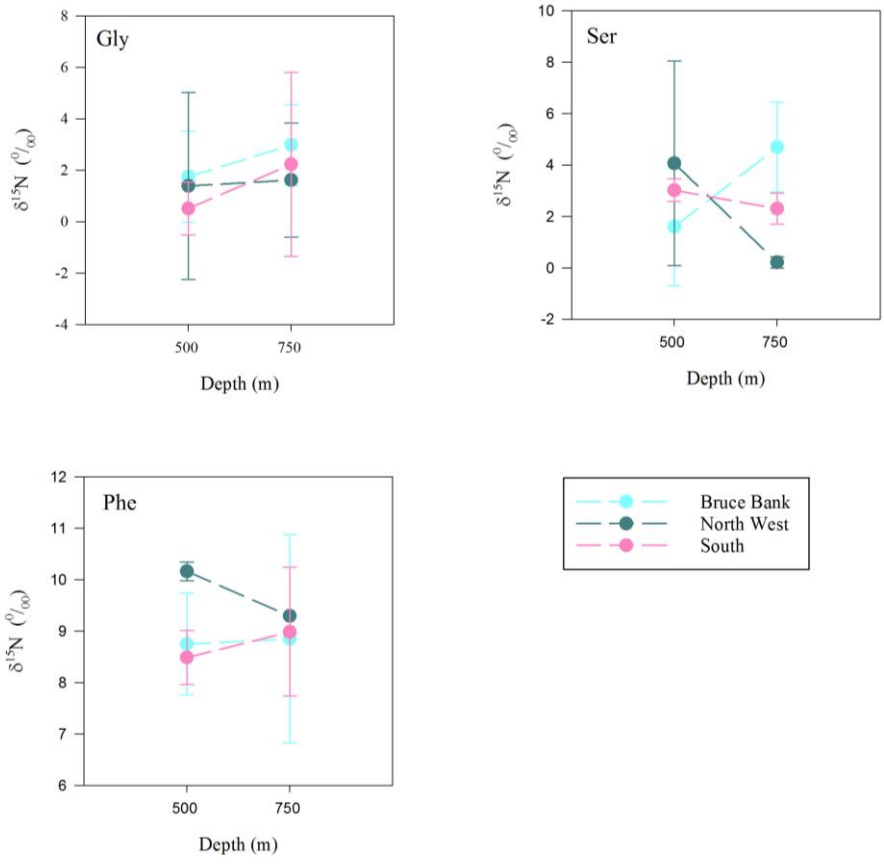


Figure 6-6 The mean total nitrogen (TN) and $\delta^{15}N_{Bulk}$ values, with standard deviation for the striped morphotype at each site with depth.

The pattern recorded with depth at each location for the $\delta^{15}N_{Bulk}$ signatures was also recorded in the trophic amino acids (Figure 6-7). The most enriched $\delta^{15}N$ values for each amino acid were consistently recorded at the Bruce bank site at 750 m depth, however the only significant interactive effects were recorded for leucine and serine (Table 6-7). Although not significant, the difference in $\delta^{15}N$ enrichment between sites was greatest in alanine and glutamic acid.

	Site	Depth	Site*depth
Total Nitrogen	$F_{(2,12)} = 1.90$, P = 0.191	$F_{(1,12)} = 0.10$, P = 0.749	$F_{(2,12)} = 0.27$, P = 0.773
Bulk	$F_{(2,13)} = 1.10$, P = 0.361	$F_{(1,13)} = 0.33$, P = 0.574	**$F_{(2,13)} = 5.33$, P = 0.020**
Glutamic acid	$F_{(2,14)} = 0.53$, P = 0.598	$F_{(1,14)} = 4.31$, P = 0.566	$F_{(2,14)} = 1.33$, P = 0.295
Alanine	$F_{(2,14)} = 1.50$, P = 0.257	$F_{(1,14)} = 0.10$, P = 0.758	$F_{(2,14)} = 3.30$, P = 0.067
Valine	$F_{(2,14)} = 1.87$, P = 0.191	$F_{(1,14)} = 0.53$, P = 0.480	$F_{(2,14)} = 3.29$, P = 0.067
Leucine	$F_{(2,14)} = 1.46$, P = 0.265	$F_{(1,14)} = 2.31$, P = 0.150	**$F_{(2,14)} = 6.45$, P = 0.010**
Threonine	$F_{(2,14)} = 1.37$, P = 0.286	$F_{(1,14)} = 0.72$, P = 0.410	$F_{(2,14)} = 0.50$, P = 0.618
Aspartic acid	$F_{(2,14)} = 0.85$, P = 0.448	$F_{(1,14)} = 1.02$, P = 0.329	$F_{(2,14)} = 1.18$, P = 0.334
Glycine	$F_{(2,14)} = 0.53$, P = 0.560	$F_{(1,14)} = 1.33$, P = 0.268	$F_{(2,14)} = 0.32$, P = 0.733
Serine	$F_{(2,14)} = 0.09$, P = 0.918	$F_{(1,14)} = 0.00$, P = 0.992	**$F_{(2,14)} = 3.78$, P = 0.048**
Phenylalanine	$F_{(2,6)} = 0.88$, P = 0.460	$F_{(1,6)} = 0.00$, P = 0.957	$F_{(2,6)} = 0.27$, P = 0.771

Table 6-7 Two-way ANOVA results of comparing total nitrogen (%), bulk $\delta^{15}N$ and individual $\delta^{15}N_{AA}$ values (‰) between site and location from the 'Stripe' polynoid specimens. Significant values in bold type.



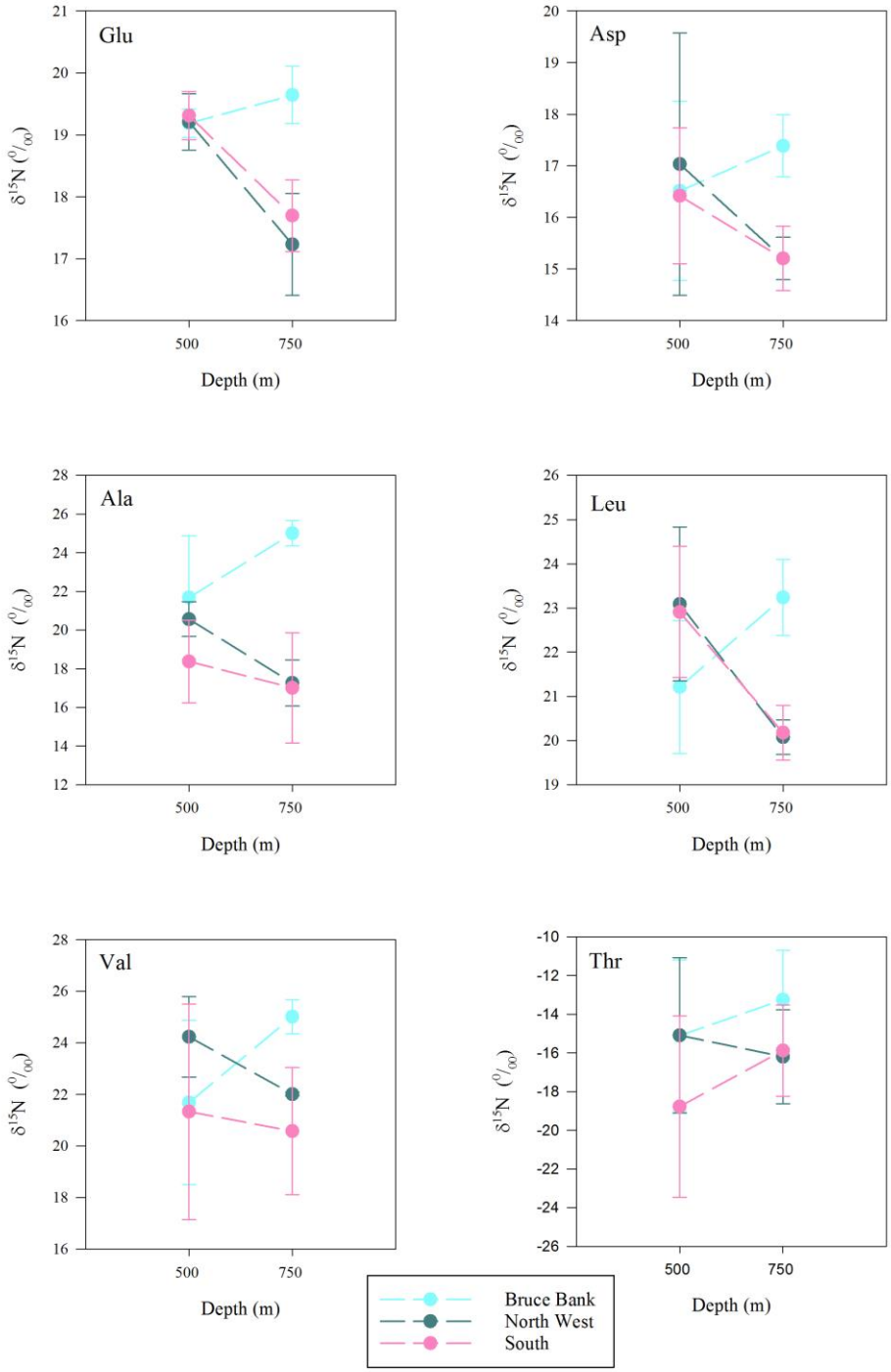


Figure 6-7 The mean amino acid δ15N values with standard deviation for the striped morphotype at each site, with depth. With source amino acids (overleaf) Gly = glycine, Phe = phenylalanine and Ser = serine and trpophic amino acids Glu = glutamic acid, Ala = alanine, Val = valine, Asp = aspartic and Leu = leucine, and Thr = Threonine.

6.3.4 Defining symbioses using stable isotope analysis

6.3.4.1 Comparison to free-living species

To provide additional insight into the trophic ecology of the symbiotic polynoids and their functional relationship with their coral hosts, the $\delta^{15}N$ values from each amino acid were compared to values obtained from free living benthic polychaetes collected at similar depths within the Western Antarctic on the BIOPEARL and JR275 expeditions (Figure 6-8).

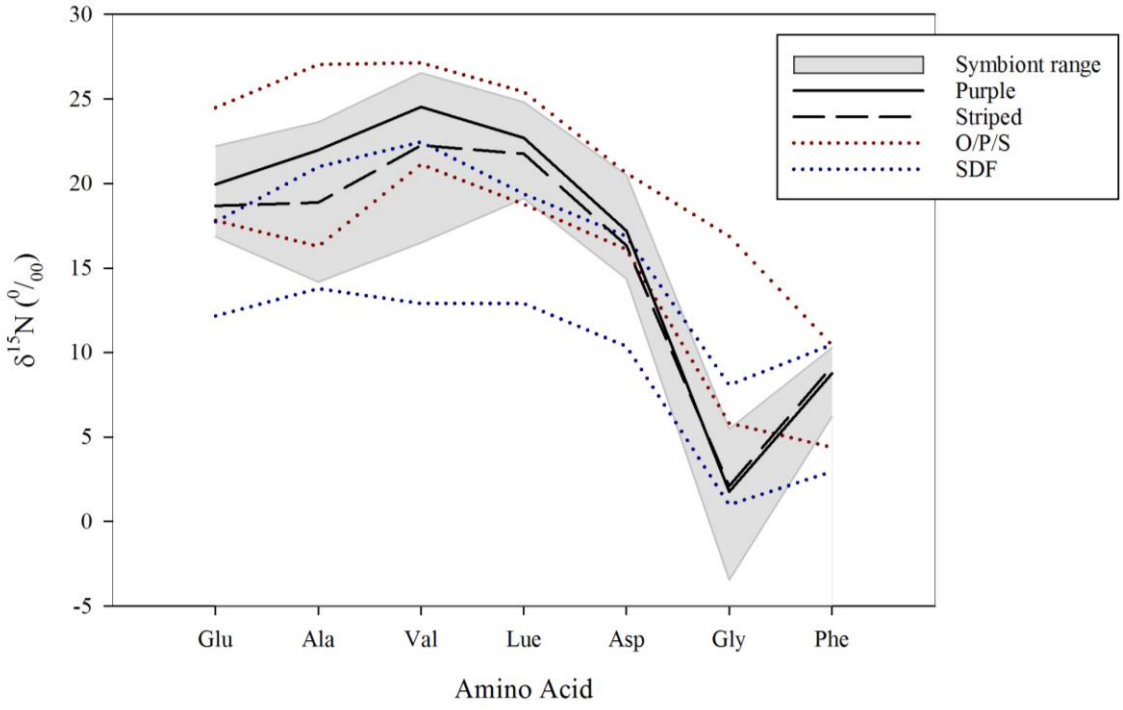


Figure 6-8 The observed range and mean of amino acid $\delta^{15}N$ values in polynoid symbionts (both morphotypes) within the South Orkney region with reference to the range observed in omnivore, predator scavenger species (O/P/S) and surface deposit feeders (SDF) from the BIOPEARL expeditions to the Scotia Sea, Amundsen Sea (BIOPEARL) and Weddell Sea (JR275).

In comparison to the free-living species, the mean trophic amino acid $\delta^{15}N$ values for the symbiotic specimens fell within the lower range of the omnivore/predator/scavenging species and, for alanine and valine and aspartic this overlapped with the upper range of the surface deposit feeders' $\delta^{15}N$ values. As observed in this study there was variation in the $\delta^{15}N$ values within the categorical feeding types which may be associated with species, location and depth (Chapter 5). Trophic amino acids signatures in free-living polychaetes on average ranged from 7.0‰, the greatest range was recorded in alanine at 10.8‰. For the symbionts, the average

range for trophic amino acid signatures was similar to the free-living categories at 6.8‰ with the greatest range in valine at 10.5‰.

The average source amino acid signatures of the polynoid symbionts were closest to the lower values recorded in free-living surface deposit feeders. Ranging from 6.23 to 10.25, and, -3.45 to 5.48, for phenylalanine and glycine respectively (Figure 6-8).

6.3.4.2 Trophic level estimates

Trophic level estimates varied across the four equations used, the lowest estimates were from the $TL_{Glu/Phe}$ (Equation 6-3) that ranged from 1.5 to 2.4. TL_{Bulk} (Equation 6-2), $TL_{Glu/Gly}$ (Equation 6-4) and $TL_{TAA/SAA}$ (Equation 6-5) estimates for all individuals produced trophic values greater than 2.4. The highest trophic level estimate for any individual was 4.0 for a purple specimen using the bulk equation (Figure 6-9). On average, trophic level estimates for the purple morphotype were higher than the striped morphotype at the same location. A significant difference in estimated trophic level was recorded between the morphotypes at Bruce Bank (Table 6-8).

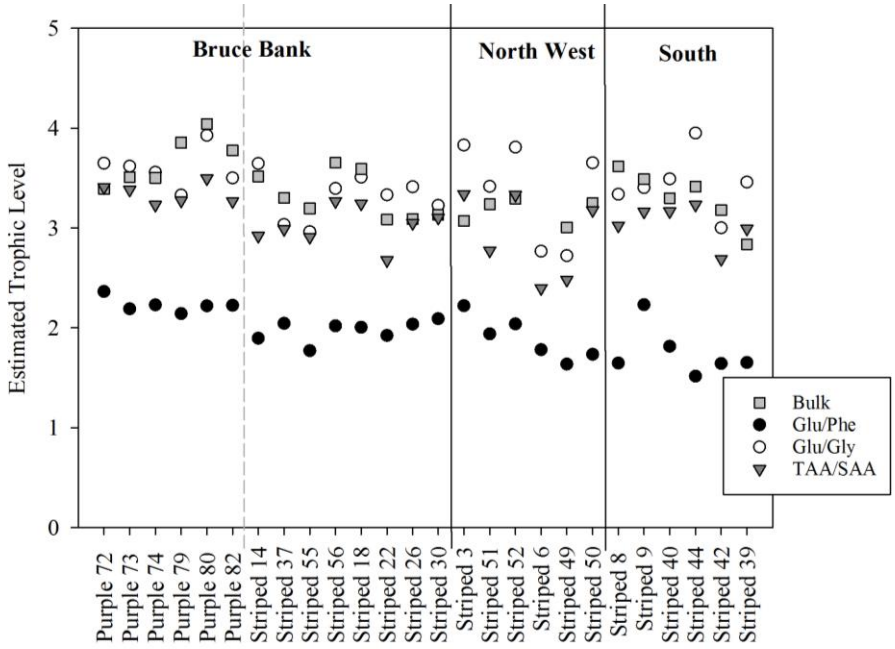


Figure 6-9 Estimated trophic level for each specimen using Equations 6-2 to 6-5 where 6-2) bulk, 6-3) Glu/phe, 6-4) Glu/Gly and 6-5) T_{AA}/S_{AA}. Note that bulk $\delta^{15}N$ data was not available for Poly 6 and thus there is no estimates for this Bulk TL with this individual.

