The Application of DNA-based Methods to the Diet of Antarctic Krill (*Euphausia superba*)

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Declaration of originality

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Abraham Passmore, 26th of November, 2008

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Abstract

Studying the diet of Antarctic krill (*Euphausia superba* Dana) is important for modelling the flow of energy and nutrients through the Southern Ocean food web. Previous studies have demonstrated that krill consume a diverse range of prey, but, have failed to detect or quantify the contribution of important prey groups. The aim of this thesis was to examine whether new DNA based methods can contribute to the analysis of krill diet.

Initial work developed methods for preserving, extracting and analysing prey DNA derived from krill stomachs. These methods were shown to be capable of preserving large amounts of intact prey DNA and generating reproducible diet data. However, two problems with the method were identified.

The first problem was the presence of a large amount of predator DNA in diet samples that competed with prey DNA during PCR amplification. Further work developed methods that removed predator DNA prior to PCR, or, blocked predator DNA amplification during PCR. These methods were successful when applied to a simplified test system but failed when applied to real field samples.

The second problem was a discrepancy between the results obtained with DNA and concurrent results obtained with microscopy. This suggested the initial method suffered from bias that skewed results for some prey groups. Subsequent work attempted to resolve this problem by changing the approach from quantifying various prey within individual krill stomachs to quantifying the presence or absence of various prey groups in a large number of krill. When applied to field samples this approach correctly identified the same prey groups as microscopy, and, suggested that gastropods are a more important component of krill diet than previously recognised. However, there were still issues regarding the quantification of prey.

The remaining work focused on fundamental issues related to the longevity and quantification of prey DNA in krill stomachs. In krill stomachs, prey DNA was found to: be stable for several hours after ingestion, vary in quantity over six orders of magnitude; and, exit the stomach faster when krill continued to engage in feeding activity. Overall the results were promising and the application of DNA methods to krill diet warrants further investigation.

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Chapter 1
General Introduction

Antarctic krill (*Euphausia superba*) swimming in a tank at the Australian Antarctic Division krill facility.

Photograph by Rob King.

1.1 Overview

The introduction is divided into two major sections. The first section begins by reviewing the general biology of Antarctic krill. The section continues with a description of potential food sources for krill and examines previous attempts to characterise krill diet. The first section concludes by arguing that new methods are required to determine krill's trophic position within the Southern Ocean foodweb. The second section identifies DNA diet analysis as a potentially valuable approach for examining krill diet. The section then describes previous studies that have applied DNA diet analysis to invertebrate species.

1.2 Krill Biology

1.2.1 The importance of krill

The Antarctic krill (*Euphausia superba*) is an abundant pelagic crustacean that is central to the Southern Ocean ecosystem, one of the world's largest marine ecosystems. With an estimated biomass of 55 - 297 million tonnes (Voronina, 1998; Nicol et al., 2000a; Siegel, 2005) krill are also one of a handful of species that dominate metazoan animal biomass on planet earth. Within the Southern Ocean food web krill are important both as a consumer of plankton and as a major food source for fish, seals, squid, whales, penguins and other sea birds (Hopkins, 1985; Laws, 1985). This makes krill the critical link that allows the energy generated by photosynthesis to be transferred to higher trophic levels. Research on krill is driven by their abundance and status in the Southern Ocean food web, interest in quantifying the flow of carbon and other nutrients through the Southern Ocean ecosystem (e.g. Treguer and Jacques, 1992; Froneman et al., 1996; Froneman et al., 2000), and the need to ensure that an active krill fishery is well managed (Croxall and Nicol, 2004).

1.2.2 General Characteristics

Krill are a member of the phylum Crustacea (Class: Malacostraca) with an exoskeleton and body plan that is similar to a shrimp or lobster. *Euphausia superba* are among the largest of the 85 krill species and range in size from eggs that are < 1mm in diameter to adults that reach 60 mm in length. Krill have six pairs of thoracic appendages (thoracopods) that are used primarily for feeding and five pairs of abdominal appendages (pleopods) that provide locomotion (Mauchline and Fisher, 1969; Hamner, 1988).

1.2.3 Krill life cycle

Krill have a long and complex lifecycle. Reproduction is via broadcast spawning and is timed to coincide with the seasonal pulse of algal blooms that occur during the spring and summer (Spiridonov, 1995, Quetin and Ross, 2003). Spawning can occur on the continental shelf but there is evidence that the majority of the spawning stock moves offshore from the shelf break to spawn in deeper water (Siegel, 1988; Lascara et al., 1999; Nicol et al., 2000b). Females release batches of hundreds to thousands of fertilised eggs directly into the water column in multiple spawning events (Cuzin-Roudy, 2000; Ross and Quetin, 2000). The eggs then sink to a depth of 500 - 2000 m before hatching into larvae (Hempel and Hempel, 1986). Larvae undergo a 'developmental ascent' passing through a series of stages (Nauplius stages I - II, Metanauplius, Calyptopsis I - III, Furcilia I - VI) as they swim back to the surface and commence feeding (Marr, 1962). Krill enter their first winter as furcilia larvae before developing into juveniles the following spring (Fraser, 1936). Juveniles progress to sexually mature adults in their third or fourth summer (Siegel and Loeb, 1994; Ross and Quetin, 2000) and may have multiple breeding seasons (Quetin and Ross, 2001, 2003) during an estimated lifespan of 5 - 7 years (Siegel and Nicol, 2000).

1.2.4 Krill range and distribution

Krill have a vast circumpolar range that extends from the Antarctic coast to ~ 52°S (Figure 1-1, Atkinson et al., 2004; Siegel et al., 2004). Most of their range is within the Continental Shelf and Seasonal Ice zones but in the Scotia Sea their range extends beyond the maximum extent of winter sea ice into the Permanently Open Ocean Zone (Hofmann and Murphy, 2004) (zones described in Treguer and Jacques, 1992). These regions experience large seasonal fluctuations in solar irradiance and ice cover which has a significant impact on the local biota.

The distribution of krill within their range is heterogeneous. High densities of krill occur along the West Antarctic Peninsula and extend north-east into the Scotia Sea with estimates suggesting that these regions contain > 50% of the total Southern Ocean krill stock. Around the rest of the continent krill densities are generally low to moderate (Nicol et al., 2000a; Atkinson et al., 2004). At the regional level krill distribution is patchy. Krill tend to be

concentrated around island groups and along the continental shelf and shelf slope where they often aggregate into high density swarms (Marr, 1962; Nicol, 2006).

Krill have historically been described as a species that lives in the epipelagic zone (0 - 200 m) throughout the year (e.g. Marr, 1962; Mauchline and Fisher, 1969, Miller and Hampton, 1989). Numerous acoustic and net surveys conducted during the summer have suggested that krill are concentrated in the top 100 m of the water column (Siegel, 1988; Trathan et al., 1993; Lascara et al., 1999; Pauly et al., 2000; Siegel et al., 2004). During winter, larval stages are associated with the underside of the sea ice and depend on the consumption of sea ice biota for their winter survival (Daly, 1990; 2004; Ross et al., 2004). Due to the difficulties associated with sampling under the sea ice the distribution of adult krill during winter is less clear. Some observations suggest adults are associated with the underside of sea ice similar to larval stages (Marschall, 1988; O'Brien, 1988, Stretch et al., 1988). However, other studies have found adults distributed throughout the epipelagic zone of the water column (Nordhausen, 1994; Lascara et al., 1999; Lawson, 2008). Other potential patterns include migration onto the continental shelf combined with a shift to slightly deeper water (depth range ~ 100 - 350 m) (Siegel, 1988; Lascara et al., 1999; Taki et al., 2005; Lawson, 2008) and migration to the sea floor (Kawaguchi et al., 1986, Quetin et al., 1996; Ligowski, 2000). It is possible that adult krill utilise all of these strategies at various times depending on local conditions (Quetin et al., 1996; Lawson, 2008).

Theories on the vertical distribution of krill may be about to undergo a paradigm shift. Recent observations from remotely operated vehicles during summer have provided evidence of adult krill associated with the sea floor on both the continental shelf (~ 450 m) (Gutt and Siegel, 1994) and on the abyssal plains (3500 m) (Clarke and Tyler, 2008). Research effort focused in the epipelagic zone may have missed a substantial proportion of the krill population and underestimated the importance of vertical migration (Brierley, 2008; Clarke and Tyler, 2008).

1.2.5 Krill diet – Potential food sources

Krill are versatile feeders that are capable of exploiting a diverse range of food. Krill feed primarily by filtering particles from the water column. Particles are collected with the thoracopods which are lined with fine comb-like setae (Figure 1-2). The thoracopods are

extended forward and laterally to create a feeding basket and are then drawn back towards the mouth. As the thoracopods draw back water is extruded from the basket and the particles are retained by the setae (Hamner, 1988). Feeding experiments suggest that krill can efficiently retain particles down to approximately 6 µm in diameter (Boyd et al., 1984; Quetin and Ross, 1985). Krill also use their thoracopods to scrape algae from the underside of sea ice (Marschall, 1988, Stretch et al., 1988; Daly, 1990) and to capture and hold large food items including metazoan prey (> 200 µm) (Price et al., 1988). Krill are therefore capable of feeding on items that range in size over several orders of magnitude. The following sections examine potential food sources that are available to krill.

1.2 5.1 Potential food sources for krill – Autotrophic protists (Algae)

Eukaryotic algae are the primary producers that form the base of the Southern Ocean pelagic food web. The algal community is derived from a taxonomically diverse range of autotrophic protists. Diatoms (Bacillariophyceae) are a key group that often dominate summer phytoplankton blooms. Diatom species diversity is high with more than 100 species described in Antarctic waters (Marchant and Scott, 2005). Important diatom genera include *Chaetoceros*, *Corethron*, *Fragilariopsis*, *Proboscia*, *Rhizosolenia* and *Thalassiosira* (Smetacek et al., 2004). Other groups of autotrophic protists with varying degrees of species diversity include Chrysophyceae (Golden Algae), Cryptophyceae (Cryptomonads), Dinophyceae (Dinoflagellates), Prasinophyceae (e.g. *Pyramimonas*) and Prymnesiophyceae (e.g. *Phaeocystis*) (Marchant and Scott, 2005). These groups make significant and sometimes dominant contributions to summer phytoplankton assemblages (Kopczynska et al., 1986; Kang and Lee, 1995; Kopczynska et al., 1995; Bidigare et al., 1996; Fiala et al., 1998, Arrigo et al., 1999; Waters et al., 2000; Rodriguez et al., 2002; Buesseler et al., 2003; Garibotti et al., 2003; Kopczynska et al., 2007). During winter the sea-ice algae community is comprised of the same taxonomic groups that occur in the water column during the summer but can differ with regard to the dominant species, particularly late in the season (Garrison and Close, 1993; Palmisano and Garrison, 1993; Lizotte, 2001). Autotrophic protists range in size from approximately 1 – 200 µm and may exist as single cells or as chains/aggregations of cells. A significant proportion of the algal biomass is less that 6 µm in diameter (Hewes et al., 1990; Detmer and Bathmann, 1997; Mengesha et al., 1998; Waters et al., 2000) and would not be efficiently filtered by krill thoracopods.

In the high latitude regions of the Southern Ocean primary production is distinctly seasonal (Arrigo et al., 1998; Moore and Abbott, 2000). Peak production occurs during the austral summer when long days and receding sea ice allow maximum light to penetrate the water column. This fuels a seasonal pulse of phytoplankton blooms that are most intense at the receding ice edge, around islands, at ocean fronts that separate large scale water masses, and along the continental shelf (Arrigo et al., 1998; Moore and Abbott, 2000; Boyd, 2002). Algal biomass during the summer is highly variable between sites (0.5 – 1900 mg C m^{-3}) but has average values of 25 – 150 mg C m^{-3} [1] up to a depth of 50 m (Treguer and Jacques, 1992; Smith and Dierssen, 1996; Wright and van den Enden, 2000; Landry et al., 2002; Holm-Hansen et al., 2004). During winter, short days and extensive ice cover lead to low levels of primary production. Algal biomass in the water column approaches zero and the remaining primary production is associated with the sea ice (Smetacek et al., 1990). The biomass of algae within sea ice can be extremely high (> 200 mg C m^{-3}) (Arrigo, 2003), but during winter the total biomass integrated over the entire epipelagic zone (0 – 200 m) is low compared to summer phytoplankton blooms (Arrigo and Thomas, 2004). The availability of algae is further reduced by the fact that only a proportion of the algae resides on the underside of the sea ice where it is available to grazers, with the remainder embedded within the ice or trapped within brine channels (Palmisano and Garrison, 1993; Arrigo, 2003; Arndt and Swadling, 2006). Algae are therefore an abundant, but seasonally variable food source. Animals that rely on phytoplankton blooms during the summer must find other food sources or use alternative strategies to survive through the winter.

1.2.5.2 Potential food sources for krill – Heterotrophic protists

Heterotrophic protists consume bacteria, algae and other heterotrophic protists as part of the microbial network that recycles nutrients in the epipelagic zone (Azam et al., 1983; Hewes et al., 1985; Sherr et al., 1988; Smetacek et al., 1990). Heterotrophic protist assemblages are typically dominated by Dinophyceae (Dinoflagellates) and ciliates such as Oligotrichia (Oligotrichs) and Choreotrichia (Tintinnids). Groups such as Foraminifera, Radiolaria, and Choanoflagellates are also present (Burkill et al., 1995; Becquevort, 1997; Klaas, 1997, Becquevort et al., 2000; Waters et al., 2000; Landry et al., 2002). Sea ice assemblages

[1] Chlorophyll-a values converted to carbon using a chlorophyll-a carbon ratio of 50 as in Hofmann, E E and Lascara, C. M. (2000). Modeling the growth dynamics of Antarctic krill Euphausia superba. Modeling the growth dynamics of Antarctic krill Euphausia superba 194 219-231.

contain the same taxonomic groups that occur in the water column but may differ with regards to dominant species (Garrison and Gowing, 1993; Palmisano and Garrison, 1993). Heterotrophic protists range in size from ~ 2 – 200 µm (Garrison and Gowing, 1993, Smetacek et al., 2004). Single cells at the smaller end of the size range would not be efficiently filtered by krill thoracopods.

Heterotrophic protist biomass is correlated to phytoplankton biomass (Burkill et al., 1995) and ranges from 0 – 67 mg C m^{-3} equating to ~ 30 – 40 % of the summer phytoplankton biomass (Garrison, 1991b; Nothig et al., 1991; Burkill et al., 1995, Becquevort, 1997, Klaas, 1997; Becquevort et al., 2000; Landry et al., 2002) and 6 – 49 % of sea-ice community biomass (Garrison, 1991a). Heterotrophic protists are therefore a potentially significant food source for krill.

1.2.5.3 Potential food sources for krill – Metazoan zooplankton

Metazoan zooplankton are an important component of the pelagic food web that consume protists and other metazoan zooplankton. The metazoan zooplankton community in the Seasonal Ice Zone and the Scotia Sea is diverse. Copepods are generally the dominant component of the community in terms of abundance and biomass (Atkinson et al., 1996; Voronina, 1998, Atkinson and Sinclair, 2000; Hosie et al., 2000; Pakhomov and Froneman, 2004a; Ward et al., 2004). Copepod species diversity is high with 346 species having been identified in Antarctic waters (Razouls et al., 2000). Generally small (< 1 mm) cyclopoid copepods (*Oithona* spp.) are the most abundant, but in terms of biomass are rivalled by larger (1 - 3 mm) calanoid species (*Calanoides acutus*, *Calanus simillimus*, *Calanus propinquus*, *Rhincalanus gigas* and *Metridia gerlachei*) (Atkinson et al., 1996; Voronina, 1998; Atkinson and Sinclair, 2000; Hosie et al., 2000; Pakhomov and Froneman, 2004a; Ward et al., 2004). Euphausids are also important in terms of biomass (Voronina, 1998). *Euphausia superba* reaches very high densities but *Thysanoessa macrura* is also widespread and abundant (Hosie et al., 2000, Lancraft et al., 2004; Pakhomov and Froneman, 2004a; Ward et al., 2004) and *Euphausia crystallorophias* becomes prevalent close to the Antarctic coast (Boysen-Ennen et al., 1991; Hosie and Cochran, 1994). Salps are more prevalent in ice-free open ocean regions but their range extends into the seasonal ice zone and Scotia Sea (Voronina, 1998; Hosie et al., 2000; Ward et al., 2004). Other

metazoan groups that can contribute up to 50 % of metazoan zooplankton biomass at some sites include amphipods, chaetognaths, cnidarians, ctenophores, pteropods (Gastrapoda) and polychaetes (Siegel et al., 1992, Schnack-Schiel and Mujica, 1994, Atkinson et al., 1996; Chiba et al., 2000; Hosie et al., 2000; Pakhomov, 2004; Ward et al., 2004). Metazoan zooplankton range in size from ~ 0.5 - > 40 mm. The adult stages of some metazoan zooplankton are probably too large to be consumed by krill, but egg and larval stages are potentially vulnerable. The early life stages of larger mid-level predators such as fish and squid may also be consumed by krill. Egg and larval stages may provide a highly nutritious and highly seasonal food source.

Metazoan zooplankton biomass in the Seasonal Ice Zone is typically in the range of 0 - 15 mg C m^{-3} [2] (Boysen-Ennen et al., 1991; Chiba et al., 2000, Pakhomov and Froneman, 2004a). During the summer much of the metazoan zooplankton is concentrated in the epipelagic zone (0 – 200 m) to take advantage of the seasonal phytoplankton blooms. During winter mortality leads to a reduction in metazoan zooplankton biomass. Some species remain at the surface associated with sea-ice but others, including some biomass-dominant copepods (*Calanoides acutus*, *Calanus simillimus* and *Rhincalanus gigas*), descend deeper into the water column (> 400 m) where they enter diapause (Quetin et al., 1996; Atkinson and Sinclair, 2000; Schnack-Schiel et al., 2001). Although metazoan biomass is less significant than other food sources it is potentially a more nutritious food source that is available in all seasons.

1.2.5.4 Potential food sources for krill – Detritus

Detritus is the non-living organic matter present at all depths of the water column. Detritus is composed of many items including dead organisms, faecal pellets from protozoan and metazoan zooplankton, crustacean exuvia, discarded polysaccharide feeding houses of thaliaceans, and secretions from algae and bacteria. These components often aggregate into larger particles described as marine snow which in turn play host to a variety of living organisms including phytoplankton, bacteria and protozoa (Knox, 2007).

[2] Dry weight values converted to carbon using a dry weight to carbon ratio of 0.5 as in Gifford, D. and Caron, D. (2000) Biomass and abundance. In. R. Harris, P. Wiebe, J. Lenz, H. Skjoldal, and M. Huntley, eds ICES Zooplankton Methodology Manual Academic Press, pp. 83-174

Detritus is difficult to quantify because it is generally only measured as part of the particulate organic matter (POC) in filtered water samples which includes both living and dead plants and animals (Knox, 2007). However, the organic carbon content of detritus probably exceeds the combined carbon content of bacteria, protists and metazoan zooplankton (Knox, 2007). Detritus is often recycled within the water column but in some locations sinking phytoplankton blooms deposit a seasonal pulse of rich organic matter on the sea bed (Turner and Owens, 1995; Gutt et al., 1998, Garrity et al., 2005; Lam and Bishop, 2007). Detritus in the water column and on the sea bed are to some extent available throughout the year. Although the nutritional value of detritus will vary, the large volume of material available makes detritus a potentially important food source.

1.2.6 Krill diet - Previous research

Over the past seventy years it has become clear that the summer blooms of phytoplankton are an important food source for krill. Evidence to support this view comes from numerous microscopic analyses of krill gut contents that have detected large numbers of diatom frustules and remnants of other phytoplankton groups (e.g. Hart, 1934; Pavlov, 1974, Genhai, 1993). Further support is provided by studies that have detected significant levels of the photosynthetic pigment chlorophyll-a in krill digestive tracts (gut pigment analysis) (Pakhomov et al., 1997; Perissinotto et al., 1997; Perissinotto et al., 2000). There is also evidence that the timing of krill spawning around the Antarctic continent varies in relation to the regional timing and intensity of phytoplankton blooms (Spiridonov, 1995).

The major question that remains with regards to krill diet is whether krill also utilise non-phytoplankton food sources to a significant degree. Opinions on this issue are wide-ranging and include the following (i) krill are predominately herbivorous and mainly feed during the summer (Marr, 1962; Quetin et al., 1994), (ii) krill are herbivores during the summer and switch to feeding on copepods and/or detritus during the winter (Boyd et al., 1984; Kawaguchi et al., 1986; Huntley et al., 1994), (iii) krill are omnivores that mainly utilise phytoplankton and heterotrophic protists (Schmidt et al., 2006), or (iv) krill are 'true' omnivores that consume phytoplankton, heterotrophic protists, detritus and metazoans throughout the year (Perissinotto et al., 2000; Pakhomov et al., 2002). Evidence to support a

significant heterotrophic component in krill diet comes from a diverse range of sources that are critically examined in the following sections.

1.2.6.1 Evidence for omnivory - Energy budget models

There have been several attempts to model the energy requirements of krill on a regional scale and relate this to the quantity of food available in the environment. The models include terms to describe the size of the krill population and the energy costs for activities such as feeding, growth and respiration. The models also include seasonally variable descriptions of food sources such as phytoplankton and sea-ice algae, and a large number of terms to describe aspects of the Antarctic environment. Analyses using these models suggest that, in both the West Antarctic Peninsula and the Scotia Sea, the energy requirements of the krill population exceeds the energy available in the form of phytoplankton and sea ice algae. This implies that krill require non-algal food sources to meet their energy needs (Hofmann and Lascara, 2000; Fach et al., 2002; Fach et al., 2006). However, the extent to which the models are vulnerable to error or bias is unclear.

The estimates of krill energy requirements are thought to be conservative because they do not include terms for energetically expensive activities such as reproduction. This supports the conclusion that krill must be omnivorous. On the other hand, the accuracy of terms that describe the energetic cost of activities such as respiration and feeding are uncertain because they are based on measurements taken from krill kept in captivity where they do not swim, feed or respire at normal rates (Quetin et al., 1994; Ritz, 2000; Atkinson et al., 2006). It is unclear whether this leads to over or under estimates of energy requirements (compare Quetin et al., 1994; Ritz, 2000). Furthermore, it is unclear whether the phytoplankton food source available to krill is accurately described by regional averages (Hofmann and Lascara, 2000), or by satellite derived Chlorophyll-a data (Fach et al., 2002; Fach et al , 2006). Krill may be capable of maintaining themselves in areas with higher than average phytoplankton density (Holm-Hansen and Huntley, 1984), or feeding on deep phytoplankton blooms that are not detected via satellite (Fach et al., 2006). In addition, these studies convert phytoplankton chlorophyll-a values to carbon ratios using a carbon . chlorophyll-a ratio of 50 (Hofmann and Lascara, 2000). This value is at the low end of the range of phytoplankton carbon : chlorophyll-a ratios that have been measured in Antarctic waters (46-144) (Hewes

et al., 1990) and may underestimate the carbon ration obtained from phytoplankton (Atkinson et al., 1996; Pakhomov et al., 2002). Thus, computational modelling suggests that krill must be omnivorous, but such analyses lack power because of the uncertainty associated with many fundamental aspects of krill biology in the Southern Ocean.