A significant interactive effect of site and depth on the bulk trophic level estimates of the striped morphotype was recorded (Table 6-9, Figure 5-9). This was a result of the increase in bulk trophic level estimates with depth at Bruce Bank and a decline in TL with depth North West and South sites, reflecting the differences recorded in the bulk values $\delta^{15}N$. Across depths there was a significant difference in the trophic levels from the $TL_{Glu/Gly}$ estimate, which declined at each station. The noticeably higher $TL_{Glu/Phe}$ estimate for striped individual 9 was associated with higher trophic amino acid values. This was a result of the increase in bulk trophic level estimates with depth at Bruce Bank and a decline with depth North West and South sites.

	Purple vs Stripe
TL_{Bulk}	**$F_{(1,12)} = 7.61, P = 0.017$**
$TL_{Glu/Phe}$	**$F_{(1,12)} = 26.36, P < 0.001$**
$TL_{Glu/Gly}$	**$F_{(1,12)} = 5.72, P = 0.030$**
$TL_{TAA/SAA}$	**$F_{(1,12)} = 9.89, P = 0.008$**

Table 6-8 One-way ANOVA results bulk, individual and trophic amino acid trophic levels between the purple and striped polynoid morphotypes collected from Bruce Bank. Significant values in bold type.

	Site	**Depth**	**Site*depth**
TL_{Bulk}	$F_{(2,14)} = 1.13, P = 0.353$	$F_{(1,14)} = 0.33, P = 0.575$	**$F_{(2,14)} = 5.37, P = 0.020$**
$TL_{Glu/Phe}$	$F_{(2,14)} = 2.87, P = 0.090$	$F_{(1,14)} = 4.40, P = 0.054$	$F_{(2,14)} = 1.52, P = 0.253$
$TL_{Glu/Gly}$	$F_{(2,14)} = 0.29, P = 0.754$	**$F_{(1,14)} = 6.71, P = 0.021$**	$F_{(2,14)} = 0.99, P = 0.396$
$TL_{TAA/SAA}$	$F_{(2,14)} = 0.46, P = 0.641$	$F_{(1,14)} = 2.07, P = 0.173$	$F_{(2,14)} = 3.14, P = 0.075$

Table 6-9 Two-way ANOVA results comparing trophic level between site and location from the striped polynoid specimens. Significant values in bold type.

6.4 Discussion

6.4.1 Genetic diversity of polynoid symbionts

The DNA barcodes of the symbiotic polynoids from the South Orkney region were closely related to those of *Polyeunoa laevis* (McIntosh, 1885). *P. laevis,* a described coral symbiont, is recorded to be widely distributed within the Southern Ocean and the southern regions of surrounding oceans (Barnich et al., 2012). The *P. laevis* sequences included in the phylogenetic and distance analyses presented here were collected from individuals found on corals in the South-West Indian Ocean and the Ross Sea (Heimeier et al., 2010, Serpetti et al., 2016). Given the K2P and geographic distance between the South-West Indian specimens and those from the South Orkneys, it could be that these populations are isolated from one another and thus we consider the South Orkney morphotypes to be *Polyeunoa* cf. *laevis*. The potential for distinct lineages and high variability of this morphospecies was previously put forward by Barnich et al. (2012) and Alvaro et al. (2014).

There were two distinct morphotypes of the South Orkney *Polyeunoa* cf. *laevis* specimens easily identifiable by their colour. The occurrence of more than one colour pattern in a single taxon can indicate more than one species of polychaete present (e.g. Nygren et al., 2005) and Pleijel et al. (2009)). Although there was genetic variation between the purple and striped morphotypes in this study, they were not deemed to be separate species. Currently there are no rules or threshold values in the determination of species by genetic difference. In previous genetic analyses of polychaetes, genetic variation of 5% was enough to discriminate between sympatric cryptic species using COI (Paxton and Åkesson, 2010, Wiklund et al., 2009). However genetic level species discrimination is also associated with a distinct barcoding gap (Hebert et al., 2003, 2004, Meyer and Paulay, 2005). Here we find considerable overlap in the genetic variation between and within morphotypes and the 'barcoding gap' is minimal or non-existent. Furthermore various colour morphs have been reported (Alvaro et al., 2014) and were included in the redescription of *Polyeunoa laevis* (Barnich et al. (2012), Sequence matches with larvae from the Ross Sea suggest that at least the striped clade may be widely distributed within the Western Antarctic (Heimeier et al., 2010).

Colour morphisms have been recorded in other polychaete taxa (Pleijel et al., 2009, Nygren et al., 2011). For example, *Harmothoe imbricata,* a well distributed species recorded from the Arctic, the North Pacific, and the North Atlantic including the Mediterranean Sea, iss found on various substrates, either free-living, or together with tubicolous polychaetes or pagurids (Barnich and Fiege, 2009). Nygren et al. (2011) sequenced mitochondrial and nuclear DNA of 57 individuals from 10 colour morphs and found no evidence for speciation between morphotypes.

The shared haplotypes between the purple and striped individuals (Poly#11 and 81) suggests there may be interbreeding between the colour morphs. This supports that *P. laevis* contains polymorphs and is not comprised of several species. However given the genetic differences between populations within the South West Indian Ocean, it is possible that this symbiotic association is promoting speciation (e.g. Faucci et al., 2007, Schiaparelli et al., 2015). Within the SOISS MPA region, the absence and reduced numbers of the purple compared to the striped morphotype at locations other than Bruce Bank suggests that there may be some functional difference between these two morphotypes. This could be associated with functional traits that promote increased habitat selectivity within the purple morphotype, reduced larval dispersal or

that the striped morphotype has greater functional flexibility, and/or that the striped morphotype is able to outcompete the purple morphotype.

6.4.2 Trophic trait variability in Polynoidae symbionts

Free-living polynoidae are generally regarded as predator scavenger species (Fauchald and Jumars, 1979). Given the habitat and behvioural differences between free-living and symbiotic species it is possible that symbionts may exhibit very different trophic traits to those described for their family. The SIA and CSIA data from the SOISS MPA region revealed significant differences between the isotopic signatures of the different morphotypes and with location and depth. Morphological and genetic differences between the purple and striped individuals could have functional implications, which may have influenced nitrogen signatures. For example, differences in the jaw structures and/or particle selectivity could influence the particle size ingested, which may well have different isotopic signatures (Fauchald and Jumars, 1979, Hutchings, 1998, Mueller et al., 2013). Diet and selectively could be linked to differences in body size, as recorded in other marine taxa. The range of prey sizes consumed can be related to predator body size (Scharf et al., 2000). The dry weight of each specimen was recorded prior to SIA analysis as a proxy for body size. No significant difference was found in the dry weight between the two morphospecies ($F_{(1,12)} = 0.13$, $P = 0.722$).

Nitrogen signatures can vary with location and can be associated with surface water nitrate availability, nutrient cycling, primary productivity or other processes that influence nitrogen chemistry on regional spatial scales (Sigman et al., 1999, 2000, Stowasser et al., 2012, Ward et al., 2012). Samples collected from different regions of the Southern Ocean within the same sampling programme have varied by 10‰ (Schmidt et al., 2003). Depth dependent $\delta^{15}N$ trends are often attributed to bacterial action, whereby the biochemical processes during bacterial degradation result in the release of ^{14}N and thus an enriched $\delta^{15}N$ value of the residual material (Saino and Hattori, 1980, Macko and Estep, 1984, Macko et al., 1986, Wada et al., 1987). The increase in $\delta^{15}N$ POM values between 0 and 1000 m depth ranges from 5 to 10‰ (Altabet and Francois, 2001, Biggs et al., 1988, Rau et al., 1991, Saino and Hattori, 1980). Such factors should be considered when determining the trophic traits of deep-sea organisms (Mintenbeck et al., 2007). Thus, the enrichment in $\delta^{15}N$ at Bruce Bank with depth could well be a result of microbial activity but this would not explain the depletion in $\delta^{15}N$ at the other locations. Anomalous depletion in $\delta^{15}N$ may be related to local biogeochemical processes such as nitrogen fixation (Peters et al., 1978, Anderson and Fourqurean, 2003). If present, the effects

of nitrogen fixation by cyanobacteria could vary spatially (Ambrose, 1991). The combination of both site and depth dependent differences could have resulted in the significant interactive effects on $\delta^{15}N$ signatures.

Isotopic signatures may also vary with host species; the polynoid symbionts were collected from multiple hosts, a trait observed in other symbionts in Antarctica (Schiaparelli et al. 2011) and polynoid coral symbionts (Buhl-Mortensen and Mortensen, 2004). If the hosts are selective feeders i.e. by particle size, the POM available for the polynoid symbionts may differ between hosts (Shimeta and Koehl, 1997, Buhl-Mortensen et al., 2001, Mueller et al., 2013). At Bruce Bank all of the purple individuals were collected from *Acanthogorgia* hosts, whilst the striped individuals were mostly collected from other octocoral species (Table 6-1). In general octocorals are considered to share feeding behaviours, capturing particulate organic matter and zooplankton in by the tentacles and pinnules (Lewis, 1982). However, observations of polyp activity have also shown that even the same coral species in the same area may have different feeding responses or behaviours (Lin et al. 2002). Thus different octocorals may capture or select different prey items which could be reflected in the symbiont diet if, they are feeding on the host captured or unselected material. Additionally, it is uncertain whether the polynoids are feeding directly on the corals. Differences in the host's diet as well as skeletal compositions will influence the isotopic signatures of the host (Williams and Grottoli, 2010), thus if the symbiont is feeding on the host this would also be reflected in their isotopic signatures.

The trophic level estimates need to be interpreted with care. The equations are based on POM data from a different year and trophic enrichment factors not specific to this species (Popp et al., 2007, Chikaraishi et al., 2009, Stowasser et al., 2012, McMahon and McCarthy, 2016). Three of the four trophic level estimates suggest that the polynoids are omnivorous potentially feeding on both POM as well as other organisms that may come into contact with, or live on the coral. The lower trophic estimates from the Glu/Phe equation suggest that the polynoids could be more herbivorous ingesting POM which settles on the host species. With minimal knowledge of the source material and technical difficulties with the GC/C/IRMS discussed in Chapter 5, it is difficult to determine which may be the most reliable. Defining a trophic level also implies that the benthic food web is a linear system. In reality it is a complex web of interactions highly influenced by many seasonal, regional and biological factors (Iken et al., 2001, Stowasser et al., 2012). Insight from observations, previous studies and the SIA and CSIA data presented here may be more useful in defining this relationship.

The significant differences in total nitrogen, $\delta^{15}N$ signatures and trophic level estimates between morphotypes and with depth and location within the striped specimens were variable. For example, significant differences were recorded between morphotypes for total nitrogen, $\delta^{15}N_{Bulk}$ and TLs but not all amino acids. For the striped specimens significant interactive effects of site and depth were recorded in $\delta^{15}N_{Bulk,}$ TL_{Bulk} as well as leucine and serine and, a significant effect of depth on $TL_{Glu/Gly}$. The significant differences observed in bulk data compared to amino acid data could indicate the higher resolution of compound specific methods preventing false positives. Otherwise it may be related to the relative abundance of each amino acid and their proportionate contribution to bulk ratios (Hayes, 2004, Styring et al., 2010). It should also be noted that the use of $p = 0.05$ as a significance level has recently been questioned. Colquhoun (2014) suggests that to keep false discovery rates below 5% a $p \leq 0.001$ should be used. If this approach were to be taken only one result would still be considered 'significant'. For this chapter, given the potential factors that could influence trophic traits the variability is embraced but caution taken when considering the 'significance' of these data.

6.4.3 Classifying symbiotic relationships

The abundance of deep-water coral invertebrate symbionts is considered to be greater than for that of shallow-water species (Roberts, 2005, Mueller et al., 2013). This difference is probably associated with the decreasing food availability with depth, which leads to increased ecological benefits for a symbiont. Similar suggestions have been made for the occurrence of symbioses at high latitudes as food webs may be simplified as well as food limited and thus the physiological benefit to the symbiont is greater (Schiaparelli et al., 2010). To determine the extent of their importance requires an understanding of the functional relationships between the host and its symbiont. Even within relatively well-studied symbioses research suggests both mutualistic and parasitic relationships for the same species (Buhl-Mortensen et al., 2001, Roberts, 2005).

The results presented within this chapter represent the first biochemical investigation into the functional relationship between polynoid symbionts and their coral hosts within the Southern Ocean. Although stable isotopes are widely used in both marine and terrestrial ecology to define trophic relationships, CSIA has only recently been used to determine the symbiotic relationships in terrestrial ecology (Sabadel et al., 2016). In comparison to the free-living polynoid species studied in Chapter 5, the trophic signatures indicate that the symbiotic polynoids show a preference for herbivory over omnivory. Given their lifestyle traits, and the

more depleted amino acid signatures i.e. at the lower end of the free-living O/P/S range, it is likely that their primary food source is organic matter that been settled on the coral host. Whether this organic matter is intercepted or stolen from the host species is impossible to tell but could affect the relationship between the host and symbiont. However, without more detailed biochemical analysis of the source amino acids and/or isotopic signatures of the POM it is not possible to rule out that they predate or scavenge on organisms that come into contact with their coral hosts or the polyps of the host itself.

It is not possible to determine the impact of the relationship on the host species from the present data, although there may be some benefit by 'cleaning', or predation by the symbionts, as observed between scale worms and *Lophelia* (Roberts, 2005). This would imply that the symbioses between polynoids and their host corals are either mutualistic or commensal. The polynoids in this study may be able to survive in the absence of their host; if so the species are facultative symbionts. These findings conform with the conclusions of Serpetti et al. (2016) which deemed the closely related polynoid, *P. laevis*, to be a facultative commensal species. With certainty, the polynoids are a polyxenous species, found on at least 11 coral hosts within the SOISS MPA region.

6.4.4 Data implications for marine management of the SOISS MPA

The genetic analyses and morphological observations presented here show high genetic variability within the SOISS MPA for this species. It is difficult to establish whether this is a reflection of genetic diversity in other free-living species. However, this region has been recorded as an area of high genetic diversity (Linse et al., 2007, Allcock et al., 2011, Brasier et al., 2016). Genetic similarity between striped specimens from different locations and depths suggests that the populations within the MPA and its vicinity are connected. The connectivity of populations could be maintained, both by larval biology and oceanographic currents aiding dispersal as well as other populations within the area (Young et al., 2015). This is important when assessing the likely supply and retention of larvae within and around the MPA and the recovery of damaged habitats and/or population loss through fishing activities.

Given their abundance and their structuring role in marine communities, symbiotic associations are of functional significance to marine ecosystems (Hay et al., 2004). Other symbiotic relationships observed within the SOISS MPA include holothurians and bivalves found on the spiny urchin *Sterechinus neumayeri*. Given the abundance of symbiotic species within the

SOISS MPA and other areas of the Southern Ocean within designated and proposed MPA domains (CCAMLR, 2016, Teschke et al., 2013), they should be considered and included in environmental assessments regarding the impacts of human activity on benthic ecosystems. Special attention should be placed on impact assessments using only camera-based surveys where symbiotic species may be overlooked. As described in Brasier et al. (2017) symbiotic species were not visible from downward facing camera surveys but certainly would be impacted by trawling and longlining activities. Furthermore, the recovery time of the symbiont needs to be taken into consideration, especially if the symbiont is obligate as this may be longer than for free-living species because of the host population would need to recover first (Buhl-Mortensen and Mortensen, 2004).

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7 Synopsis

7.1 Summary of findings and implications

The work presented in Chapters 2 to 6 has addressed the key aims of this thesis, which were to:

- Compare, using a subsample from a large Antarctic sample set, the number of polychaetes species obtained from morphological and genetic analyses
- Analyse the distribution of genetically distinct populations and cryptic species of polychaetes
- Determine the effects of different preservation methods on the $\delta^{15}N$ bulk and amino acid signatures of polychaetes using *Arenicola marina*
- Define the trophic traits of polychaetes using stable isotope analysis and how these might vary with depth and location

The key findings of this thesis and their contributions to current research are explained within this section (7.1) whilst revisiting the overarching hypotheses. In sections 7.2 to 7.5 the topics covered in this thesis are discussed within a wider ecological context with regard to diversity studies, trait based research, and future environmental change, as well as marine exploitation and management within the Antarctic.

7.1.1 Species diversity of Antarctic polychaetes

Early studies proposed that there would be a decline in the number of species with increasing latitude (Rosenzweig, 1995, Brown and Lomolino, 1998, Lomolino, 2001). However, as a result of increased benthic sampling and species documentation in the Southern Ocean, the level of benthic diversity within Antarctica is considered comparable to that of lower latitudes (Brandt et al., 2007, De Broyer et al., 2014). Representing almost 10% of the world's oceans, the Southern Ocean contributes hugely to global biodiversity, and by defining trends in Antarctic diversity, we increase our ability to assess global levels (Convey et al., 2014, Chown et al., 2015). Additionally, via deep-water connections, the Southern Ocean may serve as a 'diversity pump'. The formation of Antarctic deep water in the Weddell Sea could act as a dispersal mechanism driving fauna northward into neighbouring oceans (Brandt, 2005). At present, a major factor in the ever increasing number of species documented in Antarctica is

the abundance of newly discovered cryptic species (Grant et al., 2011). The glacial history of Antarctica allowed for the isolation of populations during glacial maxima, promoting speciation and driving enhanced cryptic diversity.

Within Chapter 2, the use of DNA barcoding following morphological analyses found a 233% increase in the number of potential polychaete species from 15 to 35. In 10 cases this was a result of potential cryptic species or genetically distinct clades. The remaining 10 additional species were considered to be different morphospecies previously overlooked or undescribed during the original identifications. Although the determination of species is complicated with no genetic cut-offs or universal rules, the results clearly show that the resolution of polychaete diversity studies can be greatly increased by incorporating genetic methods of identification (Nygren, 2014). These results also suggest that the first hypothesis of this thesis is true:

> *Hypothesis 1: As a result of cryptic species, estimates of Antarctic polychaete diversity are greater from combined morphological and genetic analysis than morphological analysis alone.*

However, as shown in Chapter 6, DNA barcoding may also uncover polymorphisms and subsequently reduce the number of species. The polynoids collected within the South Orkney Island Southern Slope (SOISS) Marine Protected Area (MPA) were identified to two distinct morphospecies then sequenced using the same barcoding methods as described in Chapter 2. Comparison to DNA barcodes within the GenBank database indicated that these species were closely related to another polynoid symbiont from the Southwest Indian Ridge (Serpetti et al., 2016). However the lack of a 'barcoding gap' between the two polynoid morphospecies meant that they were considered to belong to the same species. Thus DNA barcoding can equally identify polymorphic species and prevent an overestimation of diversity (Nygren et al., 2011).

7.1.2 Biogeography of Antarctic polychaetes using DNA

The glacial history, oceanography and benthic topography of the Southern Ocean may have, and still does influence the population connectivity of marine species. The impact of Antarctica's glacial history on the extinction or survival of species either as shelf refugia or deep-sea colonisers has resulted in biogeographic structuring of the Southern Ocean benthos (Pierrat et al., 2013). Likewise, the circumpolar current system, including the ACC and its counter current, may have also influenced species dispersal and connectivity. Since the introduction of molecular techniques and the identification of cryptic species, few taxa appear

to be truly circum-Antarctic. Most studies have revealed multiple species or lineages living within more restricted ranges than their original morphospecies.

In Chapter 3, the DNA barcodes of 23 of the genetically identified species in Chapter 2 were used to assess their distribution and, if available, compare their distribution with that described from morphological taxonomic records. Using the definition in Schüller and Ebbe (2014) the majority of species appear to be circum-Antarctic, collected from at least three regions within the Southern Ocean. Only one species, *Aricidea beligcae,* a cryptic species, was observed to be geographically separated, and this result may be an artefact of under-sampling. Most cryptic species existed sympatrically although in some cases one clade appeared restricted to certain regions whilst other clades were more widespread. Family level trait data of the widespread and potentially circum-Antarctic species suggest that many of them may have pelagic larval stages (Faulwetter et al., 2014). With dispersal facilitated via the ACC and its counter current, such traits may have promoted their widespread distributions within Antarctica (Brasier et al., 2017b). As the majority of cryptic species investigated in this study were sympatric and widespread within the West Antarctic, the second hypothesis of this thesis is rejected:

> *Hypothesis 2: Genetically divergent or cryptic clades have geographically isolated from one another with more restricted distributions than their original morphospecies.*

With more sequences and additional phylogenetic investigation, statistical analyses could be conducted to determine the level of connectivity between populations. Although the insights from Chapter 3 were useful in assessing species distributions, with the limited number of specimens from each species ($n < 10$ in most cases), it was not possible to estimate population connectivity. As found in cephalopods, even closely related species with comparable life histories and similar patterns of genetic differentiation over large geographic scales can have contrasting genetic patterns over regional scales (Strugnell et al., 2017). Many unique haplotypes were found at the Scotia Arc (e.g. Brandão et al., 2010, Allcock et al., 2011, Brasier et al., 2017b), and this area has been recorded as a location of potential diversification (Linse et al., 2007). A combination of population statistics, larval trait data and oceanographic modelling could provide a very powerful tool to aid us in the understanding of Antarctic biogeography, but for the majority of species and taxa, it is the lack of data that prevents reliable results and interpretation (Young et al., 2015, Strugnell et al., 2017).

7.1.3 Defining trophic traits of Antarctic polychaetes using stable isotope analysis

Following method development on non-Antarctic polychaetes, the use of compound specific stable isotope analysis (CSIA) provided insight into the trophic traits and variability in the isotopic composition of benthic Antarctic polychaetes (Chapters, 4, 5 and 6). This is considered to be one of the first applications of CSIA to define the trophic traits and trophic levels of any organism in the Antarctic benthos. Despite the successful use of CSIA in pelagic systems and published methodologies, there are several caveats in the collection of $\delta^{15}N$ values of source amino acids, and thus the calculation of trophic position. To estimate trophic level using CSIA data requires reliable source amino acid data and a trophic enrichment factor reflective to the fractionation of amino acids within the target taxa (McMahon and McCarthy, 2016). The trophic enrichment factor cannot be calculated without organic source material, which was not available in this study. Therefore, the estimated trophic positions in Chapter 5 and 6 should be interpreted with caution.

High variation was recorded in the CSIA signatures of free living polychaetes in Chapter 5, which could have been related to multiple factors reflecting the complexity of benthic biological systems. In general, the SIA and CSIA results in Chapters 4, 5 and 6 did reflect the categorical trophic traits of their families with detectable differences between categorical feeding traits and evidence of omnivory within species. Within the data presented it is unclear if the trophic variation was related to depth or location and thus at this point it is not possible to accept or reject the fourth hypothesis. In Chapter 6 there were significant differences in some of the $\delta^{15}N$ signatures with depth and site, subsequently there is both evidence for and against the third hypothesis:

> *Hypothesis 3: The SIA and CSIA trophic traits of Antarctic polychaetes reflect that of their categorical feeding traits but vary with depth and location.*

It is also unclear whether trophic variation could be related to cryptic diversity. With limited samples of cryptic specimens and the need to combine individuals to obtain enough tissue for CSIA, any differences between cryptic species could not be tested statistically (Chapter 5). In Chapter 6 significant differences were recorded between $\delta^{15}N$ signatures from the distinct polynoid morphotypes, however the lack of genetic differentiation between morphotypes deemed them the same species. Further investigation would be needed to test the fourth

hypothesis and understand the relationship between genetic or morphological differentiation and functional diversity under the influence of multiple environmental variables.

> *Hypothesis 4: Genetic diversity is reflected in functional diversity, i.e. cryptic species have different isotopic signatures.*

7.1.4 Contribution to Antarctic research

A combined total of 354 COI and 16S DNA barcodes collected in Chapter 2 were deposited in GenBank upon the publication of Brasier et al. (2016). These data will now be available for public use in any future diversity, phylogenetic or biogeographic study. The implications of using DNA to identify Antarctic polychaetes were also discussed in Neal et al. (2017), a large scale analysis of polychaete diversity within the BIOPEARL regions. Taxonomic contributions have also come from these DNA analyses; two species of *Macellicephala* which were discovered in Chapter 2 have been described by Neal et al. (In Review). From the biogeographical analysis, a selection of the species haplotype networks presented in Chapter 3 were used in conjunction with particle tracking analyses to help explain the influence of the circum-Antarctic current systems on Antarctic larval dispersal and species distribution in Brasier et al. (2017b).

The SIA and CSIA *Arenicola marina* dataset in Chapter 4 will contribute to a methods publication discussing the collection of $\delta^{15}N_{AA}$ data from benthic organisms, Jeffreys et al. (In Prep). The CSIA data from the free living Antarctic polychaetes in Chapter 5 will be submitted for publication with the polynoid symbiont data to *Antarctic Science*, Brasier et al. (In Prep). The genetic and CSIA analysis of the polynoids within the SOISS MPA will also contribute to the SOISS MPA review and CCAMLR reports, Brasier et al. (2017a). All SIA and CSIA will also be deposited within the SO DIET database upon publication of the manuscripts in preparation.

7.2 Species diversity *vs.* functional diversity

This thesis aimed to understand diversity on several levels, from the number of species (Chapter 2) to the diversity of trophic traits within the same taxa (Chapter 5). This reflects current trends in the literature from the traditional taxonomic diversity studies to whole ecosystem functional analysis (Bremner et al. 2003, Bremner, 2008). Historically, measures of diversity have had a taxonomic focus, revolving around the number of species within a

particular habitat or environment. However, in reality, diversity can be measured on many scales, from genes to ecosystem services. From an ecological perspective, species have various functional traits that determine when and where they can exist, how they interact with individuals from other species, and their ecological 'role' within their environment (McGill et al., 2006). Traits can be defined as a measureable feature of an individual that could affect its performance or fitness (Chown, 2012). Examples of traits measured in marine species include physical (e.g. body size), feeding guild (e.g. photosynthetic, chemosynthetic, heterotrophic etc), behavioural (e.g. scavenging), temporal or phenological (e.g. pelagic vs non-pelagic larval stages; (Marshall et al., 2006)). Traits that influence environmental tolerances and habitat selection can affect species distributions. Traits including trophic interactions and competition can affect species relationships, and traits that directly affect nutrient cycling, bioturbation activities or reef building can contribute directly to ecosystem function via physical and chemical processes. With the increased focus on trait research and expanding trait databases, how ecologists measure diversity is changing (Fukami et al., 2005, Tyler et al., 2012, Stuart-Smith et al., 2013). Ecologists can now quantify trait variation or multivariate trait differences within a community, generically referred to as 'functional diversity' (Cadotte et al., 2011). Although this thesis did not include a full-scale collection of functional trait data or a quantification of 'functional diversity', the collection of isotopic signatures and the calculation of trophic level can still be discussed with relevance to the potential use of functional trait data in Antarctic research, conservation, and management.

A recent workshop held by the Research Programme Antarctic Thresholds – Ecosystem Resilience and Adaptation of the Scientific Committee on Antarctic Research (SCAR) discussed the challenges in identifying and applying cross-disciplinary approaches in the Antarctic. It was highlighted that knowledge on species-specific traits and environmental requirements is essential for most, if not all, approaches to assess the thresholds and responses of species, as well as the resilience of ecosystems to environmental change. "Understanding the impacts of Antarctic climate change on marine and terrestrial organisms ultimately depends on understanding the specific tolerance of species to changes in their current environment. To define where and when organisms will first experience conditions that threaten their future persistence therefore requires intimate knowledge of species traits and their tolerance" (Gutt et al., 2017). Further discussion on the priorities for future Antarctic research is included in Section 7.5.

Variable amino acid signatures and trophic levels were recorded within trophic groups and species. Earlier work had deemed many species of Antarctic benthic invertebrates, as well as polychaetes from other regions, to be 'omnivorous' (Fauchald and Jumars, 1979, Knox, 1994). The widespread use of this term could lead to the interpretation that diets were unknown, rather than the species were feeding on a variety of prey species. The trophic signatures presented in Chapter 5 reinforce the high degree of omnivory amongst benthic polychaetes. However, in Chapter 6, the subtle differences between individuals within the same symbiotic species of polynoidae shows that in some cases, species are selective feeders. Despite the caveats, defining the trophic traits of species using SIA and CSIA can provide evidence of the potential for more flexible diets. These data could be useful in future trait analysis and determining the functional roles of different species when combined with categorical trait data. Understanding the species-specific traits is essential when considering, predicting or modelling ecosystem response to changing climatic conditions.

7.3 The role of diversity in 'ecosystem function' within changing benthic environments

Within Chapters 5 and 6 the importance of functional studies to understand the role of diversity within marine ecosystems was introduced. The term 'ecosystem function' has been used loosely and broadly within the literature under varying or limited definitions (Hooper et al., 2005, Petchey and Gaston, 2006, Bremner, 2008). Considered an 'umbrella term' for the operating of an ecosystem (Loreau, 2008), ecosystem function encompasses various physical, chemical and biological factors. For example, as defined in Bremner et al. (2006), 'ecosystem function' can be defined as a process (e.g. nutrient cycling) and properties (e.g. stability) of ecosystems, or the flow of energy and materials through the abiotic and biotic components of the ecosystem'. Understanding the role of diversity in ecosystem function is normally conducted using experimental conditions, modelling or multivariate trait analysis.

Across global ecosystems there are an increasing number of changes in species diversity, and we are currently struggling to address the problem of biodiversity loss (Tittensor et al., 2014). In Antarctica changes in species diversity and distribution have been predicted and observed as a result of changing environmental conditions (Barnes et al., 2009, Chown et al., 2015, Griffiths et al., 2017). Within Antarctica the abundance of benthic marine fauna lacking a planktonic larval phase may also impact the ability of species to migrate to suitable habitats

(Marshall et al., 2012). Either way, the impact of a decline in diversity through migration or local extinction will depend on the functional importance of the particular species lost. When modelled, the effect of species loss on bioturbation under different extinction scenarios (random extinction, by size, by rarity and sensitive species) demonstrated that a loss of diversity was related to a loss of ecosystem function (Solan et al., 2004). However, in reality, extinction is dependent on trait-based probabilities, the extinction driver, and interacting ecological factors (Naeem, 2006). Furthermore, under changing conditions there may not be a loss in diversity but a change in species composition. This may or may not impact ecosystem function depending on the functional traits of the 'old' and 'new' species (Ieno et al., 2006, Hewitt et al., 2008, Solan et al., 2008). In some cases non-native species could enhance secondary production and have a positive effect within ecosystems through, for example, elevated filtration, nutrient cycling and increased biogenic structures (Ruesink et al., 2006). However, in Antarctica king crabs are reinvading shallower habitats, resulting in the introduction of 'new' predatory traits, which can lead to community imbalance and could ultimately impact ecosystem functioning (Smith et al., 2012), discussed in Section 7.4.