1.2.6.2 Evidence for omnivory - Gut pigment analysis

Several studies have used gut pigment analysis to estimate the rate at which krill ingest phytoplankton and used this data to argue that krill are omnivores. Gut pigment analysis estimates phytoplankton ingestion rates by measuring levels of chlorophyll-a extracted from the gut of individual krill (e.g. Atkinson and Snyder, 1997; Pakhomov et al., 1997; Perissinotto et al., 1997; Pakhomov and Froneman, 2004b). Phytoplankton ingestion rates were generally found to be insufficient to meet krill's basic metabolic requirements implying that krill require non-algal food sources to meet their energy needs (Atkinson and Snyder, 1997; Pakhomov et al., 1997; Perissinotto et al., 1997; Pakhomov et al., 2002). Other indicators of omnivory derived from gut pigment analysis are: (1) evidence that egestion rates based on faecal pellet production are three times higher than phytoplankton ingestion rates, which implies that two thirds of the egested material is derived from heterotrophic sources (Pakhomov et al., 1997); and (2) experiments that have measured krill's total carbon consumption (without the ability to identify the source) and subtracted the amount of carbon derived from phytoplankton. The results suggest that on average 79% of krill dietary carbon is derived from heterotrophic sources (Perissinotto et al., 2000).

Gut pigment analysis is an indirect method of determining that krill are omnivores, and there are several potential problems with this approach. Pigment studies have relied on estimates of krill's energy requirements that are similar to those used in energy budget models and therefore have the same potential flaws. Another concern is the fact that chlorophyll-a is rapidly degraded within the digestive system of krill. Estimates suggest that 58 – 98 % of chlorophyll-a pigment is not recovered (Perissinotto and Pakhomov, 1996; Pakhomov et al., 1997, Perissinotto et al., 1997). Although gut pigment studies use average pigment destruction figures to correct for this problem, the estimate of the amount of phytoplankton consumed is very sensitive to the exact degree of pigment destruction (Atkinson and Snyder,

1997). If methodological problems lead to a consistent underestimation of the amount of phytoplankton ingested then the argument for significant omnivory is undermined.

1.2.6.3 Evidence for omnivory – Field Observations

Several zooplankton surveys have noted that areas with high krill densities have low densities of other metazoan zooplankton (Hosie and Cochran, 1994; Atkinson et al., 1999; Hosie et al., 2000). One potential explanation that was given for these distribution patterns is that krill consume other metazoan zooplankton (Atkinson et al., 1999). The high energy demand of individual krill, the high densities of krill within aggregations and the vast size of some krill aggregations suggests that within aggregations very little would go uneaten (Nicol, 2006). However, the survey studies do not provide direct evidence that favours predation over other explanations such as predator avoidance (Atkinson et al., 1999).

Several observational studies have provided evidence of krill eating sea bed detritus when associated with the sea floor. Krill have been observed during summer at the sea floor of the continental shelf (~ 450 m) (Gutt and Siegel, 1994) and further offshore in the abyssal plains (~ 3500 m) (Clarke and Tyler, 2008) by remotely operated vehicles. On the abyssal plains krill were observed feeding on sea bed detritus (Clarke and Tyler, 2008). During winter, krill collected by nets and light traps near the sea-floor in shallow coastal waters were found to have diatoms and other material of benthic origin in their guts (Kawaguchi et al., 1986; Ligowski, 2000). Feeding on sea-bed detritus has been suggested as a winter survival strategy (e.g. Kawaguchi et al., 1986; Ligowski, 2000). However, due to the limited number of observations, the extent to which krill engage in benthic feeding is unclear (Clarke and Tyler, 2008).

1.2.6.4 Evidence for omnivory - Lipid Analysis

Studies that have analysed the lipid profile of krill have suggested that the presence of specific lipids is evidence of omnivory. The lipid biomarker approach relies on the theory that lipids unique to particular prey groups are transferred into the predator's own lipid profile (Dalsgaard et al., 2003). There is increasing evidence from field studies and feeding experiments that krill lipid profiles are to some degree affected by diet (e.g. Alonzo et al., 2005; Schmidt et al., 2006; Hagen et al., 2007). Much of the work has focused on lipid markers associated with autotrophs and few studies have examined the question of

omnivory. High levels of the ratio of polyunsaturated fatty acids (PUFA) to saturated fatty acids (SFA) has been suggested as a indicator of carnivory. This is based on evidence that metazoans contain high levels of PUFA, and from experiments where krill that were fed copepods had an increase in their PUFA/SFA ratio (Cripps and Atkinson, 2000). However, the use of this ratio has been questioned because some diatoms and flagellates also contain high levels of PUFA, and the PUFA/SFA ratio varies in relation to total lipid content (Stubing and Hagen, 2003).

More recently the presence of C_{20} and C_{22} monounsaturated fatty acids that are characteristic of copepods were detected in krill collected in the field. These results were supported by microscopic gut contents analysis that found evidence of copepod mandibles and appendages in krill collected from the same site (Atkinson et al., 2002; Schmidt et al., 2006). While these results are potentially significant, the low amounts of lipid detected, and doubts about the ability of krill to assimilate these lipids (Stubing et al., 2003), suggest that more work is required to confirm the value of C_{20} and C_{22} monounsaturated fatty acids as indicators of trophic links (Schmidt et al., 2006).

The lipids 18:4(n-3) and 22.6(n-3) have been suggested as indicators of feeding on "flagellates" (Stubing and Hagen, 2003; Schmidt et al., 2006), but these markers do not discriminate between autotrophs and heterotrophs and are therefore unsuitable as indicators of omnivory. This highlights a general problem with the lipid biomarker approach that the conservation of lipid metabolism across large groups of organisms often prevents lipids from differentiating between prey sources. It is also becoming clear that lipid assimilation is a complex process that is affected by life stage, sex, season, metabolism and de novo synthesis (Clarke, 1980; 1984; Virtue et al., 1996; Hagen et al., 2001; Stubing et al., 2003; Alonzo et al., 2005). The current understanding of how these factors influence krill lipid profiles is limited and further work is required to demonstrate that lipid profiles are useful as indicators of trophic links.

Lipid analysis has been used effectively to show that adult krill generate a lipid store of triacylglycerols and phosphatidylcholine during the summer and utilise this store during the winter (Hagen et al., 1996; Falk-Petersen et al., 2000; Hagen et al., 2001; Lee et al., 2006). This work demonstrates that lipid storage is part of the krill's strategy for surviving the winter

when algae becomes scarce. These findings are relevant to the question of whether krill need to resort to omnivory during the winter. It has been suggested that winter survival is achieved primarily through reducing body metabolism (~ 71 %) and that other strategies play a limited role, such as lipid stores (~11%), body shrinkage (~4%), feeding (~1-2%), and unknown (~10%) (Quetin and Ross, 1991). Other calculations have suggested that reduced metabolism and lipid stores may be sufficient for adult krill to survive the winter (Atkinson et al., 2002). An alternative view is that feeding during winter is important and that adult krill switch to feeding on sea-ice biota, copepods or detritus (e.g. Kawaguchi et al., 1986; Marschall, 1988; Huntley et al., 1994; Atkinson et al., 2002). However, the evidence for winter omnivory is limited to a small number of observations (Quetin and Ross, 1991). The fact that krill utilise their lipid store during winter reduces the need for them to resort to winter omnivory.

1.2.6.5 Evidence for omnivory - Isotope Analysis

A small number of studies have used isotope ratios to estimate the trophic position of krill. The approach relies on the theory that metabolic processes concentrate heavier isotopes of nitrogen (N^{15} as opposed to N^{14}) in organisms at higher trophic levels. Trophic position (i.e. primary producer, herbivore, omnivore and carnivore) is determined by comparing an animal's isotopic ratios with a trophic baseline (e.g. particulate organic matter) and other zooplankton in the ecosystem.

Early isotope studies in krill were inconsistent with some results suggesting a correlation between heavy isotope abundance and trophic position (Wada et al., 1987) and others finding no correlation (Rau et al., 1991). Large regional and seasonal variation in the isotope ratios of the particulate organic matter baseline, and the slow assimilation of dietary signals in krill, have been suggested as potential explanations for variable results (Schmidt et al., 2003; Schmidt et al., 2004). More recent work has shifted from sampling whole animals to targeting specific tissues and amino acids that are responsible for the majority of diet related isotope enrichment (Schmidt et al., 2004, Schmidt et al., 2006). Using these improvements the isotope ratios obtained from krill collected in the field suggested that the krill were feeding omnivorously with some differentiation in isotope ratios between sites that was consistent with the local availability and diversity of phytoplankton (Schmidt et al., 2006).

Feeding trials are still required to demonstrate changes in isotope ratios occur in response to diet (Schmidt et al., 2006). Isotope ratios are unable to differentiate between predation on heterotrophic protists and metazoans (Schmidt et al., 2006). Overall the evidence for omnivory provided by isotope ratios remains limited.

1.2.6.6 Evidence for omnivory - Feeding experiments

Tank-based feeding experiments have demonstrated that krill are capable of feeding on heterotrophic protists and copepods at rates that are similar to, or greater than, the rate at which they consume phytoplankton (Price et al., 1988; Graneli et al., 1993, Atkinson and Snyder, 1997; Atkinson et al., 2002). These feeding experiments provide some of the strongest evidence that krill are omnivores. However, there are concerns about whether feeding experiments accurately reflect krill feeding behaviour in the field. Feeding rates can be affected by stress due to capture and container-effects such as wall collisions (Price et al., 1988; Atkinson et al., 2002). It is also difficult to generate mixed assemblages that accurately reflect feeding conditions in the field (Atkinson and Snyder, 1997, Atkinson et al., 2002). One aspect of this problem is that confining copepods in tanks may limit their escape responses leading to unrealistically high predation rates. It has been noted that tank-based feeding rates on copepods far exceed field-based evidence for copepod predation (Schmidt et al., 2006).

1.2.6.7 Evidence for omnivory - Microscopic diet analysis

Microscopic analysis of krill gut contents has provided evidence that krill are to some degree omnivores. Microscopic gut analysis involves the extraction of the digestive tract and the identification of taxonomically distinct items. Gut contents are typically examined in water either in glass cavity mounts or on slides with magnification ranging from 10 X – 600 X. Prey identification ranges from broad taxonomic categories down to species specific identification and depends on what structures survive ingestion. In general microscopy is significantly better than pigments, lipids and isotopes for the identification and differentiation of prey.

Microscopy has provided direct field-based evidence that, in addition to phytoplankton, krill consume a diverse range of heterotrophic protists including choanoflagellates, dinoflagellates, foraminiferans and radiolarians. Evidence for the consumption of heterotrophic protists has been reasonably consistent over the history of microscopic diet

studies but their contribution remains poorly quantified (Table 1-1). Microscopy has also detected metazoan zooplankton groups including copepods, ostracods, polychaetes, coenlenterates and pteropods. In general the rates of metazoan prey detection have been low. The small number of studies that have reported high incidences of metazoan prey are balanced by studies that have analysed large samples of krill and detected very few metazoans (Table 1-1).