Quantifying ecosystem function is a developing area of research that can provide useful insight into the potential changes that may occur within marine environments. Species specific traits will have an important role in determining any future community or ecosystem change within the Antarctic benthos (Peck, 2002, Peck et al., 2009). However, this will be compounded by indirect food-web effects if there are changes in the overlying primary production (Norkko et al., 2007). Changes in phytoplankton blooms will have cascading effects on both pelagic and benthic food webs, and therefore must be incorporated when considering the vulnerability or stability of benthic communities (Gutt et al., 2017).

7.4 Impacts of climate change on Antarctic food webs

This thesis investigated the use of stable isotopes to determine the trophic traits of Antarctic polychaetes. As well as understanding current ecosystem function, the data provided insight to the trophic flexibility of these taxa. Under predicted future scenarios the indirect impacts of climate change as well as the exploitation of Southern Ocean fisheries will impact the Antarctic benthic communities. Predicted climate driven changes at the base of the food web, the removal of species by fisheries and species invasions under warming conditions will impact both community structure and function (Gutt et al., 2017, Pinnegar et al., 2000, Schofield et al., 2010).

The majority of benthic communities within the Southern Ocean are fuelled by export primary production. As previously discussed, the food web dynamics of the Southern Ocean are inextricably linked to sea ice conditions, which affects the timing and productivity of phytoplankton blooms and the primary food sources available to higher trophic levels (Norkko et al., 2007). Some reviews have suggested that, at present, interannual variability in ocean productivity and the relatively short duration of time series data, limit our ability to recognise or interpret long term trends (Henson et al., 2010). However, global changes in ocean productivity are predicted under future climate change scenarios, due to its impact on physical oceanography and biogeochemical cycling (Bopp et al., 2001, Gehlen et al., 2006, Hoegh-Guldberg and Bruno, 2010). Projections for the Southern Ocean suggest that a longer growing season (associated with a decline in sea ice) will result in increased primary and export production. For example, a 25% decrease in sea ice cover may result in a 10% increase in primary production as a result of increased photo-synthetically active radiation in an enlarged area of open water (Bracegirdle et al., 2008). Although primary production may increase, the major source of secondary production, krill, may be negatively influenced by changes in sea ice (discussed in section 7.5) and phytoplankton communities (Arrigo and Thomas, 2004, Murphy et al., 2012). To date there have been noticeable shifts from large to small celled algae around the West Antarctic Peninsula, which has had knock on effects for zooplankton communities, with an increase in those more efficient at grazing on smaller cells such as salps and a decline in those favouring larger phytoplankton (krill) (Atkinson et al., 2004, Montes-Hugo et al., 2009).

Away from the sea ice zone at the northern limits of the Southern Ocean, the polar frontal system is an area of regionally high primary production (Moore and Abbott, 2000). Any movement in the position of the frontal system would result in a change in the physical environment but also a shift in the composition and functioning of the local pelagic communities along the frontal zone (Cheung et al., 2009). Although the position of the polar front can vary interannually (Moore et al., 1999), increasing sea surface temperatures are likely to result in a southward shift of the polar front, and the impact to the pelagic system is expected to be severe (Gutt et al. (2017). Again, the dependence of benthic communities on export production is such that any change within the pelagic may impact the underlying benthos, even within the open ocean.

Understanding trophic interactions and trophic cascades within marine habitats is essential for predicting the future dynamics of marine ecosystems under changing conditions (Schofield et al., 2010). Trophic cascades occur where a change in a single component of the food web has consequences on the neighbouring trophic levels. Depending on the altered component, cascades can either be top down or bottom up (Pinnegar et al., 2000). For example, a bottom up cascade could be triggered by a change in the base of the Antarctic food web in terms of timing, intensity and community structure of the phytoplankton bloom. Top down cascades are triggered by the loss or removal of apex predators (e.g. by fisheries) from an environment. To date, trophic cascades within Antarctica have been studied with regard to meso and apex predators (Boveng et al., 1998, Ainley et al., 2006, Ainley et al., 2015). For example, a seasonal cascade in the Ross Sea occurred when a grounded iceberg inhibited krill dispersal that led to a shift in penguin foraging from a krill to silverfish based diet (Ainley et al., 2006).

Using the isotopic composition of seafloor taxa from multiple trophic levels, Norkko et al. (2007) investigated the importance of sea ice and advected primary food sources to the structure of benthic food webs in coastal Antarctica. The study suggested that the seasonal flux of exported primary production was dampened by detrital food sources. Many omnivorous species exhibited a shift from the consumption from freshly produced algal material to detrital matter with changes in proximity to sea-ice cover. The use of detrital food sources was also reported in Mincks et al. (2008) from isotopic investigations of the benthic communities of the West Antarctic Peninsula. The slow degradation of summer bloom-derived phytodetritus formed a sediment ‘‘food bank’’ and the primary supply of organic matter for benthic detritivores. Whilst some selectivity and seasonal variability was recorded at a species level, for example in the deposit-feeding holothurian *Protelpidia murrayi*, at the community scale the seasonal variability in the isotopic composition of particulate organic matter was not reflected in the sediments. These findings suggest that many species within benthic ecosystems may be able to integrate the variability of primary production in the water column above, or are only weakly coupled with specific food sources (Gillies et al., 2013). An overlap in the $\delta^{15}N$ signatures of benthic omnivores and deposit feeders from the Weddell Sea again emphasizes trophic flexibility and a continuum of trophic positions rather than stepwise levels (Quiroga et al., 2014). With a high level of omnivory amongst benthic species recorded within both the literature and Chapter 5, any trophic cascades within the benthos may depend more on the quantity rather than quality of food sources available.

In addition to a change in source material or secondary production, altered predator-prey relationships will occur with species loss and the invasion of "new" species facilitated by changing environmental conditions such as increased temperature. If interacting species respond differently to climatic impacts, the dynamics of their relationship could become unbalanced (Walther et al., 2002). One group likely to alter species interactions are the aforementioned king crabs, which are becoming increasingly abundant in shallow regions of the Antarctic from which they were previously absent (Sahade et al., 2015). With an increase in the abundance of these shell crushing predators, the benthic communities which evolved in their absence could be drastically altered by top-down predatory control (Aronson et al., 2007, Smith et al., 2012, Griffiths et al., 2013). This could result in one or more species becoming rare or disproportionately abundant (Van der Putten et al., 2010). The removal of species by fishing activity may also alter species interactions and the stability of marine food webs (Pinnegar et al., 2000, Baum and Worm, 2009). In Antarctica the rise and management of krill fisheries could have serious implications on many non-commercial marine species (Constable et al., 2000, Trathan and Agnew, 2010).

7.5 Future of Antarctic marine living resources and marine management

The management and monitoring of Antarctic marine resources was touched on in Chapter 6. The impact of fishing activity on the future of Antarctic ecosystems is as important as the impacts of climate change. A change in fishery production, catch quotas and marine protection will have many impacts on non-commercial species.

The productivity of any fishery is limited by its primary food sources, and thus any change in primary production, including regional shifts or a decline in productivity, will need to be considered when managing catch limits and maintaining sustainable fishing effort. An additional complication in both Antarctic and Arctic fisheries is the close coupling of primary productivity with sea ice cover. In the Arctic, significant changes within the pelagic food web have already been observed (Trembley and Gagnon, 2009). These observations are a likely result of a change in sea ice dynamics and are predicted to impact ecosystem services related to natural resources and fish stocks (Post et al., 2009). Within Antarctica, the recruitment of commercially important krill species is driven largely by the extent of winter sea-ice. Krill are dependent on sea ice for reproduction and recruitment, however their distribution also corresponds to their main food source, large diatoms (Murphy et al., 2007, Bernard et al., 2012). Given the predicted changes in sea ice extent and primary production (an increase in smaller

celled species in which krill are inefficient at grazing upon), understanding the impacts of these changes on krill populations is urgent (Flores et al., 2012). If krill fisheries are not managed effectively and stocks are over exploited, this could have a serious impact on Antarctic ecosystems via trophic links (Constable, 2001, Trathan and Agnew, 2010).

Ecosystem modelling is currently being used to estimate the impacts of environmental change on fisheries production. A range of modelling approaches, including size based ecosystem models, are currently being implemented to estimate the biomass and future production of marine communities over the Kerguelen Plateau (a commercially important region in the Indian Sector of the Southern Ocean) under different future scenarios (Blanchard et al., 2012, Melbourne-Thomas et al., 2013). Considerable research effort has been put into ensuring these models capture ecosystem dynamics, particularly for commercially important species such as Patagonian toothfish that are known to forage in both benthic and pelagic zones. Given the complex oceanographic features, community structure and biological interactions of Southern Ocean ecosystems (Murphy et al., 2012, Constable et al., 2014), it is important to evaluate how accurately these models reflect the unique ecosystem properties of the region. Successful applications can then be used for other areas of the Southern Ocean undergoing changing or exploitation.

The management of current commercial fisheries, especially finfish such as Patagonian toothfish and mackerel icefish, can directly impact Antarctic benthic communities. Patagonian toothfish are a large, long-lived deep-water species. The exploitation of this species as a part of the mixed bottom-trawl fishery began in the 1970s around sub-Antarctic Islands (Constable et al., 2000). Long-lining fishing methods were introduced in 1987 and are now the principle fishing method, however, trawl fishing is still active in some areas of the Southern Ocean. Some of the direct effects to the benthos include scraping and ploughing of the substrate, physical removal of species, sediment resuspension, destruction of benthos, and dumping of processing waste (Jones, 1992, Dayton et al., 1995, Pham et al., 2014).

Following physical disturbance, slow growing benthic communities may have long recovery times. The use of Vulnerable Marine Ecosystem (VME) indicator taxa is helping to identify areas that should be protected from bottom trawling. Although there are no established guidelines as to how a VME is quantified in the Southern Ocean, CCAMLR agreed that a minimum of 10 kg of VME taxa over a 1200 m (the length of a longline) would be substantial evidence for a possible VME (Jones and Lockhart, 2011). Research at various locations has

shown that the presence of VMEs are related to bottom type, however, spatial variability on local scales may limit our ability to predict VME locations without biological surveys (Post et al., 2010, Brasier et al., 2017a, Post et al., 2017). If VME 'thresholds' are found by either fishing or research operations, these data should be presented to the CCAMLR Scientific Committee and Commission for VME registry, and steps can then be taken to minimise the impacts of bottom fishing within that area.

The establishment of MPAs in Antarctic waters will assist in maintaining species diversity and ecosystem function. Although management may vary from 100% no take (e.g. South Orkney Islands Southern Slope) to partial closures (Ross Sea), the removal or reduced exploitation and disturbance will protect benthic VMEs. To date the establishment of MPAs within Antarctica has been slow in comparison to the designation of terrestrial regions. This is associated with the inability of CCAMLR nations to agree on their designation (Chown 2015). As discussed in the next section within regard to research, international collaboration, as well as inclusion of policy considerations within interdisciplinary research, should help accelerate the development of future MPAs. For example, by incorporating findings such as genetic evidence for asymmetric migration within the Scotia Arc and potential source locations could be used as evidence to protect marine habitats (Strugnell et al., 2017). Additionally, the multiple drivers of diversity including physical and biological (often species specific) factors highlight the difficulty in managing benthic species and Antarctic regions on single species data.

7.6 Future directions in Antarctic biological research and monitoring

The strong coupling between Antarctic ecosystem function and the physical environment puts Antarctic research at the forefront of important scientific challenges. Applying holistic approaches that combine systematic assessments of key physical predictors and key biota is critical in understanding the ecological consequences of climate change (Gutt et al., 2017). The importance of continuing and improving scientific investigations is constantly being re-emphasized, with significant evidence of climate change both within the research communities and in the global media, including the calving of the Larsen C iceberg (BAS, 2017). The recent discussions within the Antarctic science community have identified some of the highest priority scientific questions for future Antarctic research as a part of the Horizon Scan process (Sutherland et al., 2011), and highlighted clear research themes with a call for inter-disciplinary research and cooperation (Gutt et al., 2017). The outlined research topics from the SCAR Horizon Scan discussed in Kennicutt et al. (2014), (2015) include: atmospheric and global

connections, changes in oceanography, sea ice dynamics, biological systems and anthropogenic impacts. Investigating these topics effectively will require major collaborative research.

Despite successful advances in single disciplines, including genomic research, remote sensing and new remotely operated technologies, the Antarctic research community often lacks cross disciplinary approaches and collaborations (Gutt et al., 2017). By combining the research efforts of different scientific disciplines, we stand the best chance of understanding, monitoring and mitigating the anthropogenic impact on Antarctic ecosystems, Figure 7-1. The importance of inter-disciplinary research was demonstrated within this thesis and corresponding publications. For example, to understand the biogeography of polychaetes oceanographic data was needed, and the interpretation of trophic trait data was limited without knowledge of the nitrogen chemistry and primary production between locations.

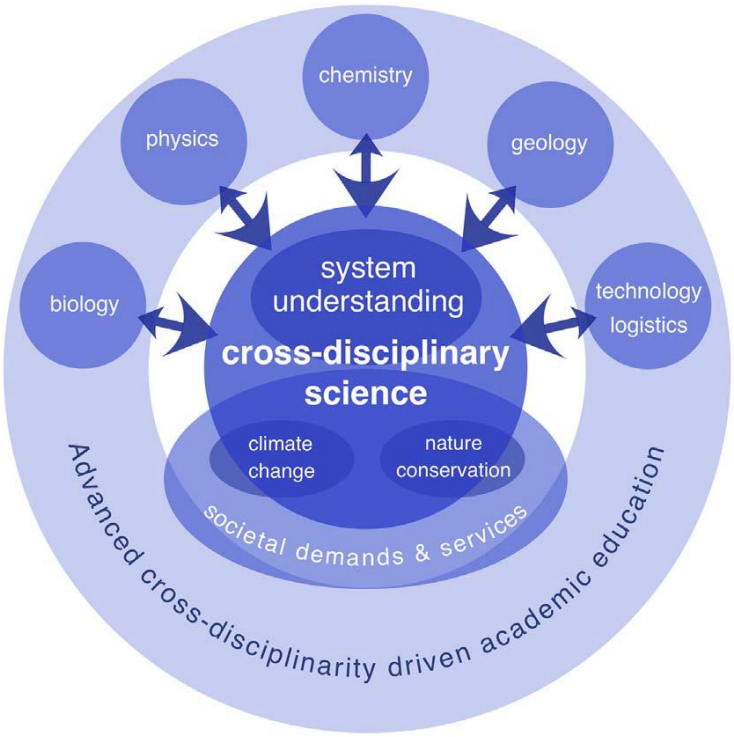

Figure 7-1 Schematic overview of how to achieve advanced cross-disciplinary research. Different scientific disciplines can contribute through cross-disciplinary coordination and management to improved scientific and societal approaches. This strategy includes modern cross-disciplinary academic education. From Gutt et al. (2017).

Priorities for benthic research that can contribute to interdisciplinary analysis include a continuation of basic biological data collection such as taxonomic and diversity information which is still lacking from many regions including the deep-sea (Kaiser et al., 2013, Xavier et al., 2016). To enable the prediction of biological responses to future climatic changes, trait data

such as dispersal, physiological tolerance, feeding ecology, growth and reproduction should also be collected (IPCC 2013). By combining these ecological findings with physical oceanographic data and climate predictions, vulnerable regions can be identified, and once known, such regions can become a focus future sampling to ground truth modelling predictions with in situ observations (Gutt et al., 2017).

From a trophic perspective, and as discussed in previous sections, the understanding of biological change in the waters overlying benthic communities is essential in understanding any cascade or linked impacts. Thus, these data should also be incorporated into the identification of vulnerable habitats. In the majority of ecological studies benthic and pelagic systems have been studied in isolation. Exceptions include several food web analyses in the Antarctic including Mincks et al. (2008) and Smith Craig et al. (2017). The importance of this integration has also been noted in Arctic research by Hobson et al. (1995) and Tamelander et al. (2006). Additionally, modelling studies are beginning to include benthic-pelagic coupling, capturing the energy flux and production across these two interacting systems (Blanchard et al., 2012, Jennings and Collingridge, 2015, Blanchard et al., 2017). With the heightened discussion and brainstorming workshops setting out to influence the course of Antarctic research, there is hope that the scientific community will increase interdisciplinary research efforts. This approach will benefit our scientific understanding, ecosystem monitoring and management and inevitably the future of the Antarctic ecosystem.

7.7 References

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8 Appendices

8.1 Appendix 1

Species identification (ID) information used in Chapters 2, 3 and 5. Including the original ID published in Neal et al. 2017, genetic ID in Brasier et al. 2016 (modification of Chapter 2), sample location, DNA extraction number, GenBank Accession Numbers and, for those specimens not used for CSIA, Darwin Core ID numbers for the Natural History Museum, London (NHM) collection. *specimen used for CSIA, no material available.

Species ID in Neal et al 2017	Species ID in Brasier et al 2016	Sample Location	MB DNA extraction#	GenBank COI Accession#	GenBank 16S Accession#	Darwin core ID for NHM collection
Aglaophamus cf. *trissophyllus*	*Aglaophamus* cf. *tissophyllus* (MBa)	ST-EBS-4-S	259	KX867389	KX867143	*
Aglaophamus cf. *trissophyllus*	*Aglaophamus* cf. *tissophyllus* (MBa)	LI-AGT-4b	279	KX867391	KX867144	*
Aglaophamus cf. *trissophyllus*	*Aglaophamus* cf. *tissophyllus* (MBb)	SG-EBS-5-E	195/387	KX867381	KX867145	*
Aglaophamus cf. *trissophyllus*	*Aglaophamus* cf. *tissophyllus* (MBb)	SG-EBS-5-E	245/390	KX867382	KX867146	*
Aglaophamus cf. *trissophyllus*	*Aglaophamus* cf. *tissophyllus* (MBc)	EI-AGT-2	274	KX867383	KX867147	*
Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB2)	BIO6-EBS-3D	13	KX867384	KX867117	*
Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB2)	LI-AGT-1	282	KX867386	KX867119	*
Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB2)	BIO4-EBS-3A-Epi	20	KX867385	KX867139	*
Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB3)	SG-EBS-3-E	219	KX867387	KX867120	*
Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB3)	BIO5-EBS-2A	240	KX867388	KX867121	*
Aglaophamus cf. *trissophyllus*	*Aglaophamus tissophyllus* (MB)	LI-AGT-4	268	KX867390		*
Aglaophamus cf. *trissophyllus*	*Aglaophamus* cf. *trissophyllus* (MB)	SG-EBS-4-S	209/393		KX867142	NHMUK_BIOPEARL_2018.6562
Aglaophamus cf. *trissophyllus*	*Aglaophamus* cf. *trissophyllus* (MB)	LI-AGT-4	376		KX867140	NHMUK_BIOPEARL_2018.9300
Aglaophamus cf. *trissophyllus*	*Aglaophamus* cf. *trissophyllus* (MB)	LI-AGT-4b	285		KX867141	NHMUK_BIOPEARL_2018.9301
Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB)	BIO3-EBS-1B	261		KX867123	NHMUK_BIOPEARL_2018.9302
Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB)	LI-AGT-4	434		KX867124	NHMUK_BIOPEARL_2018.9303

Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB)	BIO4-EBS-3A	410	KX867125	NHMUK_BIOPEARL_2018.9304
Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB)	JR275-23	444	KX867126	NHMUK_BIOPEARL_2018.9305
Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB)	JR275-45-EBS	463	KX867127	NHMUK_BIOPEARL_2018.9306
Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB)	BIO5-EBS-3D	184	KX867128	NHMUK_BIOPEARL_2018.9307
Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB)	BIO4-EBS-3D	295	KX867129	NHMUK_BIOPEARL_2018.9308
Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB)	BIO4-EBS-3A	316	KX867130	NHMUK_BIOPEARL_2018.9309
Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB)	BIO6-EBS-3A	326	KX867131	NHMUK_BIOPEARL_2018.9310
Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB)	JR275-810	399	KX867132	NHMUK_BIOPEARL_2018.9311
Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB)	JR275-196	415	KX867133	NHMUK_BIOPEARL_2018.9312
Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB)	JR275-91	441	KX867134	NHMUK_BIOPEARL_2018.9313
Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB)	BIO4-EBS-3D-Epi	128/379	KX867135	NHMUK_BIOPEARL_2018.9314
Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB)	BIO4-EBS-3B	447	KX867136	NHMUK_BIOPEARL_2018.9315
Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB)	Jr275-50-EBS	461	KX867137	NHMUK_BIOPEARL_2018.9316
Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB)	LI-AGT-1	272	KX867138	NHMUK_BIOPEARL_2018.9317
Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB2)	BIO4-EBS-3D-Epi	42	KX867118	NHMUK_BIOPEARL_2018.9318
Aglaophamus cf. *trissophyllus*	*Aglaophamus* sp. (MB4)	BIO6-EBS-3E-Epi	46/382/394	KX867122	NHMUK_BIOPEARL_2018.9319
Aricidea simplex	*Aricidea* cf. *beligcea* (MB1)	BIO5-EBS-3B	255	KX867148	*
Aricidea simplex	*Aricidea* cf. *beligcea* (MB1)	BIO3-EBS-1B	258	KX867149	*
Aricidea simplex	*Aricidea* cf. *beligcea* (MB1)	BIO3-EBS-1B	294	KX867150	*
Aricidea simplex	*Aricidea* cf. *beligcea* (MB1)	BIO4-EBS-3D	356	KX867151	*
Aricidea simplex	*Aricidea* cf. *beligcea* (MB1)	BIO5-EBS-2A	361	KX867152	*
Aricidea simplex	*Aricidea* cf. *beligcea* (MB1)	BIO4-EBS-3B-Epi	139	KX867153	*
Aricidea simplex	*Aricidea* cf. *beligcea* (MB1)	BIO5-EBS-2A	385	KX867154	*
Aricidea simplex	*Aricidea* cf. *beligcea* (MB1)	BIO4-EBS-2A	264	KX867155	*
Aricidea simplex	*Aricidea* cf. *beligcea* (MB1)	BIo3-EBS-1B	432	KX867156	*
Aricidea simplex	*Aricidea* cf. *beligcea* (MB1)	BIO5-EBS-2A-Sup	159	KX867157	*

Aricidea simplex	*Aricidea simplex (MB)*	JR275-23	402	KX867165	*
Aricidea simplex	*Aricidea simplex* (MB)	JR275-45-EBS	423	KX867166	*
Aricidea simplex	*Aricidea simplex* (MB)	JR275-40-EBS	457	KX867167	*
Aricidea simplex	*Aricidea simplex* (MB)	JR275-89-EBS	462	KX867168	*
Aricidea simplex	*Aricidea simplex* (MB)	BIO6-EBS-3D	192	KX867169	NHMUK_BIOPEARL_2018.6248
Aricidea simplex	*Aricidea simplex* (MB)	BIO6-EBS-3E	312	KX867170	NHMUK_BIOPEARL_2018.6251
Aricidea simplex	*Aricidea* cf. *pulchra* (MB)	EI-EBS-2-E	337	KX867161	NHMUK_BIOPEARL_2018.6255
Aricidea simplex	*Aricidea simplex* (MB)	EI-EBS-2-E	148	KX867162	NHMUK_BIOPEARL_2018.6256
Aricidea simplex	*Aricidea* cf. *beligcae* (MB2)	LI-EBS-4E	331	KX867159	NHMUK_BIOPEARL_2018.6261
Aricidea simplex	*Aricidea* cf. *beligcae* (MB2)	LI-EBS-4-E	207	KX867158	NHMUK_BIOPEARL_2018.6262
Aricidea simplex	*Aricidea simplex* (MB)	SR-EBS-6-E	126	KX867163	NHMUK_BIOPEARL_2018.6273
Aricidea simplex	*Aricidea* cf. *beligcea* (MB3)	Jr275-50-EBS	406	KX867160	NHMUK_BIOPEARL_2018.9320
Aricidea simplex	*Aricidea simplex* (MB)	JR275-99-EBS-E	284	KX867164	NHMUK_BIOPEARL_2018.9321
Chaetozone setosa	*Chaetozone* sp. (MB2)	BIO5-EBS-3A	224	KX867186	NHMUK_BIOPEARL_2018.6178
Chaetozone setosa	*Chaetozone* sp. (MB1b)	BIO6-EBS-3A	305	KX867182	NHMUK_BIOPEARL_2018.6190
Chaetozone setosa	*Chaetozone* sp. (MB1c)	EI-EBS-1-E	252	KX867184	NHMUK_BIOPEARL_2018.6194
Chaetozone setosa	*Chaetozone* sp. (MB1a)	EI-EBS-2-E	243	KX867180	NHMUK_BIOPEARL_2018.6196
Chaetozone setosa	*Chaetozone* sp. (MB1a)	LI-EBS-4E	189	KX867176	NHMUK_BIOPEARL_2018.6206
Chaetozone setosa	*Chaetozone* sp. (MB1a)	LI-EBS-4S	345	KX867177	NHMUK_BIOPEARL_2018.6207
Chaetozone setosa	*Chaetozone* sp. (MB1c)	PB-EBS-1E	266	KX867183	NHMUK_BIOPEARL_2018.6208
Chaetozone setosa	*Chaetozone* sp. (MB5)	PB-EBS-3-E	262	KX867191	NHMUK_BIOPEARL_2018.6210
Chaetozone setosa	*Chaetozone* sp. (MB1a)	PB-EBS-4-S	238	KX867178	NHMUK_BIOPEARL_2018.6212
Chaetozone setosa	*Chaetozone* sp. (MB1a)	BIO4-EBS-3A	422	KX867175	NHMUK_BIOPEARL_2018.9324
Chaetozone setosa	*Chaetozone* sp. (MB1a)	BIO4-EBS-3B	364	KX867179	NHMUK_BIOPEARL_2018.9325
Chaetozone setosa	*Chaetozone* sp. (MB1a)	BIO5-EBS-3B	214	KX867181	NHMUK_BIOPEARL_2018.9326
Chaetozone setosa	*Chaetozone* sp. (MB2)	JR275-99-EBS-E	401	KX867187	NHMUK_BIOPEARL_2018.9327
Chaetozone setosa	*Chaetozone* sp. (MB3)	BIO4-EBS-3A	228	KX867185	NHMUK_BIOPEARL_2018.9328
Chaetozone setosa	*Chaetozone* sp. (MB4)	BIO6-EBS-2A	311	KX867188	NHMUK_BIOPEARL_2018.9329
Chaetozone setosa	*Chaetozone* sp. (MB4)	BIO6-EBS-2A	202	KX867189	NHMUK_BIOPEARL_2018.9330
Chaetozone setosa	*Chaetozone* sp. (MB5)	JR275-99-EBS-E	287	KX867190	NHMUK_BIOPEARL_2018.9331

Euphrosinella cirratoformis	*Euphrosinella* cf. *cirratoformis* (MB1)	BIO4-EBS-3E-Epi	131		KX867192	NHMUK_BIOPEARL_2018.3373
Euphrosinella cirratoformis	*Euphrosinella* cf. *cirratoformis* (MB2)	BIO3-EBS-1A	14/381		KX867204	NHMUK_BIOPEARL_2018.3837
Euphrosinella cirratoformis	*Euphrosinella* cf. *cirratoformis* (MB1)	BIO4-EBS-3A	322		KX867201	NHMUK_BIOPEARL_2018.3843
Euphrosinella cirratoformis	*Euphrosinella* cf. *cirratoformis* (MB1)	BIO4-EBS-3A	366		KX867196	NHMUK_BIOPEARL_2018.3844
Euphrosinella cirratoformis	*Euphrosinella* cf. *cirratoformis* (MB1)	BIO4-EBS-3B	199		KX867199	NHMUK_BIOPEARL_2018.4032
Euphrosinella cirratoformis	*Euphrosinella* cf. *cirratoformis* (MB1)	BIO4-EBS-3B	249		KX867200	NHMUK_BIOPEARL_2018.4033
Euphrosinella cirratoformis	*Euphrosinella* cf. *cirratoformis* (MB1)	BIO4-EBS-3B	411		KX867197	NHMUK_BIOPEARL_2018.4034
Euphrosinella cirratoformis	*Euphrosinella* cf. *cirratoformis* (MB1)	SR-EBS-4-E	180		KX867193	NHMUK_BIOPEARL_2018.6564
Euphrosinella cirratoformis	*Euphrosinella* cf. *cirratoformis* (MB1)	SR-EBS-4-E	289		KX867194	NHMUK_BIOPEARL_2018.6565
Euphrosinella cirratoformis	*Euphrosinella* cf. *cirratoformis* (MB1)	SG-EBS-4-E	336		KX867195	NHMUK_BIOPEARL_2018.9332
Euphrosinella cirratoformis	*Euphrosinella* cf. *cirratoformis* (MB1)	BIO6-EBS-3D	153		KX867198	NHMUK_BIOPEARL_2018.9333
Euphrosinella cirratoformis	*Euphrosinella* cf. *cirratoformis* (MB2)	BIO6-EBS-2A	265		KX867202	NHMUK_BIOPEARL_2018.9334
Euphrosinella cirratoformis	*Euphrosinella* cf. *cirratoformis* (MB2)	BIO6-EBS-2A	187		KX867203	NHMUK_BIOPEARL_2018.9335
Flabelligena sp. A	*Flabelligena* sp. A (MB)	BIO4-EBS-3D-Sup	24	KX867406	KX867213	*
Flabelligena sp. A	*Flabelligena* sp. A (MB)	BIO4-EBS-3B-Sup	28	KX867407	KX867214	*
Flabelligena sp. A	*Flabelligena* sp. A (MB)	BIO5-EBS-3D	233	KX867410	KX867217	*
Flabelligena sp. A	*Flabelligena* sp. A (MB)	BIO5-EBS-3B	391	KX867411	KX867218	*
Flabelligena sp. A	*Flabelligena* sp. A (MB)	BIO5-EBS-3D	445		KX867219	*
Flabelligena sp. A	*Flabelligena* sp. A (MB)	BIO5- EBS-3A-Sup	10		KX867220	*
Flabelligena sp. A	*Flabelligena* sp. A (MB)	BIO5-EBS-3E Sup	154		KX867221	*
Flabelligena sp. A	*Flabelligena* sp. A (MB)	BIO4-EBS-3E	278		KX867222	*
Flabelligena sp. A	*Flabelligena* sp. A (MB)	BIO4-EBS-3B-Epi	196	KX867409		*
Flabelligena sp. A	*Flabelligena* sp. A (MB)	BIO5-EBS-3A-Epi	185		KX867223	NHMUK_BIOPEARL_2018.6568
Flabelligena sp. A	*Flabelligena* sp. A (MB)	BIO5-EBS-3D-Sup	19	KX867405	KX867212	NHMUK_BIOPEARL_2018.6569
Flabelligena sp. A	*Flabelligena* sp. A (MB)	BIO6-EBS-3A	32	KX867408	KX867215	NHMUK_BIOPEARL_2018.9337
Flabelligena sp. B	*Flabelligena* sp. B (MB)	BIO4-EBS-3D-Epi	129	KX867413	KX867224	*
Flabelligena sp. B	*Flabelligena* sp. B (MB)	BIO5-EBS-3A-Epi	242	KX867414	KX867225	*