Microscopy suffers from two methodological problems that have limited the application of this approach. The first is bias towards prey with identifiable structures that survive ingestion (Atkinson and Snyder, 1997; Perissinotto et al., 2000). The extent to which this hard-part bias has skewed perception of krill diet is unclear, but there is a range of soft-bodied autotrophs and heterotrophs that would not be detected with microscopy. The second problem is that microscopy is labour intensive (Boyd et al., 1984; Perissinotto et al., 2000) which has often limited microscopic studies to small sample sizes and qualitative or semi-quantitative descriptions of the prey consumed (Table 1-1, Boyd et al., 1984; Schmidt et al., 2006). These problems have prevented microscopy from providing quantitative data on the scale required to effectively answer diet related questions.

1.2.7 Krill diet - Summary and Conclusions

Studying the diet of Antarctic krill is important for understanding the flow of nutrients through the Southern Ocean ecosystem (e.g. Treguer and Jacques, 1992; Froneman et al., 1996, Froneman et al., 2000) and may also explain the distribution patterns of organisms affected by krill predation (Smetacek et al., 2004; Smith and Lancelot, 2004). Although recent opinion has suggested that krill are omnivores the evidence to support this position is limited. Much of the evidence comes from indirect methods that need further support to verify their findings (i.e. modelling, zooplankton surveys, feeding experiments and gut pigment analysis). Direct methods applied to animals collected in the field have struggled to detect, identify and quantify the heterotrophic component of krill diet (i.e. lipids, isotopes and microscopy). Where field-based evidence for omnivory has been obtained it has been limited to small sample sizes and qualitative assessments. As a result, the role of heterotrophic material in krill diet remains unclear (Perissinotto et al., 2000).

The analysis of krill diet may benefit from the application of new diet analysis methods. Previous work provides several criteria for meaningful advances in understanding krill diet. New methods need to develop the capacity to detect and differentiate a broader range of potential prey groups, particularly heterotrophs. New methods should focus on analysing krill collected in the field to provide direct evidence of diet without artefacts. Finally, previous studies have identified that krill consume a diverse range of prey and the replication of these findings on small samples of krill with new methods is of little benefit. New methods need to develop the capacity to provide quantitative data from sample sizes that are ecologically relevant.

1.3 DNA diet analysis

DNA is an attractive option as a dietary biomarker because: (1) it is present in all types of prey, (2) it enables a high degree of differentiation between prey groups, and, (3) it has the potential for high sample throughput. DNA therefore fulfils many of the criteria identified above as important for advancing the analysis of krill diet. DNA diet analysis is part of a broader field of research that involves the extraction and analysis of DNA from environmental samples. Most of the pioneering work in this area has been in environmental microbiology where DNA has been used to characterise bacterial communities from terrestrial and marine environments and investigate their role in driving important biological processes (e.g. van Hannen et al., 1999; Moon-van der Staay et al., 2001; Sogin et al., 2006). Environmental microbiologists have developed many of the techniques used in DNA diet analysis and highlighted many of the pitfalls associated with these approaches (von Wintzingerode et al., 1997). In the analysis of ancient DNA extracted from field samples, researchers have grappled with the problems of amplifying small amounts of highly degraded DNA, and the important issue of sample contamination (Cooper and Poinar, 2000).

DNA diet analysis has previously been applied to both vertebrate and invertebrate predators. All studies have used the polymerase chain reaction (PCR) to amplify prey DNA for analysis. The first successful application of DNA diet analysis was the PCR amplification of a 356 bp fragment of plant chloroplast DNA from the scat of a brown bear (Hoss et al., 1992). Since then work on a range of vertebrate and invertebrate predators has continued to produce positive results (reviewed in Symondson, 2002; Sheppard and Harwood, 2005). Studies on

vertebrate predators have generally focused on the development of non-invasive methods based on the analysis of scats. Concerns about invasive sampling generally do not extend to invertebrates and these studies typically extract prey DNA from intact predators or from part of their digestive system. In most other respects the techniques applied to vertebrates and invertebrates have been similar and the work on vertebrates has been reviewed elsewhere (Symondson, 2002; Sheppard and Harwood, 2005).

The first successful application of DNA diet analysis to an invertebrate predator [3] described the detection of stone flounder in sand shrimps (Asahida et al., 1997). The experiment was a feeding trial where shrimp were fed pieces of flounder and then kept under starvation conditions. Following the feeding trial DNA was extracted from shrimp stomachs and assayed for the presence of flounder DNA with PCR. The PCR assays targeted the mitochondrial genome because it occurs in multiple copies per cell. The study developed two sets of PCR primers. The first primer set amplified a 2500 - 2800 base pair (bp) DNA fragment from a range of fish. The second primer set amplified a 1460 bp fragment and was specific to flounder. Both primer sets detected flounder for up to 5 hours after feeding but the primer set that amplified the shorter DNA fragment provided more consistent detection. Similar feeding experiments have since been conducted on a range of terrestrial and marine invertebrates including beetles, copepods, mites and spiders (Table 1-2). Three common themes have emerged from these studies. First, all studies used PCR-based presence / absence detection assays that target a single prey species. Second, prey detection was improved by targeting genes that are present in multiple copies per cell usually the ribosomal genes from the nuclear genome or a variety of genes from the mitochondrial genome. Third, targeting short regions of DNA (generally < 400 bp) improves prey detection (e.g. Agustí et al., 2000; Chen et al., 2000; Hoogendoorn and Hempel, 2001). Across the studies the length of time that prey could be detected after feeding varied widely from 0 - 72 hours (Agustí et al., 1999, Zaidi et al., 1999; Cuthbertson et al., 2003) . This variation probably reflects differences in assay systems, the lengths of DNA fragments that were targeted, and, differences between the predator's digestive systems.

[3] In the context of this thesis the terms predator and prey are used in the broadest sense to describe one organism consuming another organism. No distinction is made between animals that consume plants, fungi, protists or other animals.

Several studies have progressed from the laboratory into the field. The first field based application of DNA diet analysis to invertebrates examined spider predation on collembolans in cereal crops (Agustí et al., 2003a). The authors developed three species specific PCR assays to detect three species of Collembola and used these assays to screen 50 spiders for the presence of collembollen prey. By comparing the frequency of collembollans detected in spiders with the frequency of collembolans in the field, the authors were able to show that the spiders had a preference for feeding on a particular collembolan species. Several studies have since applied DNA diet analysis to invertebrates collected in the field (Table 1-2). Methods of preserving and extracting prey DNA varied widely and no clear consensus on the best method emerges. The majority of field studies have utilised single species detection assays similar to the laboratory studies described earlier.

Two studies have moved beyond single species detection assays to the detection of multiple prey. An innovative study on the diet of wasps applied PCR to meals brought back to the nest in the jaws of foraging wasps (Kasper et al., 2004). The study used 'universal' PCR primers that targeted the mitochondrial genome and were capable of amplifying a 500 - 600 bp DNA fragment from a diverse range of species. PCR amplified DNA fragments were sequenced and then identified by matching them to sequences in the GenBank sequence database. This approach allowed a large range of prey to be identified including several orders of insects and flesh from vertebrates including chicken and kangaroo. Identifying prey with GenBank avoided the need to characterise all prey prior to diet analysis but relied on GenBank to provide coverage of consumed prey and accurate identification through BLAST matching (BLAST algorithm is described in Altschul et al., 1990). By collecting material from returning wasps, the study avoided the problem of having to separate out a mix of PCR amplicons derived from different prey. For most invertebrate predators this is not an option. Samples extracted from the predator and amplified with universal primers will contain DNA from multiple prey species that must be separated before they can be identified. The second study examined ground beetles consuming aphids, weevils, earthworms and slugs (Harper et al., 2005). The study developed several sets of PCR primers that amplified DNA from groups of related species (group specific primers) or were species specific, each primer set was labelled with a different fluorescent sequencing dye. PCR with all primers sets was conducted in a single multiplex PCR reaction. Amplified DNA fragments were separated on a

gel-based sequencing apparatus by size and dye colour and identified by comparing the resulting bands with known standards. This approach effectively dealt with multiple prey items in a system with low to moderate prey complexity. In addition, the use of automated sequencing technology allowed rapid sample screening. The approach required prior characterisation of all prey to allow accurate scoring of the results and with only four sequencing dyes available the method can only cope with a maximum of four prey types that produce DNA fragments of a similar size (Harper et al., 2005). The study did not address the issue of assay sensitivity or discuss the relative sensitivity of multiple PCR assays.

In summary, studies that have examined DNA diet analysis have demonstrated that the prey DNA can be detected in a wide variety of invertebrate predators. To date, DNA diet studies have relied on PCR to amplify prey DNA fragments for analysis. Several studies have shown that targeting short multi-copy regions of DNA provides optimal detection. Most studies have focused on assays that detect a single prey species. Assay systems that detect multiple prey species are still in the early stages of development.

1.4 Thesis outline

The work in this thesis examines the application of DNA diet analysis to Antarctic krill. Much of the focus is on assessing whether this approach is capable of answering important biological questions related to krill diet. The four chapters showing results (Chapters 2-5) are written in the style of independent scientific papers. Chapter 2 describes a feasibility study which examines whether krill are amenable to DNA diet analysis. The work describes the practical application of DNA diet analysis to krill collected in the field and explores methods for preserving, extracting and analysing prey DNA through the use of DNA clone libraries. The amount of prey DNA extracted from krill was also determined to assess how robust the approach was likely to be. Finally the DNA results were compared with results from microscopic diet analysis to assess the accuracy of the DNA data. Chapter 3 examines an issue raised in Chapter 2 that relates to the presence of predator DNA in diet samples and problems this causes in relation to efficient processing and analysis of dietary DNA. The study explores potential methods of removing predator DNA or preventing predator DNA amplification during PCR. Chapter 4 returns to the detection of prey in krill stomachs and explores the use of incidence based approaches to provide quantiative data on krill diet. The

study applies multiple sets of PCR primers targeting a range of protists and invertebrates that are potential krill prey. Comparisons with microscopic diet analysis were again utilised to assess whether results obtained were an accurate representation of diet. Chapter 5 examines the stability of prey DNA in krill stomachs to verify that prey DNA extracted from krill stomachs was representative of feeding behaviour for a significant period of time prior to collection. The work describes a feeding trial where the dynamics of prey DNA accumulation and decay in krill stomachs was tracked through time using quantitative PCR. The study also identified some of the factors involved in these processes. The thesis concludes with a final discussion (Chapter 6) on the current state of DNA diet analysis as applied to Antarctic krill and outlines topics for future research.