Flabelligena sp. B	*Flabelligena* sp. B (MB)	BIO5-EBS-3D	421		KX867226	*
Flabelligena sp. B	*Flabelligena* sp. B (MB)	BIO5-EBS-3E	424		KX867227	*
Flabelligena sp. B	*Flabelligena* sp. B (MB)	BIO5-EBS-3D	212		KX867228	*
Flabelligena sp. B	*Flabelligena* sp. B (MB)	BIO5-EBS-3B	263/380/384		KX867229	*
Flabelligena sp. B	*Flabelligena* sp. B (MB)	BIO4-EBS-3E	253		KX867230	*
Flabelligena sp. B	*Flabelligena* sp. B (MB)	BIO4-EBS-3E	275		KX867231	NHMUK_BIOPEARL_2018.9338
Glycear capitata	*Glycera* sp. (MB1)	SG-EBS-3-E	30	KX867394	KX867234	*
Glycear capitata	*Glycera* sp. (MB1)	BIO6-EBS-3D-Epi	124		KX867235	*
Glycear capitata	*Glycera* sp. (MB1)	JR275-89-EBS	459		KX867237	*
Glycear capitata	*Glycera* sp. (MB1)	BIO6-EBS-3D-Epi	350		KX867238	*
Glycear capitata	*Glycera* sp. (MB1)	SG-EBS-3E	269		KX867239	*
Glycear capitata	*Glycera* sp. (MB1)	JR275-40-EBS	456		KX867241	*
Glycear capitata	*Glycera* sp. (MB1)	JR275-94-EBS	386		KX867242	*
Glycear capitata	*Glycera* sp. (MB1)	SG-EBS-3E	370		KX867243	*
Glycear capitata	*Glycera* sp. (MB1)	SR-EBS-4-E	375		KX867244	*
Glycear capitata	*Glycera* sp. (MB1)	JR275-94-EBS	318		KX867245	*
Glycear capitata	*Glycera* sp. (MB1)	SG-EBS-3E	280		KX867246	*
Glycear capitata	*Glycera* sp. (MB1)	BIO6-EBS-3D-Epi	78	KX867395		*
Glycear capitata	*Glycera* sp. (MB2)	BIO5-EBS-3B-Epi	8	KX867396	KX867247	*
Glycear capitata	*Glycera* sp. (MB2)	BIO4-EBS-3B-Sup	22	KX867397	KX867248	*
Glycear capitata	*Glycera* sp. (MB2)	BIO6-EBS-2A-Epi	64/116	KX867399	KX867250	*
Glycear capitata	*Glycera* sp. (MB2)	BIO5-EBS-2A-Epi	67/118	KX867400	KX867251	*
Glycear capitata	*Glycera* sp. (MB2)	BIO3-EBS-1B-Epi	70/122	KX867402	KX867253	*
Glycear capitata	*Glycera* sp. (MB2)	BIO6-EBS-3D	201	KX867403	KX867254	*
Glycear capitata	*Glycera* sp. (MB2)	BIO5-EBS-3B	227	KX867404	KX867255	*
Glycear capitata	*Glycera* sp. (MB2)	BIO6-EBS-2A-Epi	1		KX867257	*
Glycear capitata	*Glycera* sp. (MB2)	BIO4-EBS-3D	334		KX867260	*
Glycear capitata	*Glycera* sp. (MB2)	BIO6-EBS-3A	327		KX867261	*
Glycear capitata	*Glycera* sp. (MB2)	BIO4-EBS-2A-Epi	69/120	KX867401	KX867252	NHMUK_BIOPEARL_2018.2313
Glycear capitata	*Glycera* sp. (MB1)	EI-EBS-4-S	5	KX867392	KX867232	NHMUK_BIOPEARL_2018.2319

Glycear capitata	*Glycera* sp. (MB2)	BIO4-EBS-1B-Sup	26	KX867398	KX867249	NHMUK_BIOPEARL_2018.2715
Glycear capitata	*Glycera* sp. (MB2)	ST-EBS-1-E	57/142		KX867262	NHMUK_BIOPEARL_2018.2737
Glycear capitata	*Glycera* sp. (MB2)	ST-EBS-1-E	338		KX867258	NHMUK_BIOPEARL_2018.2738
Glycear capitata	*Glycera* sp. (MB1)	EI-EBS-4-E	15		KX867233	NHMUK_BIOPEARL_2018.3049
Glycear capitata	*Glycera* sp. (MB1)	EI-EBS-4-E	53/114		KX867240	NHMUK_BIOPEARL_2018.3050
Glycear capitata	*Glycera* sp. (MB1)	SR-EBS-4-E	51/112		KX867236	NHMUK_BIOPEARL_2018.3516
Glycear capitata	*Glycera* sp. (MB2)	JR275-23-EBS TOT	307		KX867256	NHMUK_BIOPEARL_2018.9339
Glycear capitata	*Glycera* sp. (MB2)	JR275-83	453		KX867259	NHMUK_BIOPEARL_2018.9340
Glycear capitata	*Glycera* sp. (MB2)	JR275-99-EBS-E	286		KX867263	NHMUK_BIOPEARL_2018.9341
Harmothoe fuligineum	*Harmothoe fuligineum* (MB)	SG-EBS-3-E	91	KX867415	KX867264	*
Harmothoe fuligineum	*Harmothoe fuligineum* (MB)	BIO6-EBS-3A-Epi	44	KX867416	KX867265	*
Harmothoe fuligineum	*Harmothoe fuligineum* (MB)	BIO4-EBS-3D	98	KX867417	KX867266	*
Harmothoe fuligineum	*Harmothoe fuligineum* (MB)	BIO3-EBS-1B-Epi	103	KX867418	KX867267	*
Harmothoe fuligineum	*Harmothoe fuligineum* (MB)	BIO4-EBS-3D-Sup	166	KX867420	KX867269	*
Harmothoe fuligineum	*Harmothoe fuligineum* (MB)	BIO5-EBS-3B-Epi	158		KX867270	*
Harmothoe fuligineum	*Harmothoe fuligineum* (MB)	BIO4-EBS-3E	225		KX867271	*
Harmothoe fuligineum	*Harmothoe fuligineum* (MB)	BIO5-EBS-3B-Epi	161		KX867272	*
Harmothoe fuligineum	*Harmothoe fuligineum* (MB)	SG-EBS-4-E	169		KX867273	*
Harmothoe fuligineum	*Harmothoe fuligineum* (MB)	BIO6-EBS-3E	218		KX867274	*
Harmothoe fuligineum	*Harmothoe fuligineum* (MB)	BIO6-EBS-3B	197		KX867275	*
Harmothoe fuligineum	*Harmothoe fuligineum* (MB)	SG-EBS-4-E	320		KX867276	*
Harmothoe fuligineum	*Harmothoe fuligineum* (MB)	BIO4-EBS-3D	330		KX867277	*
Harmothoe fuligineum	*Harmothoe fuligineum* (MB)	BIO4-EBS-3E-Epi	47	KX867278		*
Harmothoe fuligineum	*Harmothoe fuligineum* (MB)	BIO4-EBS-3D Epi	409		KX867268	NHMUK_BIOPEARL_2018.9245
Harmothoe fuligineum	*Harmothoe fuligineum* (MB)	BIO5-EBS-3E-Epi	150	KX867419		NHMUK_BIOPEARL_2018.9342
Hesionidae sp. A	*Hesionidae* sp. (MB)	FT-EBS-1E	358		KX906561	NHMUK_BIOPEARL_2018.2350
Hesionidae sp. A	*Hesionidae* sp. (MB1)	ST-EBS-1-E	200	KX867424	KX906543	NHMUK_BIOPEARL_2018.2353
Hesionidae sp. A	*Hesionidae* sp. (MB1)	PB-EBS-3-E	217	KX867425	KX906544	NHMUK_BIOPEARL_2018.2774
Hesionidae sp. A	*Hesionidae* sp. (MB)	SG-EBS-3-S	203		KX906554	NHMUK_BIOPEARL_2018.2776
Hesionidae sp. A	*Hesionidae* sp. (MB1)	BIO6-EBS-2A-Epi	23	KX867422	KX906541	NHMUK_BIOPEARL_2018.3094

Hesionidae sp. A	*Hesionidae* sp. (MB)	BIO6-EBS-2A	363		KX906557	NHMUK_BIOPEARL_2018.3095
Hesionidae sp. A	*Hesionidae* sp. (MB1)	BIO4-EBS-3A-Sup	93	KX867423	KX906542	NHMUK_BIOPEARL_2018.3546
Hesionidae sp. A	*Hesionidae* sp. (MB2)	BIO4-EBS-3A-Sup	107	KX867430	KX906548	NHMUK_BIOPEARL_2018.3547
Hesionidae sp. A	*Hesionidae* sp. (MB2)	BIO6-EBS-3A	299	KX867433	KX906551	NHMUK_BIOPEARL_2018.3566
Hesionidae sp. A	*Hesionidae* sp. (MB)	SR-EBS-4-E	321		KX906559	NHMUK_BIOPEARL_2018.3591
Hesionidae sp. A	*Hesionidae* sp. (MB2)	SG-EBS-3-E	41	KX867428	KX906547	NHMUK_BIOPEARL_2018.3801
Hesionidae sp. A	*Hesionidae* sp. (MB)	SR-EBS-6E	369		KX906555	NHMUK_BIOPEARL_2018.3807
Hesionidae sp. A	*Hesionidae* sp. (MB2)	BIO4-EBS-3D-Supra	136	KX867431	KX906549	NHMUK_BIOPEARL_2018.9237
Hesionidae sp. A	*Hesionidae* sp. (MB1)	BIO4-EBS-3D Epi	12	KX867421	KX906540	NHMUK_BIOPEARL_2018.9238
Hesionidae sp. A	*Hesionidae* sp. (MB2)	PB-EBS-4-E	9	KX867426	KX906545	NHMUK_BIOPEARL_2018.9239
Hesionidae sp. A	*Hesionidae* sp. (MB2)	BIO5-EBS-3D-Epi	27	KX867427	KX906546	NHMUK_BIOPEARL_2018.9239
Hesionidae sp. A	*Hesionidae* sp. A	SG-EBS-3E	353		KX906552	NHMUK_BIOPEARL_2018.9243
Hesionidae sp. A	*Hesionidae* sp. (MB)	BIO3-EBS-1B	271		KX906553	NHMUK_BIOPEARL_2018.9343
Hesionidae sp. A	*Hesionidae* sp. (MB)	BIO4-EBS-3D	348		KX906558	NHMUK_BIOPEARL_2018.9344
Hesionidae sp. A	*Hesionidae* sp. (MB)	BIO3-EBS-1A-Epi	89		KX906560	NHMUK_BIOPEARL_2018.9345
Hesionidae sp. A	*Hesionidae* sp. (MB)	BIO4-EBS-3A	325		KX906562	NHMUK_BIOPEARL_2018.9346
Hesionidae sp. A	*Hesionidae* sp. (MB2)	BIO5-EBS-3A	222	KX867432	KX906550	NHMUK_BIOPEARL_2018.9347
Hesionidae sp. A	*Hesionidae* sp. A	PB-EBS-4-S	45	KX867429	KX906556	NHMUK_BIOPEARL_2018.9348
Laonice weddelia	*Laonice* cf. *antarctica* (MB)	JR275-23-EBS TOT	308		KX867279	*
Laonice weddelia	*Laonice* cf. *antarctica* (MB)	JR275-23-EBS	454		KX867280	*
Laonice weddelia	*Laonice* cf. *antarctica* (MB)	JR275-45-EBS	420		KX867283	*
Laonice weddelia	*Laonice* cf. *vieitezi* (MB)	BIO5-EBS-3E Supra	179		KX867287	*
Laonice weddelia	*Laonice* cf. *vieitezi* (MB)	BIO5-EBS-3D	328		KX867288	*
Laonice weddelia	*Laonice* cf. *vieitezi* (MB)	BIO5-EBS-3D-Sup	3		KX867289	*
Laonice weddelia	*Laonice weddellia* (MB)	ST-EBS-4S	97	KX867440	KX867295	*
Laonice weddelia	*Laonice weddellia* (MB)	BIO6-EBS-2A-Sup	21	KX867437	KX867292	*
Laonice weddelia	*Laonice weddellia* (MB)	ST-EBS-4E	135	KX867442	KX867297	*
Laonice weddelia	*Laonice weddellia* (MB)	BIO6-EBS-2A	349		KX867300	*
Laonice weddelia	*Laonice weddellia* (MB)	BIO6-EBS-3E	355		KX867301	*

Laonice weddelia	*Laonice weddellia* (MB)	ST-EBS-3-E	397		KX867306	*
Laonice weddelia	*Laonice weddellia* (MB)	JR275-40-EBS	460		KX867307	*
Laonice weddelia	*Laonice weddellia* (MB)	BIO6-EBS-3E-Epi	173		KX867308	*
Laonice weddelia	*Laonice weddellia* (MB)	JR275-99-EBS-E	288		KX867309	*
Laonice weddelia	*Laonice weddellia* (MB)	BIO4-EBS-3B	297		KX867310	*
Laonice weddelia	*Laonice weddellia* (MB)	Jr275-50-EBS	451		KX867311	*
Laonice weddelia	*Laonice weddellia* (MB)	BIO3-EBS-1B-Epi	25		KX867313	*
Laonice weddelia	*Laonice weddellia* (MB)	JR275-99-EBS-E	407		KX867314	*
Laonice weddelia	*Laonice* cf. *antarctica* (MB)	BIO4-EBS-3E	332		KX867282	NHMUK_BIOPEARL_2018.2007
Laonice weddelia	*Laonice weddellia* (MB)	BIO5-EBS-2A	211	KX867444	KX867299	NHMUK_BIOPEARL_2018.2033
Laonice weddelia	*Laonice* sp. (MB1)	BIO6-EBS-2A Epi	177		KX867290	NHMUK_BIOPEARL_2018.2176
Laonice weddelia	*Laonice* cf. *vieitezi* (MB)	LI-EBS-1E	94	KX867285	KX86728	NHMUK_BIOPEARL_2018.2240
Laonice weddelia	*Laonice weddellia* (MB)	LI-EBS-3-S	7	KX867312	KX867312	NHMUK_BIOPEARL_2018.2244
Laonice weddelia	*Laonice weddellia* (MB)	PB-EBS-1-S	130	KX867441	KX867296	NHMUK_BIOPEARL_2018.2245
Laonice weddelia	*Laonice weddellia* (MB)	PB-EBS-4-E	84/119	KX867439	KX867294	NHMUK_BIOPEARL_2018.2250
Laonice weddelia	*Laonice* cf. *vieitezi* (MB)	EI-EBS-1-S	80/115		KX867286	NHMUK_BIOPEARL_2018.2651
Laonice weddelia	*Laonice weddellia* (MB)	ST-EBS-1-E	77/111	KX867438	KX867293	NHMUK_BIOPEARL_2018.2667
Laonice weddelia	*Laonice weddellia* (MB)	SG-EBS-3A	378		KX867304	NHMUK_BIOPEARL_2018.3019
Laonice weddelia	*Laonice weddellia* (MB)	LI-EBS-4S	368		KX867302	NHMUK_BIOPEARL_2018.4116
Laonice weddelia	*Laonice* sp. (MB2)	PB-EBS-3-E	186	KX867436	KX867291	NHMUK_BIOPEARL_2018.4260
Laonice weddelia	*Laonice weddellia* (MB)	PB-EBS-3-S	383		KX867305	NHMUK_BIOPEARL_2018.4261
Laonice weddelia	*Laonice* cf. *antarctica* (MB)	EI-EBS-4-E	29	KX867434	KX867284	NHMUK_BIOPEARL_2018.9236
Laonice weddelia	*Laonice* cf. *antarctica* (MB)	SG-EBS-2-E	90	KX867435		NHMUK_BIOPEARL_2018.9240
Laonice weddelia	*Laonice weddellia* (MB)	SG-EBS-3E	373		KX867303	NHMUK_BIOPEARL_2018.9241
Laonice weddelia	*Laonice weddelia* (MB)	BIO4-EBS-3E Epi	170	KX867443	KX867298	NHMUK_BIOPEARL_2018.9349
Lumbrineris cingulata	*Lumbrineris* sp. (MB)	BIO5-EBS-3E Supra	168		KX867316	NHMUK_BIOPEARL_2018.2366
Lumbrineris cingulata	*Lumbrineris* sp. (MB)	BIO6-EBS-3A	455		KX867318	NHMUK_BIOPEARL_2018.2368
Lumbrineris cingulata	*Lumbrineris* sp. (MB)	ST-EBS-4-S	404		KX867324	NHMUK_BIOPEARL_2018.2383
Lumbrineris cingulata	*Lumbrineris* sp. (MB)	PB-EBS-4-S	151		KX867315	NHMUK_BIOPEARL_2018.2796