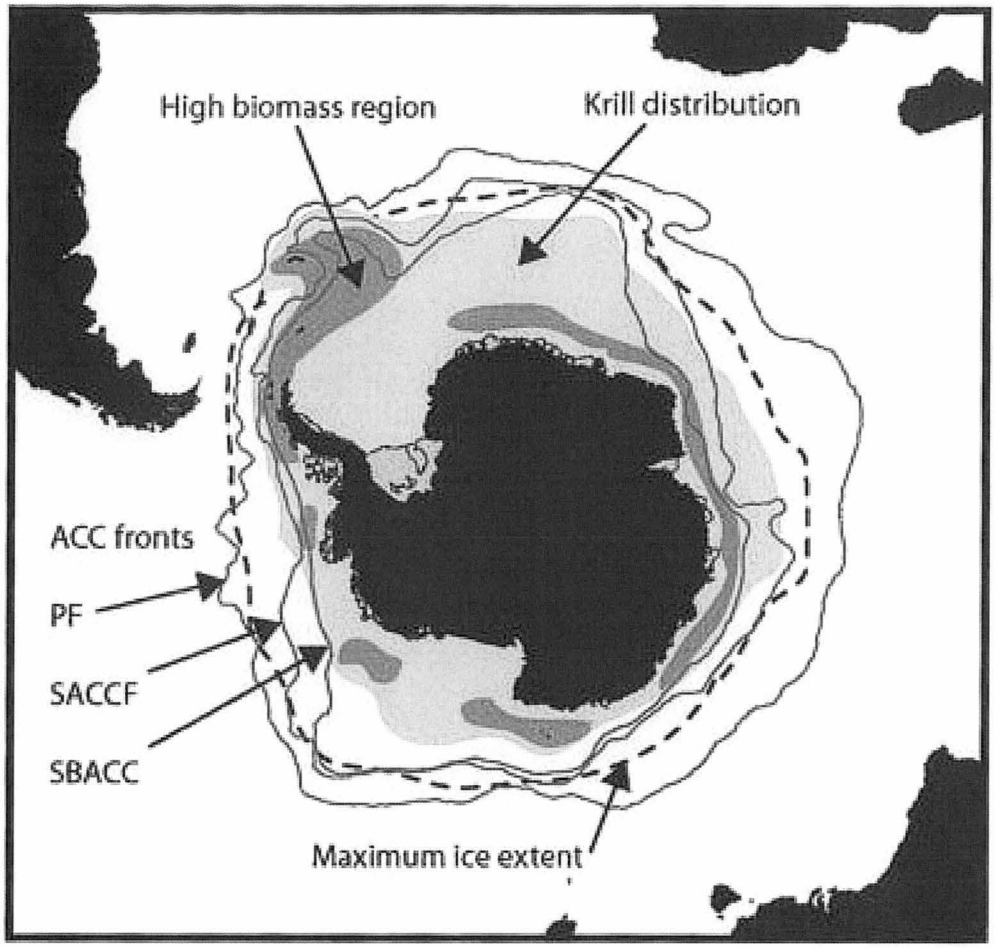

Figure 1-1 Krill distribution and abundance. Fronts are the boundaries between large scale water masses, PF = Polar front, SACCF = Southern Antarctic Circumpolar Current Front, SBACC = Southern Boundary Antarctic Circumpolar Current. Figure taken from (Hofmann and Murphy, 2004)

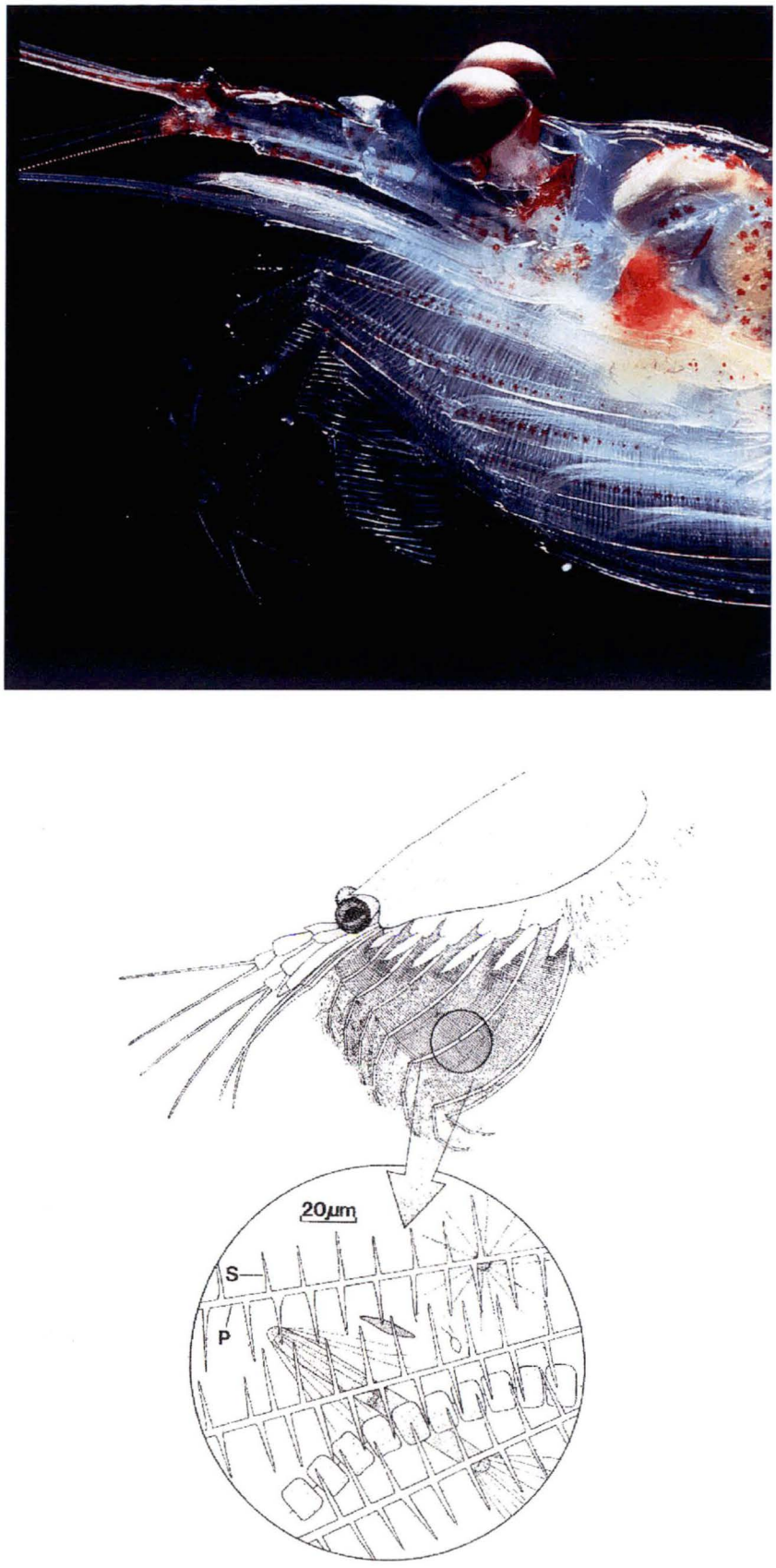

Figure 1-2 Krill feeding apparatus displaying the thoracopods lined with fine setae. Photograph by Rob King

Table 1-1 Summary of studies that have examined krill diet via the microscopic examination of their gut contents

Reference	Region	Season	# of krill	Choanoflagellate	Ciliophora	Dinoflagellata	Foraminifera	Radiolaria	Amphipoda	Ctenophora	Copepoda	Ostracoda	Polychaeta	Pteropoda	Crustacean	Comments
Hart as cited by (Marr, 1962)	?	Summer, 1937-38	40			Yes	Yes				Yes					Work unpublished. (Marr, 1962) provided description and qualitative list of groups identified by Hart. Radiolaria not recorded
Barkley, 1940 as cited by (Marr, 1962)	?	?	~1550		Yes		Yes	Yes								Study in German and was not available. (Marr, 1962) provided qualitative list of groups identified by Barkley. Crustaceans and dinoflagellates were not recorded (Schmidt et al., 2006) provided figure for number of krill analysed.
(Pavlov, 1969)	Scotia Sea	Jan-Mar, 19??	450								2					
(Pavlov, 1974)	Atlantic Sector	Feb-Mar, 1965	~600		Yes		Yes	Yes			8					English translation of Russian study published in 1971. Generally a qualitative description of stomach contents although figures for some groups provided. Evidence of cannibalism.
(Hopkins, 1985)	WAP	Mar-Apr, 1983	57		1 *			1 *			2 *		1 *			*-Data presented as number of krill that contain prey group.
(Marchant and Nash, 1986)	Indian Ocean	Summer, 1981-85	ND	Yes												Qualitative Description. Electron Microscopy.

Reference	Region	Season	# of krill	Choanoflagellate	Ciliophora	Dinoflagellata	Foraminifera	Radiolaria	Amphipoda	Ctenophora	Copepoda	Ostracoda	Polychaeta	Pteropoda	Crustacean	Comments
(Hopkins and Torres, 1989)	Weddell Sea	Mar, 1986	96		49 *	39 *	2 *	6 *		57 *	23 *	3		3 *		*-Data presented as number of krill that contain prey group Sampled adults, juveniles and larvae in similar numbers. The 30 larvae had no heterotophic material.
(Daly, 1990)	Scotia Sea	Jun-Aug, 1988	ND	Yes	Yes	Yes	Yes									Qualitative description. Light and Electron microscopy. Larval and juvenile krill
(Lancraft et al., 1991)	Scotia Sea	Jun-Aug, 1988	30		15 *	3 *		1 *			2 *		1 *			*-Data presented as number of krill that contain prey group.
(Nordhausen et al , 1992)	WAP	Jul-Aug, 1992	ND								Yes					Qualitative description of finding copepods in gut.
(Genhai, 1993)	WAP	Summer, 1986-87	340	Rare	Rare	Rare									Rare	Semi-Quantitative description. Groups defined as absent, rare, abundant or very abundant Electron Microscopy.
(Hopkins et al., 1993)	Scotia Sea	Nov-Dec, 1983	20		2 *	12 *	4 *	12 *			2 *					*-Data expressed as number of krill that contain prey items Also detected Siphonophore (1 gut) and invertebrate eggs (13 guts)
(Huntley et al., 1994)	WAP	Jul-Aug, 1994	ND								Yes					Qualitative description of finding copepods in gut.

Reference	Region	Season	# of krill	Choanoflagellate	Ciliophora	Dinoflagellata	Foraminifera	Radiolaria	Amphipoda	Ctenophora	Copepoda	Ostracoda	Polychaeta	Pteropoda	Crustacean	Comments
(Nishino and Kawamura, 1994)	Scotia Sea	Jul-Aug, 1993	130		Yes	Yes	Yes	Yes			Rare					Qualitative description. Specifically states that copepods were rarely detected. 77 animals engaged in cannibalism. Sampled mainly adults and juveniles with some larvae
(Pakhomov et al., 1997)	Scotia Sea	Feb-Mar, 1994	28	-	6 - 13	0 - 20	-	0 - 1	-	-	-	-	0 - 2	-	5 - 13	Data presented as average abundance of food items per site derived from 3 - 5 individuals per site for 6 sites. Data summarised as the range of average item abundance across the six stations. The total number of food items identified per krill ranged from 31 - 99 across the six sites.
(Perissinotto et al., 1997)	Atlantic	Jan, 1993	28	-	11 - 51	1 - 10	-	-	-	-	-	-	3 - 6		7 - 15	Data presented as average abundance of food items per site derived from 3 - 5 individuals per site for 6 sites. Data summarised as the range of average item abundance across the six stations. The total number of food items identified per krill ranged from 50 - 167 across the six sites.
(Hernandez-Leon et al , 2001)	WAP	Feb, 1993	ND												0 - 3	Data presented as crustacean fragments per krill gut. Text states mainly appendages and implies that they are copepods.

Reference	Region	Season	# of krill	Choanoflagellate	Ciliophora	Dinoflagellata	Foraminifera	Radiolaria	Amphipoda	Ctenophora	Copepoda	Ostracoda	Polychaeta	Pteropoda	Crustacean	Comments
(Atkinson et al., 2002)	Lazarev Sea	Apr, 1999	12								0 - 5				0 - >10	Copepod data is number of mandibles detected. Crustacean fragments not described in detail.
(Meyer et al., 2002)	Lazarev Sea	Apr, 1999	60													Diatom frustules were the only recognizable food item. Electron microscopy. Larval krill only.
(Schmidt et al., 2006)	Scotia Sea	Jan-Feb, 2003	60		0 - 350	251-2300					M=0 - 2 A=0 - 11					Data presented as life stage averages from 5 sites. Data summarised as range of averages. Copepod data M=Mandibles, A=appendages. Heterotrophic protists significant in terms of volume representing 5-15% of total volume overall and up to 70% at some sites.