Lumbrineris cingulata	*Lumbrineris* sp. (MB)	SG-EBS-4-S	248		KX867328	NHMUK_BIOPEARL_2018.2798
Lumbrineris cingulata	*Lumbrineris* sp. (MB)	SG-EBS-4-S	416		KX867325	NHMUK_BIOPEARL_2018.2799
Lumbrineris cingulata	*Lumbrineris* sp. (MB)	ST-EBS-3b	341		KX867322/KX867323	NHMUK_BIOPEARL_2018.2800
Lumbrineris cingulata	*Lumbrineris* sp. (MB)	BIO4-EBS-3A	438		KX867330	NHMUK_BIOPEARL_2018.3097
Lumbrineris cingulata	*Lumbrineris* sp. (MB)	BIO6-EBS-3D	458		KX867319	NHMUK_BIOPEARL_2018.3361
Lumbrineris cingulata	*Lumbrineris* sp. (MB)	BIO6-EBS-3D	306		KX867329	NHMUK_BIOPEARL_2018.3362
Lumbrineris cingulata	*Lumbrineris* sp. (MB)	PB-EBS-1-S	175		KX867320	NHMUK_BIOPEARL_2018.3365
Lumbrineris cingulata	*Lumbrineris* sp. (MB)	SR-EBS-4-E	329		KX867326	NHMUK_BIOPEARL_2018.3369
Lumbrineris cingulata	*Lumbrineris* sp. (MB)	SG-EBS-3E	324		KX867327	NHMUK_BIOPEARL_2018.3626
Lumbrineris cingulata	*Lumbrineris* sp. (MB)	ST-EBS-4E	172		KX867321	NHMUK_BIOPEARL_2018.3819
Lumbrineris cingulata	*Lumbrineris* sp. (MB)	BIO4-EBS-3E	354		KX867317	NHMUK_BIOPEARL_2018.9350
Macellicephala sp. A	*Macellicephala* sp. (MB1)	BIO3-EBS-1B	205		KX867377	NHMUK_BIOPEARL_2018.1422
Macellicephala sp. A	*Macellicephala* sp. (MB1)	BIO3-EBS-1A	310		KX867373	NHMUK_BIOPEARL_2018.1463
Macellicephala sp. A	*Macellicephala* sp. (MB1)	BIO6-EBS-3A-Epi	4	KX867447	KX867371	NHMUK_BIOPEARL_2018.211
Macellicephala sp. A	*Macellicephala* sp. (MB2)	BIO5-EBS-3D Epi	36	KX867448	KX867378	NHMUK_BIOPEARL_2018.521
Macellicephala sp. A	*Macellicephala* sp. (MB1)	BIO5-EBS-3D	414		KX867372	NHMUK_BIOPEARL_2018.522
Macellicephala sp. A	*Macellicephala* sp. (MB1)	BIO5-EBS-3D	446		KX867376	NHMUK_BIOPEARL_2018.533
Macellicephala sp. A	*Macellicephala* sp. (MB1)	BIO6-EBS-2A	221	KX867445	KX867369	NHMUK_BIOPEARL_2018.9351
Macellicephala sp. A	*Macellicephala* sp. (MB1)	BIO5-EBS-2A	291		KX867374	NHMUK_BIOPEARL_2018.9352
Macellicephala sp. A	*Macellicephala* sp. (MB1)	BIO5-EBS-2A	452		KX867375	NHMUK_BIOPEARL_2018.9353
Macellicephala sp. A	*Macellicephala* sp. (MB1)	BIO5-EBS-3A	236		KX867379	NHMUK_BIOPEARL_2018.9354
Macellicephala sp. A	*Macellicephala* sp. (MB1)	BIO5-EBS-2A	360		KX867380	NHMUK_BIOPEARL_2018.9355
Macellicephala sp. A	*Macellicephala* sp. (MB1)	BIO6-EBS-2A	210	KX867446		NHMUK_BIOPEARL_2018.9356
Macellicephaloides sp. A	*Macellicephaloides* sp. (MB1)	BIO4-EBS-3D	231		KX867331	*
Macellicephaloides sp. A	*Macellicephaloides* sp. (MB1a)	BIO4-EBS-3B	344		KX867332	*
Macellicephaloides sp. A	*Macellicephaloides* sp. (MB1a)	BIO4-EBS-3A	377		KX867333	*
Macellicephaloides sp. A	*Macellicephaloides* sp. (MB1a)	BIO5-EBS-3D	193		KX867334	*
Macellicephaloides sp. A	*Macellicephaloides* sp. (MB1a)	BIO4-EBS-3A	315		KX867335	*
Macellicephaloides sp. A	*Macellicephaloides* sp. (MB1a)	BIO4-EBS-3D	388		KX867336	*
Macellicephaloides sp. A	*Macellicephaloides* sp. (MB1a)	BIO6-EBS-2A	440		KX867337	*

Macellicephaloides sp. A	*Macellicephaloides* sp. (MB1a)	BIO6-EBS-2A	437		KX867338	*
Macellicephaloides sp. A	*Macellicephaloides* sp. (MB1a)	BIO5-EBS-3D	412		KX867339	*
Macellicephaloides sp. A	*Macellicephaloides* sp. (MB1a)	BIO6-EBS-2A	398		KX867340	*
Macellicephaloides sp. A	*Macellicephaloides* sp. (MB1a)	BIO6-EBS-3A	365		KX867341	*
Macellicephaloides sp. A	*Macellicephaloides* sp. (MB1a)	BIO4-EBS-3B	296		KX867342	*
Macellicephaloides sp. A	*Macellicephaloides* sp. (MB1b)	BIO4-EBS-3A-Epi	17		KX867343	*
Macellicephaloides sp. A	*Macellicephaloides* sp. (MB1b)	BIO6-EBS-2A Sup	33		KX867344	*
Macellicephaloides sp. B	*Macellicephaloides* sp. B	BIO6-EBS-2A	443		KX867368	NHMUK_BIOPEARL_2018.9357
Maldane sarsi	*Eupraxillella* cf. *antarctica* (MB)	BIO4-EBS-3A-Epi	191		KX867207	NHMUK_BIOPEARL_2018.6410
Maldane sarsi	*Eupraxillella* cf. *antarctica* (MB)	BIO4-EBS-3B	313		KX867209	NHMUK_BIOPEARL_2018.6410
Maldane sarsi	*Praxillella* sp. (MB)	BIO4-EBS-3B	429		KX867348	NHMUK_BIOPEARL_2018.6412
Maldane sarsi	*Eupraxillella* cf. *antarctica* (MB)	BIO4-EBS-3D	216		KX867210	NHMUK_BIOPEARL_2018.6415
Maldane sarsi	*Maldane sarsi antarctica* (MB)	PB-EBS-3-S	257		KX867345	NHMUK_BIOPEARL_2018.6425
Maldane sarsi	*Asychis amphiglyptus* (MB)	SG-EBS-3-E	204		KX867174	NHMUK_BIOPEARL_2018.6430
Maldane sarsi	*Asychis amphiglyptus* (MB)	SG-EBS-5-E	260		KX867173	NHMUK_BIOPEARL_2018.6435
Maldane sarsi	*Maldanidae* sp. (MB)	SR-EBS-4-E	251		KX867347	NHMUK_BIOPEARL_2018.6439
Maldane sarsi	*Asychis amphiglyptus* (MB)	LI-AGT-4	374		KX867171	NHMUK_BIOPEARL_2018.9322
Maldane sarsi	*Asychis amphiglyptus* (MB)	LI-AGT-3	270/371		KX867172	NHMUK_BIOPEARL_2018.9323
Maldane sarsi	*Eupraxillella* cf. *antarctica* (MB)	LI-AGT-4b	292		KX867208	NHMUK_BIOPEARL_2018.9336
Maldane sarsi	*Maldane sarsi antarctica* (MB)	LI-AGT-2	367		KX867346	NHMUK_BIOPEARL_2018.9358
Scalibgrema inflatum	*Scalibregma* sp. (MB1)	BIO5-EBS-3B	152	KX867451	KX867351	*
Scalibgrema inflatum	*Scalibregma* sp. (MB1)	BIO4-EBS-3A-Epi	162	KX867452	KX867352	*
Scalibgrema inflatum	*Scalibregma* sp. (MB1)	BIO5-EBS-3D	188	KX867453	KX867353	*
Scalibgrema inflatum	*Scalibregma* sp. (MB1)	BIO5-EBS-3E	230	KX867454	KX867354	*
Scalibgrema inflatum	*Scalibregma* sp. (MB1)	BIO5-EBS-3B	323		KX867356	*
Scalibgrema inflatum	*Scalibregma* sp. (MB1)	BIO4-EBS-3D	290		KX867357	*
Scalibgrema inflatum	*Scalibregma* sp. (MB1)	BIO4-EBS-3D	213		KX867358	*
Scalibgrema inflatum	*Scalibregma* sp. (MB1)	BIO4-EBS-3A-Epi	167		KX867359	*
Scalibgrema inflatum	*Scalibregma* sp. (MB1)	BIO5-EBS-3A	237		KX867362	*
Scalibgrema inflatum	*Scalibregma* sp. (MB3)	ST-EBS-3b-E	18	KX867456	KX867364	*

Scalibgrema inflatum	*Scalibregma* sp. (MB3)	ST-EBS-4S	92	KX867457	KX867365	*
Scalibgrema inflatum	*Scalibregma* sp. (MB3)	ST-EBS-4E	121	KX867458	KX867366	*
Scalibgrema inflatum	*Scalibregma* sp. (MB3)	ST-EBS-4E	88		KX867367	*
Scalibgrema inflatum	*Scalibregma* sp. (MB2)	EI-EBS-2-E	82/117	KX867455	KX867363	NHMUK_BIOPEARL_2018.2427
Scalibgrema inflatum	*Scalibregma* sp. (MB1)	EI-EBS-4-S	37	KX867449	KX867349	NHMUK_BIOPEARL_2018.2842
Scalibgrema inflatum	*Scalibregma* sp. (MB1)	EI-EBS-4E	346		KX867360	NHMUK_BIOPEARL_2018.2843
Scalibgrema inflatum	*Scalibregma* sp. (MB1)	LI-EBS-4-E	76/109	KX867450	KX867350	NHMUK_BIOPEARL_2018.6566
Scalibgrema inflatum	*Scalibregma* sp. (MB1)	LI-EBS-4E	339		KX867355	NHMUK_BIOPEARL_2018.6567
Scalibgrema inflatum	*Scalibregma* sp. (MB1)	JR275-23-EBS TOT	309		KX867361	NHMUK_BIOPEARL_2018.9359

8.2 Appendix 2

Minimum and maximum K2P pairwise comparison values (%) of the target species for DNA barcoding in Chapter 2 by family. Including intraspecific (MB, MB#) and intraclade (MB#a) comparisons and interspecific and interclade comparisons indicated by colour, where purple = morphospecies comparisons, blue = cryptic species comparison and green = within species clade comparisons for both the COI (left) and 16S (right) gene. * indicates no range available because only one pairwise comparison, ** indicates no pairwise comparison as only one sequence available for the relative species/clade. Note that for some families/species no COI data was obtained and in the case of Hesionidae sp. A and *Aglaophamus* spp. a greater number of species/clades were obtained from COI data than 16S. From Brasier et al. 2016.

Family	**Morphospecies (MB), Cryptic species (MB#) or Clade (MB#a)**	**COI**			**16S**			
		Intraspecific/ Intraclade K2P %	**Interspecific/ Interclade K2P %**		**Intraspecific/ Intraclade K2P %**	**Interspecific/ Interclade K2P %**		
Acrocirridae	1. *Flabelligena* sp.. A (MB)	0.00-1.90	1.	2.	0.75*	1.	2.	
	2. *Flabelligena* sp.. B (MB)	0.00-1.88	24.83-31.06		0.00-1.33	24.68-26.46		
Cirratulidae	*1. Chaetozone* sp.. (MB1a)				0.00-0.019	1.	2.	3.
	2. Chaetozone sp.. (MB1b)				**	2.72-4.35		
	3. Chaetozone sp.. (MB1c)				0.27*	5.70-7.22	4.53-4.88	
Euphrosinidae	1. *Euphrosinella* cf. *cirratoformis* (MB1)				0.00-2.42	1.	2.	3.
	2. *Euphrosinella* cf. *cirratoformis* (MB2)				0.27-0.28	5.50-7.53		

	3. *Euphrosinopsis* cf. *antarctica* (MB)				0.62*	21.11-27.44		20.41-24.83			
Glyceridae	1. *Glycera* sp.. (MB1)	0.00-0.44	1.	2.	0.00-2.74	1.			2.		
	2. *Glycera* sp.. (MB2)	0.00-0.30	17.12-18.05		0.00-1.60	8.17-12.80					
Hesionidae	1. Hesionidae sp.. (MB1)	0.15-0.51	1.	2.	0.00-2.97						
	2. Hesionidae sp.. (MB2)	0.00-1.01	10.59-12.41								
Lumbrineridae	*1. Lumbrineris kerguelensis-cingulata* (MB1a)				0.00-1.42	1.	2.	3.	4.	5.	6.
	2. Lumbrineris kerguelensis-cingulata (MB1b)				**	9.45-11.61					
	3. Lumbrineris kerguelensis-cingulata (MB1c)				0.00-0.36	9.22-11.74	5.62-7.63				
	4. Lumbrineris kerguelensis-cingulata (MB1d)				1.46*	9.35-11.97	6.24-6.70	4.38-5.88			
	5. Lumbrineris kerguelensis-cingulata (MB1e)				**	10.40-12.50	6.11*	4.57-6.10	4.86-4.88		
	6. Lumbrineris kerguelensis-cingulata (MB1f)				0.00-0.36	10.35-15.54	5.77-7.56	3.91-5.54	4.21-5.22	3.91-5.21	
Maldanidae	1. *Asychis amphiglyptus* (MB)				0.27-2.05	*1.*	*2.*	*3.*	*4.*	*5.*	
	2. *Eupraxillella* cf. *antarctica* (MB)				0.28-4.11	35.11-49.94					

	3. *Maldane sarsi antarctica* (MB)							0.26*	15.31-16.82	30.51-36.26			
	4. Maldanidae sp.. (MB)							**	35.62-40.50	20.24-24.39	37.72-33.27		
	5. *Praxillella* sp.. (MB)							**	39.39-40.48	23.08-24.83	37.47-37.65	10.81*	
Nephtyidae	1. *Aglaophamus* cf. *trissophyllus* (MB1a)	0.00-0.18	1.	2.	3.	4.	5.	0.00-1.67	*1. 2. 3.*		*4. 5.*		*6.*
	2. *Aglaophamus trissophyllus* (MB1b)	0.92*	5.33-5.75										
	3. *Aglaophamus* cf. *trissophyllus* (MB1c)	**	4.54-4.74	5.54-6.13									
	4. *Aglaophamus* sp.. (MB2)	0.16-0.18	13.55-14.01	13.09-13.34	13.31-13.54			0.00-0.80	1.63-3.38				
	5. *Aglaophamus* sp.. (MB3)	2.12*	11.56-13.06	11.52-12.97	12.45-13.08	9.64-13.32							
	6. *Aglaophamus* sp.. (MB4)							**	5.58-6.90		5.53-6.13		
Paraonidae	1. *Aricidea simplex* (MB)							0.00-1.45	*1.*	*2.*	*3.*	*4.*	*5.*
	2. *Aricidea* cf. *belgicae* (MB1)							0.00-0.61	18.70-22.63				
	3. *Aricidea* cf. *belgicae* (MB2)							0.27	18.34-21.63	0.82-1.54%			

	4. *Aricidea* cf. *belgicae* (MB3)					**	19.16-21.22	2.46-3.07%	2.45-2.78		
	5. *Aricidea* cf. *pulchra* (MB)					**	19.43-22.37	4.18-5.05%	3.89-4.18	3.05*	
Polynoidae	1. *Harmothoe fuligineum* (MB)	0.00-1.47	1.	2.	3.	0.00-2.28	1.	2.	3.	4.	5.
	2. *Macellicephala* sp.. (MB1)	0.15-0.73	27.03-30.86			0.00-1.08	34.52-47.89				
	3. *Macellicephala* sp.. (MB2)	**	25.47-26.85	12.58-12.84		0.00-0.56	31.58-36.45	2.74-4.30			
	4. Macellicephaloides sp.. (MB1a)					0.00-4.10	34.61-41.81	36.46-54.35	35.29-43.05		
	5. Macellicephaloides sp.. (MB1b)					0.58*	36.90-41.08	38.05-45.40	37.07-38.17	0.87-2.36	
Scalibregmatidae	1. *Scalibregma* sp.. (MB1)	0.14-1.48	1.	2.	3.	0.00-2.86	1.		2.		3.
	2. *Scalibregma* sp.. (MB2)	**	13.99-14.53			**	4.92-6.66				
	3. *Scalibregma* sp.. (MB3)	0.00-1.46	14.35-15.33	6.51-6.77		0.00-1.56	6.66-9.82		2.13-2.44		
Spio	1. *Laonice weddellia* (MB)	0.00-1.32	1.		2.	0.00-1.44	1.		2.		3.

	2. *Laonice* cf. *antarctica* (MB)	0.17*	22.54-24.10		0.00-0.27	16.74-19.07		
	3. *Laonice* cf. *vietezi* (MB)				0.00-1.70	14.84-17.28	16.10-18.44	

8.3 Appendix 3

Amino acid values from duplicate analyses on GC/IRMS for trophic and source amino acids with calculated trophic levels using $TL_{Glu/Phe}$, $TL_{Glu/Gly}$ and $TL_{TAA/SAA}$ equations in Chapter 5, for each species with location and categorical trophic trait. Where location; EI = Elephant Island, LI = Livingston Island, ST = Southern Thule, SG = South Georgia, OAS = Outer Amundsen Sea, IAS = Inner Amundsen Sea and WS = Weddell Sea. Trophic traits; O/PS = Omnivore/predator/scavenger, SDF = surface deposit feeder, SSDF = subsurface deposit feeder. Trophic AA; Glu = Glutamic Acid, Ala = Alanine, Val = Valine, Leu = Leucine, Thr = Threonine, Asp = Aspartic acid. Source AA; Phe = Phenylalanine, Gly = Glycine, Ser = Serine. Trophic level; Glu/Phe = Equation 5-3, Glu/Gly = Equation 5-4 and T/S = Equation 5-6.

Species	Location_ depth (m)	Trophic trait	**Trophic AA**						**Source AA**			**Trophic level**		
			Glu	Ala	Val	Leu	Thr	Asp	Phe	Gly	Ser	Glu/Phe	Glu/Gly	TAA/ SAA
Aglaophamus trissophyllus (MB1)	EI_1000	O/P/S	22.2 ± 0.9	24.8 ± 0.7	26.7 ± 0.8	24.7 ± 2.0	-15.8 ± 1.0	20.4 ± 0.8	9.5 ± 0.2	7.9 ± 1.4	6.5 ± 1.5	3.0	2.6	3.0
Aglaophamus trissophyllus (MB1)	LI_200	O/P/S	20.0 ± 1.3	19.6 ± 0.0	24.9 ± 1.5	22.5 ± 0.2	-12.5 ± 1.1	17.8 ± 0.9	7.3 ± 0.7	6.9 ± 0.6	5.7 ± 1.9	2.2	2.9	2.7
Aglaophamus trissophyllus (MB1)	SG_200	O/P/S	17.9 ± 1.0	16.5 ± 0.5	21.1 ± 0.8	18.8 ± 0.3	-12.0 ± 2.6	16.4 ± 1.2	7.6	6.7 ± 0.4	5.9 ± 1.8	2.7	2.6	2.5
Aglaophamus trissophyllus (MB1)	ST_200	O/P/S	17.8 ± 0.6	16.3 ± 0.3	22.2 ± 1.4	18.8 ± 1.1	-10.3 ± 0.2	16.1 ± 0.6	10.3 ± 1.9	7.8 ± 0.4	6.3 ± 2.0	1.3	2.4	2.1
Aglaophamus sp. (MB2)	IAS_500	O/P/S	18.7 ± 0.0	18.9 ± 0.5	23.7 ± 0.8	21.5 ± 0.9	-16.2 ± 2.1	17.3 ± 0.2	10.4 ± 1.8	8.1 ± 0.7	6.6 ± 0.4	1.7	2.5	2.3
Aglaophamus sp. (MB2)	IAS_1000	O/P/S	18.7 ± 2.1	16.5 ± 1.7	22.7 ± 0.4	20.3 ± 1.2	-14.2 ± 1.3	18.0 ± 2.3	6.2	7.1 ± 1.6	6.0 ± 3.2	1.2	2.7	2.5
Aglaophamus sp. (MB3)	SG_500	O/P/S	18.3 ± 0.9	17.4 ± 1.1	21.4 ± 0.6	21.8 ± 1.4	-11.5	16.4 ± 1.4	6.8	7.7 ± 1.3	4.7 ± 2.0	1.3	2.5	2.4
Glycera sp. (MB1)	OAS_500	O/P/S	20.3 ± 0.9	20.6 ± 0.1	25.3 ± 1.4	22.3 ± 0.1	-14.3 ± 5.6	18.9 ± 0.6	4.4 ± 0.2	15.2 ± 1.0	11.2 ± 1.8	2.7	1.7	2.4
Glycera sp. (MB1)	SG_500	O/P/S	19.1 ± 1.6	20.7 ± 0.9	22.1 ± 0.6	19.2 ± 0.7	-10.1 ± 4.0	17.0 ± 1.1	6.4	13.7 ± 0.3	12.3 ± 1.6	2.4	1.8	1.3
Glycera sp. (MB1)	WS_500	O/P/S	19.9 ± 0.3	19.6 ± 1.5	24.8 ± 2.2	21.4 ± 1.3	-11.6 ± 2.0	18.6 ± 0.1	4.6	13.1 ± 2.0	13.7 ± 0.4	2.5	2	1.5
Glycera sp. (MB2)	IAS_500	O/P/S	22.4 ± 0.4	23.5 ± 0.9	24.1 ± 1.4	21.9 ± 0.7	-16.8	19.3 ± 0.8	6.7 ± 1.5	14.1 ± 1.6	9.8	2.8	2.2	2.6

Glycera sp. (MB2)	OAS_500	O/P/S	21.8 ± 0.7	20.5 ± 0.5	23.6 ± 2.0	21.4 ± 1.0	-17.8 ± 0.8	18.4 ± 0.3	5.1 ± 1.1	12.8 ± 1.8	9.8 ± 0.15	2.6	2.3	2.6
Glycera sp. (MB2)	IAS_1000	O/P/S	24.5 ± 0.6	25.3 ± 1.7	27.0 ± 1.6	24.1 ± 0.8	-14.9 ± 1.0	20.6 ± 0.3	8.0 ± 0.5	16.9 ± 1.5	12.2 ± 0.7	2.8	2.1	2.6
Glycera sp. (MB2)	OAS_1000	O/P/S	21.0 ± 1.9	18.9 ± 0.4	22.4 ± 0.1	23.8 ± 1.6	-9.9	19.7 ± 1.0	9.6 ± 1.0	13.9 ± 0.7	11.6 ± 1.5	1.8	2	2.2
Harmothoe fuligineum	IAS_500	O/P/S	22.4 ± 1.3	22.0 ± 0.1	24.4 ± 1.1	23.7 ± 0.9	-10.1 ± 1.0	19.1 ± 1.2	6.4 ± 0.4	6.5 ± 1.8	6.2 ± 0.0	2.6	3.3	3.1
Harmothoe fuligineum	OAS_500	O/P/S	21.7 ± 1.1	22.5 ± 0.0	23.4 ± 0.7	21.5 ± 0.5	-17.25 ± 0.3	17.0 ± 1..3	6.9 ± 0.8	6.5 ± 0.8	3.6 ± 1.4	3.5	3.2	3
Harmothoe fuligineum	WS_500	O/P/S	22.0 ± 1.4	24.6 ± 1.0	24.8 ± 2.8	23.8 ± 1.0	-17.4 ± 0.9	19.0 ± 0.9	5.0 ± 0.2	10.4 ± 1.5	6.5 ± 1.3	3.1	2.7	3
Macellicephaloides sp. (MB1)	IAS_500	O/P/S	22.6 ± 0.9	22.8 ± 0.4	25.2 ± 0.4	24.5 ± 1.0	-14.5 ± 1.7	18.8 ± 0.4		6.7 ± 0.8	3.7 ± 1.3		3.3	3.1
M*acellicephaloides* sp. (MB1)	IAS_1000	O/P/S	22.8 ± 0.1	27.1 ± 0.3	27.1 ± 0.0	25.4 ± 0.3	-17.3 ± 0.3	19.1 ± 0.7	9.3 ± 0.3	6.7 ± 0.9	1.5 ± 1.0	2.9	3.3	3.1
Macellicephaloides sp. (MB1)	OAS_1000	O/P/S	24.1 ± 0.8	26.1 ± 0.2	25.5 ± 0.6	24.6 ± 0.7	-17.4 ± 0.9	19.1 ± 1.7	8.4 ± 0.4	5.8 ± 0.4	1.9 ± 1.3	2.9	3.6	3.3
Flabelligena sp. A (MB)	IAS_500	SDF	12.2 ± 1.3	13.8 ± 0.7	12.9 ± 0.4	12.9 ± 0.4	-13.1 ± 0.3	10.4 ± 0.3		5.5 ± 1.8	2.1		2.0	1.9
Flabelligena sp. B (MB)	IAS_500	SDF	16.6 ± 0.3	18.3 ± 0.7	17.1 ± 2.2	16.2 ± 1.3	-8.4 ± 0.8	15.1 ± 0.3	6.9 ± 0.8		4.7 ± 0.2	2.0	3.4	2.4
Laonice cf. *antarctica* (MB)	WS_500	SDF	14.4 ± 1.3	15.6 ± 0.0	17.5 ± 0.6	13.6 ± 0.1	-8.3 ± 1.3	13.3 ± 0.9	5.0 ± 0.8	8.0 ± 0.6	1.9 ± 0.2	1.9	1.9	2.1
Laonice cf.*vietezi* (MB)	IAS_500	SDF	15.9 ± 0.3	18.0 ± 0.6	22.5 ± 0.4	17.0 ± 1.1		16.3 ± 0.1	3.0 ± 0.6	5.3 ± 2.9	5.2 ± 0.8	2.5	2.5	2.8
Laonice weddellia	IAS_500	SDF	16.6 ± 1.2	15.3 ± 1.1	17.6 ± 1.8	18.9 ± 1.0	-11.0 ± 2.2	16.9 ± 1.5	3.2 ± 0.4	6.4 ± 2.1	6.3 ± 1.1	2.1	2.5	2.6
Laonice weddellia	OAS_500	SDF	17.2 ± 1.3	16.8 ± 0.0	16.5 ± 1.7	19.4	-11.4 ± 0.9	13.9 ± 0.1	3.8	4.1 ± 2.1		2.3	2.9	2.7
Laonice weddellia	OAS_1000	SDF	12.2 ± 0.3	14.1 ± 1.0	16.3 ± 0.4	13.1 ± 0.7	-14.8 ± 0.7	11.9 ± 0.3	6.9 ± 1.2	1.0 ± 1.4	-3.9 ± 0.1	1.5	2.6	2.2
Laonice weddellia	ST_500	SDF	17.7 ± 3.1	18.6 ± 0.1	21.3 ± 0.7	18.1 ± 2.0	-5.1 ± 3.7	16.0 ± 3.1	3.8 ± 0.0	8.1 ± 3.2	3.7 ± 2.6	2.5	2.4	2.6

Laonice weddellia	WS_500	SDF	15.7 ± 0.1	21.0 ± 0.9	22.0 ± 1.7	18.3 ± 1.5	-8.1 ± 0.7	16.3 ± 0.8	9.7 ± 1.1	5.3 ± 0.9	-0.1 ± 1.3	2	2.5	2.5
Laonice weddellia	WS_1000	SDF	12.4 ± 2.2	15.1 ± 1.9	17.4 ± 1.8	13.1 ± 2.0	-12.9 ± 2.1	10.8 ± 2.0	10.5	3.9 ± 3.0	7.2	1.2	2.2	1.8
Aricidea belgicae (MB1)	IAS_500	SSDF	21.1 ± 0.6	21.0 ± 0.7	20.2 ± 0.7	18.6 ± 0.1	-11.5 ± 4.0	16.3 ± 1.6	6.2 ± 0.9	9.3 ± 0.5	6.2 ± 2.0	2.5	2.7	2.7
Aricidea belgicae (MB1)	OAS_500	SSDF	21.1 ± 0.1	20.3 ± 0.1	21.5 ± 0.2	19.0 ±0.9	-11.2 ± 2.4	17.3 ± 0.1		9.1 ± 0.8	6.8 ± 0.3		2.7	2.5
Aricidea belgicae (MB1)	IAS_1000	SSDF	21.9 ± 1.8	22.8 ± 1.4	21.8 ± 1.5	19.5 ± 0.6	-13.1 ± 1.2	18.0 ± 2.5	5.1 ± 0.1	8.8 ± 1.1	6.3 ± 0.3	2.9	2.9	3.0
Aricidea simplex	WS_500	SSDF	15.9 ± 0.5	17.0 ± 0.3	17.8 ± 0.0	16.8 ± 0.3		14.2 ± 1.0	4.9 ± 0.7	10.0 ± 0.9	7.5 ± 0.3	2.1	1.8	1.8
Scalibregma sp. (MB1)	IAS_500	SSDF	21.5 ± 0.4	23.5 ± 0.4	23.2 ± 0.6	22.0 ± 0.4	-6.5 ± 3.9	19.6 ± 1.2	8.7 ± 0.2	8.8 ± 0.4	8.5 ± 2.5	2.5	2.8	2.8
Scalibregma sp. (MB3)	ST_500	SSDF	19.4 ± 1.3	22.8 ± 0.2	22.6 ± 0.7	20.2 ± 1.4	-7.4 ± 1.1	18.1 ± 2.1	8.6 ± 0.1	9.6 ± 2.2	8.3 ± 3.1	2.4	2.4	2.6