Table 1-2 Summary of studies that have applied DNA diet analysis to invertebrate predators

Predator	Diet items detected	Sample preservation method	Sample tested	DNA extraction method	Amount of template DNA	PCR cycles	Gene	Marker size	Sample size	Field study	Reference
Sand Shrimp *(Crangon affinis)*	Stone Flounder *(Kareius bicoloratus)*	Frozen -80°C	Stomach contents	Proteinase K+SDS modified to include 8M UREA in initial buffer	10-300 ng	30	Mitochondrial D-loop	1400, 2800	24	No	(Asahida et al., 1997)
Plant Bug *(Dicyphus tamaninii)*	Moth Eggs *(Helicoverpa armigera)*	Frozen -20°C	Whole predator	CTAB	2.5 µl	45	Nuclear? RAPD fragment	1100 (fail) 600 254	20	No	(Agustí et al., 1999)
Ground Beetle *(Pterostichus cupreus)*	Mosquito*(Culex quinquefasciatus)*	Frozen ?-20°C?	Whole predator minus appendages	SDS lysis buffer+homogenization	100 ng	31	Nuclear multicopy estrase	146, 263	70	No	(Zaidi et al , 1999)
Plant bug *(Dicyphus tamaninii)*	Whitefly *(Trialeurodes vaporariorum)*	Freezing -20°C	Whole predator	CTAB	2.5 µl	45	Nuclear? RAPD fragment	2100 (fail) 310	20	No	(Agustí et al., 2000)
Ladybird beetle & Lacewing	Cereal Aphids	Freezing -20°C	Whole predator	SDS lysis buffer+homogenization	10-100 ng	35	Mitochondrial COII	198, 246, 339	>100	No	(Chen et al., 2000)
Ladybird beetle *(Coleomegilla maculata)*	Moth eggs *(Ostrinia nubilalis)*	Frozen then 70% ethanol at -20°C	Whole predator	Ref Bender et al 1983	2 µl	30	Nuclear rDNA	100, 220, 343, 463	94	No	(Hoogendoorn and Hempel, 2001)
Linyphiid spiders	Collembola	Frozen -20°C	Whole predator	DNeasy tissue kit (Qiagen)	4 µl	35	Mitochondrial COI	211, 216, 276	82	Yes	(Agustí et al., 2003a)
Minute pirate bug *(Anthocoris tomentosus)*	Pear psylla *(Cacopsylla pyricola)*	Frozen -20°C	Whole predator	DNeasy tissue kit (Qiagen)	6 µl	35	Mitochondrial COI	188, 271	140	No	(Agustí et al., 2003b)

Predator	Diet items detected	Sample preservation method	Sample tested	DNA extraction method	Amount of template DNA	PCR cycles	Gene	Marker size	Sample size	Field study	Reference
Predatory Mite *(Anystis baccarum)*	Aphid *(Rhopalosiphum insertum)*	Some fresh some frozen - 18°C	Whole predator	high pure PCR template preparation kit	1 µl	37	Mitochondrial Span ND1 & LrRNA	283	14	No	(Cuthbertson et al., 2003)
Copepod *(Calanus finmarchicus)*	Haptophyte algae *(Emiliania huxleyi)*	Frozen -80°C	Whole predator &fecal pellets	Ultra clean soil DNA extraction kit	4-10 µg typo? ng?	35	Nuclear rDNA	58	45?	No	(Nejstgaard et al., 2003)
Wasps	Various	On ice then 70% ethanol	Prey collected from mouth parts	Puregene DNA isolation kit	10-100 ng	35	Mitochondrial 16S	500 - 650	71	Yes	(Kasper et al., 2004)
Ladybird Beetle *(Curinus coeruleus)*	Moth Catapillars	100% Ethanol or crushed and dried	Whole predator	QIAamp® DNA Stool Mini Kit (Qiagen)	2-5 µl	30-35	Mitochondrial COI	140 - 170	46	No	(Sheppard et al , 2004)
Bivalve and Amphipods	Various	Frozen -80°C and 70% Ethanol	Bivalve gut and Amphipod hindgut	DNeasy tissue kit (Qiagen) and Forensics Kit (MoBio)	4 µl	40	Mitochondrial COI and Nuclear 18S	~700	6	Yes	(Blankenship and Yayanos, 2005)
Ground beetle *(Pterostichus melanarius)*	Slugs and Aphids	Frozen -80°C	Gut tissue	QIAamp DNA stool kit (Qiagen)	4 ul	35	Mitochondrial 12S and COI	109, 254	35	No	(Foltan et al., 2005)
Ground beetle *(Pterostichus melanarius)*	Various	Frozen -80°C	Gut tissue	DNeasy tissue kit (Qiagen)	0.75 µl	35-40	Mitochondrial COI & 12S	78 - 242	>50	Yes	(Harper et al , 2005)

Predator	Diet items detected	Sample preservation method	Sample tested	DNA extraction method	Amount of template DNA	PCR cycles	Gene	Marker size	Sample size	Field study	Reference
Ground beetle larvae *(Poecilus versicolor)*	Scarab beetle larvae and eggs *(Melolontha melolontha)*	Frozen -28°C	Whole predator	Homogenization+ Proteinase K+SDS +CTAB	3 µl	35	Mitochondrial COI	175 - 585	>200	No	(Juen and Traugott, 2005)
Damsel bug and spider	Moth larvae *(Plutella xylostella)*	Frozen -20°C	Whole predator	SDS lysis buffer+homogenization	500 ng	35	Nuclear ITS-1	275	>100	Yes	(Ma et al , 2005)
Ground beetle *(Pterostichus melanarius)*	Aphids / spiders (secondary predation)	Frozen -? 20°C?	Foregut	QIAamp® DNA Stool Mini Kit (Qiagen)	4 µl	35	Mitochondrial COI	110, 245	>300	No	(Sheppard et al., 2005)
Copepod *(Pareuchaeta norvegica)*	Copepod *(Calanus helgolandicu)*	Frozen -80°C	Feacal pellets	None-direct use of feacal pellets sample in PCR	?	Nested 40 then 38	Mitochondrial COI	172	14	Yes	(Vestheim et al., 2005)
Antarctic krill (*Euphausia superba*)	Various	Frozen -70°C	Stomach contents and faecal pellets	Proteinase K+SDS	~ 10 ng	28-35	Nuclear rDNA	~240	>10?	Yes	(Martin et al., 2006)
Antarctic krill (*Euphausia superba*)	Diatoms	Frozen 80°C and 80% Ethanol	Intact stomachs	DNeasy tissue kit (Qiagen)	2 µl	30	Nuclear rDNA	~145	6	Yes	(Passmore et al., 2006)
Beetle (*Rivacindela sp.*)	Various	?	Whole predator	?	?	40	Mitochondrial cytochrome b	358	14	Yes	(Pons, 2006)
Various	Beetle (*Phyllopertha horticola*)	Frozen -28°C	Whole predator	Homogenization+ Proteinase K+SDS +CTAB	2 µl	35	Mitochondrial COI	291	332	Yes	(Juen and Traugott, 2007)

Predator	Diet items detected	Sample preservation method	Sample tested	DNA extraction method	Amount of template DNA	PCR cycles	Gene	Marker size	Sample size	Field study	Reference
Anthocorid (*Orius insidiosus*)	Various	Frozen -80°C	Whole predator	Proteinase K+SDS	?	35-45	Mitochondrial COI	160 - 281	~ 240	Yes	(Harwood et al., 2007)
Appendicularian (*Oikopleura dioica*)	Various algae	Frozen -80°C	Whole predator	DNeasy tissue kit (Qiagen)	50 ng	40	Nuclear rDNA	57 - 82	30	No	(Troedsson et al., 2007)
Various	Wasp (*Aphidius colemani*)	95% Ethanol	Whole Predator	Proteinase K + Chelex resin	2 µl	35	Elongation Factor 1 alpha	185	165	Yes	(Chacón et al., 2008)
Various	Wasp (*Lysiphlebus testaceipes*)	Frozen -24°C	Whole Predator	Proteinase K +Chelex & DNeasy blood tissue kit (Qiagen)	1.5 µl	40	Mitochondrial COI	122 - 369	~ 140	No	(Traugott and Symondson, 2008)
Ground Beetle (*Pterostichus melanarius*)	Aphid (*Sitobion avenae*)	Frozen -24°C	Foregut	DNeasy blood tissue kit (Qiagen)	3 µl	40	Mitochondrial COI	231	~ 192	No	(von Berg et al , 2008b)
Ground Beetle (Various)	Aphid (*Sitobion avenae*)	Frozen -80°C	Digestive Tract	DNeasy blood tissue kit (Qiagen)	3 µl	40	Mitochondrial COI	85 - 383	104	No	(von Berg et al., 2008a)
Spiders (Various)	Aphid (*Sitobion avenae*)	Frozen -24°C	Whole Predator	DNeasy tissue kit (Qiagen)	4.45 µl	40	Mitochondrial COI	316	493	Yes	(Birkhofer et al., 2008)
Rock Lobster (*Jasus edwardsii*)	Various	70% Ethanol	Faeces	Ultra Clean Fecal DNA Kit (Mo Bio Laboratories)	20 ng	35	Mitochondrial COI & Nuclear rDNA	~ 150	10	No	(Redd et al., 2008)

Chapter 2

DNA as a dietary biomarker in Antarctic krill (*Euphausia superba*): a feasibility study

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2.1 Abstract

The diet of Antarctic krill (*Euphausia superba*) has been studied using a variety of techniques but current methods still suffer from problems that are difficult to solve. The aim of this study was to examine the feasibility of using DNA as dietary biomarker for Antarctic krill. Methods were developed for the preservation, extraction and identification of prey DNA from krill collected in the field. Group specific PCR primers were developed to amplify diatom prey (Phylum: Bacillariophyta), a critically important food item for krill, and the results from DNA clone libraries were compared with microscopic diet analysis. DNA analysis was superior to microscopy for prey detection. However, differences in prey relative abundance estimates between the two techniques suggested some bias in the DNA-based estimates. Quantification of the amount of DNA template extracted from krill stomachs showed that large amounts of prey DNA had been successfully preserved and extracted. Overall the results suggest that the application of DNA-based diet analysis to krill warrants further investigation, particularly for prey that are difficult to study using other methods.

2.2 Introduction

The Antarctic krill (*Euphausia superba*) is central to the Southern Ocean ecosystem. Krill are major grazers of phytoplankton and have been described as predominately herbivorous (Hart, 1934; Quetin and Ross, 1991). On the other hand there is also evidence that krill also consume a range of protozoa and small zooplankton (Genhai, 1993; Atkinson and Snyder, 1997; Pakhomov et al., 1997). A recent study comparing the amounts of dietary carbon derived from autotrophic and heterotrophic sources suggested that heterotrophic carbon makes a significant and sometimes dominant contribution to krill diet (Perissinotto et al., 2000). This finding implies that important components of krill diet may have gone undetected and has prompted a call for new diet analysis techniques (Perissinotto et al., 2000).

Using DNA as a prey biomarker for krill is an alternative approach that warrants investigation. All organisms have unique DNA sequences that can be used as biomarkers for detection and identification and these can be used to study any prey species or group of interest (Symondson, 2002; Jarman et al., 2004). DNA methods also offer potential for rapid, high throughput screening of samples (e.g. Harper et al., 2005).

Prior to this work there were no studies that had applied DNA diet analysis to Antarctic krill although subsequently one study from another laboratory has been published (Martin et al., 2006). The current study describes the development of methods for the preservation, extraction, and analysis of prey DNA from krill collected in the field. Diatoms were targeted in this initial work becuase they are an important food item for krill and their presence in the diet can be verified by microscopy. Once suitable techniques were established, three krill from each of two field collection sites underwent intensive diet analysis that included microscopic diet analysis, prey DNA clone library analysis and prey DNA quantification to determine the feasibility of applying DNA diet analysis to krill. Issues with the technique and potential improvements are discussed.

2.3 Materials and Methods

2.3.1 Krill collection

Antarctic krill were collected in the Prydz bay region of the Southern Ocean during the KAOS voyage with the RSV *Aurora Australis*. Krill were collected from Site A (-66.5575, 64.0670) on the 18th January 2003 and from Site B (-66.5890, 69.6087) on the 12th February 2003 during daylight hours. Schools of krill were detected with sonar and collected in targeted rectangular midwater trawls (RMT8, nominal mouth area of 8 m^2 and mesh size of 4.5 mm) at a towing speed of ~ 1 knot. The two sites were 134 nautical miles apart and both sites were approximately 65 nautical miles from the Antarctic coast.

2.3.2 Preservation of krill for DNA diet analysis

Ship based sample collection requires sample preservation methods that are fast, simple and safe. In addition, the ideal method would preserve prey DNA within intact krill thereby avoiding the need to perform delicate and time consuming dissection procedures at sea. Two methods of preserving intact krill were examined, preservation in ethanol and preservation by freezing. The ethanol preservation method was developed based on recommendations from previous field studies that had successfully preserved animal tissue for DNA analysis (Masner, 1994; Dawson et al., 1998). Live krill were dropped into 2L plastic sample containers filled with 80% ethanol (v/v). Samples were further dehydrated by draining and replacing the original 80% ethanol twice, once 15 minutes after the initial preservation and a second time 24 hours later. Samples were subsequently stored at –20 °C. To maintain

a high concentration of ethanol, the volume of krill was not allowed to exceed a third of the container volume. Attempts were also made to preserve krill in 96% ethanol but this was abandoned because the krill tissue became too brittle for efficient dissection. For preservation by freezing, individual krill were simply inserted into cryotubes and frozen in a –80 °C freezer. All samples were left in storage until the ship returned to Australia. Samples were stored for 6 months before analysis commenced.

2.3.3 Krill dissection

Digestive tissue was extracted from individual krill to increase the proportion of prey DNA in diet samples. Stomachs were targeted for diet analysis because trial dissections led to the observation that krill stomachs were large and easy to isolate compared to other organs of the digestive tract. Also the stomach is located close to the start of the digestive tract which suggested that it may contain less degraded prey DNA than other parts of the digestive tract.

Dissections were performed using a dissecting microscope and two pairs of forceps. Individual krill were rinsed in fresh ethanol and gently dried on paper towel prior to dissection in a dry sterile Petri dish. Forceps were used to remove the carapace and expose the animal's stomach on the dorsal surface. Intact stomachs were then removed with forceps, taking care not to squeeze out the stomach contents. To prevent the transfer of contaminants from the external surface of the krill to the stomach tissue, care was taken not to make contact with the stomach during the removal of the carapace. Forceps were flame sterilized prior to the removal of the stomach and between each krill dissection.

2.3.4 Extraction and separation of DNA and 'hard parts' from krill stomachs

To facilitate a direct comparison between DNA and microscopic diet analysis a method was developed to extract and separate the DNA and 'hard part' components of a single krill stomach. Several DNA extraction methods were trialed (CTAB, Guanadinium thiocynate + silica beads, Proteinase K/SDS + NaAc precipitation, Qiagen plant DNAeasy columns) but the following method gave the best prey specific PCR amplification results (data not shown).

DNA extraction was performed on individual krill stomachs using the DNAeasy Tissue kit (Qiagen) according to the manufacturer's instructions except for the following modifications. Prior to overnight digestion in ATL buffer + Proteinase K the stomachs were disrupted

manually with a pipette tip to assist tissue digestion. Following overnight digestion, the DNA and hard parts were separated by centrifuging the samples at 2500 x *g*. The supernatant containing the DNA was transferred into a fresh tube and DNA extraction was continued on this fraction as per manufacturer's instructions. The hard part pellet was processed separately as described below. At the end of the DNA extraction procedure DNA samples were eluted in 100 µL of AE buffer and stored at 4 °C.

Following the removal of the supernatant, the hard part pellet was treated with hydrogen peroxide to remove organic matter. The pellet was resuspended in 500 µL of 30% hydrogen peroxide and incubated at 100 °C for 30 min. Samples were then centrifuged at 2500 x *g* for 1 min to repellet the sample. The hydrogen peroxide was removed and the pellet was washed 3 times in 500 µL of dH_2O. The hard part samples were then dried and stored at room temperature in preparation for microscopic diet analysis. It should be noted that this method was optimised for examining siliceous prey such as diatoms and silicoflagellates at high taxonomic resolution. The hydrochloric acid treatment would destroy some diet items which could otherwise have been identified in water mounts under low microscopic power.

2.3.5 Microscopic analysis of krill stomachs

Light microscopy was used to count and identify diatoms and silicoflagellates extracted in the 'hard part' component of krill stomachs. Diatoms and silicoflagellates are highly amenable to microscopic diet analysis because their hard silica exoskeletons often survive ingestion relatively intact. Variation in the structure of the exoskeleton allows diatoms to be identified down to genus or species level when examined at high levels of magnification.

The hard parts extracted from three krill at each site A and B were examined. Hard parts were resuspended in 500 µL of dH_20 and then diluted so that approximately 1000 hard part fragments could be spotted onto a glass coverslip. Coverslips were then dried and mounted onto glass slides using Optical Adhesive (Norland Products). All fragments on each coverslip were counted and identified at 400 X or 600 X magnification using a differential interference contrast microscope (Zeiss). All fragments that were more than half an intact diatom valve or equivalent were included in the count. The proportion of the hard part sample that was

counted was multiplied by the total number of items counted to provide an estimate of the total number of items consumed by each krill.

2.3.6 Determining the concentration of DNA extracted from krill stomachs

DNA concentrations were determined using a Picofluor fluorometer (Turner Designs) and Picogreen dsDNA quantification reagent (Molecular Probes) as per manufacturer's instructions. Stomach DNA samples were diluted to 10 ng μL^{-1} in AE buffer (Qiagen) for use in PCR.

2.3.7 PCR primer design

Identification of PCR amplicons from krill stomachs relies on matching them to a database of taxonomically identified DNA sequences. The small ribosomal subunit (SSU) gene was chosen because the current database of SSU sequences is larger and more taxonomically diverse than for any other DNA region. SSU is also a multi-copy gene (Prokopowich et al., 2003) which improves the chances of detecting prey. Initial attempts to amplify prey DNA using 'universal' SSU primers (Primers 14 and 17, Hendriks et al., 1991) failed because all amplicons were derived from krill and not their prey (data not shown). To counter this problem a pair of group specific primers (Jarman et al., 2004) that target diatoms was developed. The primers were designed to match all available diatom SSU sequences and specifically exclude amplification from krill with at least one 3' end mismatch in the equivalent region of the krill SSU sequence. The primers were designed manually using a taxonomically diverse alignment of SSU sequences obtained from The Ribosomal Database Project II (Cole et al., 2003) and the krill SSU that was sequenced as part of this study (GenBank accession no. AY672801). Primers were checked for hybridization to non-target taxonomic groups using PROBE (Cole et al., 2003) and BLAST (Altschul et al., 1990). This analysis suggested that in addition to diatoms the primers would amplify SSU sequences from several stramenopile groups closely related to diatoms and a small number of species from more distant groups including the16S gene of some chloroplasts and bacteria. Primers were checked for hairpin loops and primer dimers using PRIMER 3 (Rozen and Skaletsky, 2000). The primers generate an amplicon of approximately 143 base pairs and their sequence is: forward primer ASF1630/18 5' TACACACCGCCCGTCGCA 3', reverse primer ASR1775/22 5' CGGAAACCTTGTTACGACTTCA 3'.

The primers were tested empirically on DNA samples derived from seven cultures of diatoms (*Fragilariopsis curta, Fragilariopsis kerguelensis, Fragilariopsis sublineata, Navicula directa, Nitzschia lecointei, Phaeodactylum tricornutum, Porosira glacialis*) and four 'non-diatoms' (*Euphausia superba* - crustacean, *Polarella* sp. - dinoflagellate, *Pyramimonas* sp. - flagellate, *Geminigera cryophyllum* - cryptomonad). The primers produced amplicons from all seven diatoms and were specific to diatoms in this assay (data not shown).

2.3.8 PCR

PCR for all purposes other than quantitative PCR contained the following: 20 ng of template DNA, 3.0 mM $MgCl_2$ (Gibco), 0.125 mM dNTPs (Gibco), 0.25 uM of each primer (Geneworks), 1 X Bovine Serum Albumin (NEB), 0.5 U Amplitaq Gold (Gibco), 1 X manufacturer's PCR buffer (Gibco) and made up to 20 µL with dH_20. Samples were amplified in a PTC-200 thermal cycler (MJ Research) using the following cycling parameters: preheat at 95 °C for 3 min, 30 cycles of 95 °C for 5 s, 60 °C for 15 s and 72 °C for 15 s and a final extension at 72 °C for 5 min. No template and DNA extraction negative controls were run alongside krill stomach samples.

2.3.9 Comparing krill preservation methods

PCR results from krill preserved in ethanol and by freezing were compared to determine which preservation method was most suitable for DNA diet studies. PCR was performed on individual stomach DNA samples as described above and 5 µL of the PCR reactions were run on a 2% agarose gel stained with GelStar (Lonza). All subsequent analyses were performed on krill that had been preserved in 80% ethanol.

2.3.10 Generating clone libraries from krill stomachs

The sequence diversity of prey DNA was examined by generating a clone library of PCR amplicons from each krill. PCR reactions were performed on stomach extracts using the group specific primers ASF1630/18 and ASR1775/22 as described above. PCR reactions were then checked by gel electrophoresis (data not shown) prior to creating clone libraries with the TOPO-TA cloning kit (Invitrogen). At least 50 DNA clones from each krill were then purified and sequenced using the Mini Plasmid Prep kit (Mol Bio) and CEQ2000 Genetic Analysis System (Beckman Coulter) as per manufacturer's instructions.

2.3.11 Clone identification

DNA sequences from individual clones were grouped into 'Operational Taxonomic Units' (OTUs) for each geographic location, where sequences were considered to be a single OTU if they had < 1% sequence divergence. OTUs were then tentatively identified by finding their closest match in the GenBank database using the MEGABLAST algorithm (Zhang et al., 2000). A sequence similarity tree was then created to support the OTU identifications and examine the relationship between OTU and diatom SSU sequences. The tree contained the following collection of DNA sequences: all OTUs derived from krill stomachs; the GenBank database sequences that most closely matched each OTU sequence; all available diatom SSU sequences, and several stramenopile SSU sequences closely related diatoms or OTUs. Non-OTU sequences were edited down to the region between the two primer sites and all sequences were aligned in Clustal X (Thompson et al., 1997), with gap opening and gap extension parameters set to 10 and 0.5 respectively. Using this alignment a similarity tree was created using MEGA2 (Kumar et al., 2001). Sequence similarity was estimated using Tamura-Nei distances (Tamura and Nei, 1993) and a tree constructed using the minimum evolution algorithm with gap handling by pairwise deletion. OTUs were identified as the closest named sequence within the sequence similarity tree. Following identification, some diatom SSU sequences that were repetitious or unrelated to OTU sequences were removed to simplify the tree for publication.

Based on the available sequence data OTUs were classified into one of six categories: *Fragilariopsis* (diatom genus); *Thalassiosira* (diatom genus); *Chaetoceros* (diatom genus); Other Diatoms (sequences that matched a diatom sequence but did not match members of the previously defined groups); Dictyochophyceae (silicoflagellate class); and Other Stramenopiles (sequences that matched to other stramenopile sequences). For most OTU sequences there was complete agreement between MEGABLAST and tree-based identification but for OTUs with GenBank match scores < 97% there was sometimes a discrepancy between the two identification methods. In these cases, a conservative approach to identification classified these OTUs as either Other Diatoms or Other Stramenopiles.

2.3.12 Comparing relative abundance estimates from DNA and microscope

To compare relative abundance estimates derived from the two diet analysis methods the microscope data were grouped into the same categories defined above for OTUs. For both DNA and microscopic data the relative abundance of each prey group was calculated for individual krill and then averaged across krill within a site to estimate the mean relative abundance of consumed prey ± standard deviation (n = 3).

2.3.13 Quantifying prey DNA extracted from krill stomachs

Quantitative PCR was used to estimate how many copies of prey SSU DNA were extracted from each krill stomach. A standard for estimating prey DNA copy number was created by cloning a 143 base amplicon derived from the diatom *Fragilariopsis curta* into the TOPO-TA vector pCR2.1. The plasmid was linearised with *Nco*I (NEB) to remove supercoiling and then purified with the Qiaquick PCR purification kit (Qiagen). A series of ten-fold dilutions from 10^{-1} ng μL^{-1} to 10^{-7} ng μL^{-1} (approximately 2 2 x 10^7 to 2.2 x 10^1 copies of the plasmid) was used to generate a standard curve for quantification of the krill stomach samples ($y = -0.267x + 10.928$, $r^2 > 0.999$). Quantitative PCR was performed using the Quantitect SYBR Green RT-PCR kit (Qiagen) and reactions contained the following: 20 ng of template DNA, 3 mM $MgCl_2$, 1X BSA (NEB), 0.25 µM of each primer (ASF1630/18 and ASR1775/22), 1X Quantitect SYBR Green Master Mix and made up to 20 µL with dH_20. Samples were amplified in a Rotor Gene 2000 thermal cycler (Corbett Research) using the following cycling parameters: preheat at 95 °C for 15 min, 50 cycles of 95 °C for 20 s, 60 °C for 20 s and 72 °C for 20 s. All reactions were performed in triplicate and the results presented as an average of the three PCR reactions ± standard deviation (n = 3).

2.4 Results

2.4.1 Prey DNA Preservation

Two methods of preserving intact krill for DNA diet analysis were examined - preservation in ethanol and preservation by freezing. Krill that had been preserved in ethanol provided strong PCR amplification results using prey specific primers (Figure 2-1, lanes 1 - 5). In contrast, krill preserved by freezing gave poor PCR amplification results (Figure 2-1, lane 6 - 10). These results suggest that preserving krill in ethanol is superior to preservation by freezing for maintaining the integrity of prey DNA within intact krill.

2.4.2 Microscopic analysis of krill stomachs

Microscopic diet analysis presented a simple picture of krill diet at both sites A and B (Table 2-1). Krill from both sites had consumed a large quantity of diatoms and krill from Site A had also consumed a small number of the silicoflagellate *Dictyocha speculum*. No other prey groups were identified by microscopy. At both sites the diatom component of krill diet was dominated by a single genus (*Fragilariopsis*) and there was little variation in prey composition or relative abundance between the three individual krill analysed within each site. Site A krill consumed mainly *Fragilariopsis cylindrus* (69 % of total diet) and *F. curta* (26 %), with the next highest contribution from any single species contributing <1% of the total diet. In contrast, the diet of krill at Site B was dominated by *F. curta* (85 %) followed by *F. cylindrus* (12 %). Site B krill had also consumed a small amount of the diatom genus *Thalassiosira* (3 %). These results were consistent with previous studies that have applied microscopic diet analysis to krill collected during the Antarctic summer (e.g. Marr, 1962; Genhai, 1993; Pakhomov et al., 1997).

2.4.3 Detection and identification of prey DNA in krill stomachs

The diversity of prey DNA was examined by generating clone libraries of PCR amplicons from individual krill. Sequencing of individual clones confirmed that all OTUs generated with the group specific primers ASF1630/18 and ASR1775/22 were derived from organisms that are likely to be krill prey (Figure 2-2, Table 2-2). Most OTUs matched diatom SSU sequences, in accordance with the microscopic diet analysis. Several rare OTUs had poor percentage matches scores with GenBank sequences, reflecting the paucity of sequence information available for taxonomic groups likely to be prey for krill.

DNA diet analysis detected OTUs identical to *F. cylindrus* and *F. curta* sequences in all three krill at both sites A and B (Table 2-3). OTUs related to *Thalassiosira* sequences were also detected in all three krill at site B. DNA diet analysis therefore detected all genera identified as more than 1 % to the total diet by microscopy (compare Table 2-1 with Table 2-3).

DNA analysis also identified two groups that were not detected by microscope analysis. Site A krill produced a small number of clones from the diatom genus *Chaetoceros*. Site B krill produced clones from a non-diatom stramenopile group, possibly golden algae (Class: Chrysophyceae).

2.4.4 Comparing prey relative abundance estimates from DNA and microscope

Prey relative abundance estimates obtained from DNA clone libraries and microscope analysis showed significant differences (Figure 2-3). Site B shows a large discrepancy in the relative abundance estimates for *Thalassiosira*. The ratios of *F. cylindrus* and *F. curta* OTUs at both sites were also inconsistent with the ratios obtained by microscopic diet analysis. The observed bias was consistent for all three krill within a site, suggesting that sampling error was unlikely to be the cause of the bias.

2.4.5 Quantifying prey DNA extracted from krill stomachs

Quantitative PCR was used to determine how much prey DNA template was extracted from krill stomachs. All six krill stomachs were found to contain a large amount of prey DNA template that could be amplified with the group specific PCR primers (Table 2-4). All stomachs contained at least 100,000 copies of the intact SSU template with one stomach exceeding a million copies. These results suggest that DNA diet assays based on the SSU gene can provide robust PCR results derived from a significant amount of starting template.

Microscopic estimates suggested the total number of diatoms consumed by each krill ranged from ~ 80,000 - 370,000 diatoms per individual (Table 2-4). The ratio of prey DNA template : diatoms consumed ranged from 1.0 - 4.9, indicating that copies of SSU template recovered per diatom consumed was reasonably high.

2.5 Discussion

2.5.1 Preservation of prey DNA

A comparison of methods for preserving intact krill for DNA diet analysis suggested that preservation with ethanol is superior to preservation by freezing. Previous DNA diet studies on invertebrates have used ethanol preservation (e.g. Hoogendoorn and Hempel, 2001) but preservation by freezing has been the dominant method (e.g. Zaidi et al., 1999; Agustí et al., 2003a). Given the general preference for preservation by freezing the poor result with frozen krill was unexpected. Previous studies did not compare preservation methods so it is unclear whether alternative methods would have provided better results. In addition, most studies have examined terrestrial insect predators which may not provide a useful precedent for marine organisms like krill. Problems extracting prey DNA from frozen marine invertebrates is not unprecedented. Ashida et al. had difficulties extracting prey DNA from frozen sand

shrimps (Asahida et al., 1997). This was attributed to abundant DNA endonucleases and the problem was solved by altering the DNA extraction buffer. Freezing does not destroy DNA nucleases and successful DNA extraction from frozen tissue relies on rapid inactivation of nucleases prior to significant defrosting of the sample (Strauss, 1998). Thus, prey DNA may have been successfully preserved in frozen krill at the time of collection, but subsequently degraded during the dissection procedure because of limited but unavoidable defrosting. Krill have an extremely high water content that makes it difficult if not impossible to dissect completely frozen animals in a sterile manner. It is difficult to see a way around this problem and preserving krill by freezing may be incompatible with sterile dissection and high yields of prey DNA.

Subsequent to the work in this chapter, Martin et al. published a study that applied freezing preservation to krill and successfully extracted and amplified prey DNA (Martin et al., 2006). Dissections were performed on a cold glass plate to keep stomach samples at low temperature until they could be transferred into DNA extraction buffer (Martin et al., 2006). Prey DNA was amplified with universal primers and detected with denaturing gradient gel electrophoresis (DGGE). The amount of prey DNA extracted was not quantified but comments in the methods section suggest the amount was small. DGGE provides extremely sensitive detection, well beyond what can be achieved using agarose gels. Samples that require 25-30 cycles of amplification to be observed in agarose typically require only 15-20 cycles to be observed with DGGE (personal observation). In spite of this high level of sensitivity, several gut samples in Martin et al.'s study required 35 cycles of PCR to be detected by DGGE. Therefore the results of Martin et. al may still be consistent with the exonuclease degradation hypothesis described above.

In this study ethanol preservation successfully preserved prey DNA and provided animals that could be dissected at a leisurely pace. The superior results obtained with ethanol preservation could be due to the fact that ethanol permeates animal tissue and denatures nuclease enzymes (Flournoy et al., 1996). A potential issue with ethanol preservation is that live krill dropped into ethanol may react by swallowing ethanol during the preservation process which could introduce contamination into the diet sample. Given the large volume of ethanol per krill used in this study and the short time that it took for krill to expire (~ 3

seconds) the contamination risk from this source is probably low. However, it may be prudent to preserve and store krill individually to further minimize contamination risk.

This is the first study to compare preservation methods and the results suggest that optimising the preservation method is important. Additional work in this area could provide further improvements in prey DNA yield. In terms of applying these results to other animals it is important to recognise that the optimal preservation method may differ for different predator species. Feasibility studies should consider testing multiple preservation methods.

2.5.2 Krill dissection

The dissection method used in this study was fast and efficient but the issue of contamination warrants some discussion. Contamination is a significant issue for DNA diet analysis because PCR can amplify from extremely small amounts of DNA template. Contamination must be avoided at every step in the sample processing procedure. Previous DNA diet studies have rarely mentioned contamination and some studies advocate extracting DNA from intact predators with no regard for external sources of contamination. For aquatic predators that literally swim in their food, potential contamination can come from water carried over during collection or from material attached to the exterior of the animal, particularly food trapped amongst swimming and feeding appendages. Extracting digestive tissue must be carefully handled to prevent the transfer of external contamination to the stomach sample. It is recommended that the dissection procedure is carefully considered in relation to contamination and the dissection tools are routinely sterilised.

2.5.3 PCR assay design

The design of PCR based prey detection assays requires careful consideration due to the presence of krill DNA in the diet samples. Krill DNA swamped initial attempts to amplify prey DNA with universal primers despite the use of dissection to increase the relative concentration of prey DNA. In the current study this problem was solved by designing prey specific PCR primers that did not amplify krill DNA. The problem with this approach is that it restricts the range of prey that can be detected. Recent work suggests there may be other ways of dealing with predator DNA (Blankenship and Yayanos, 2005) and these techniques are explored in more detail in Chapter 3.

The taxonomic resolution of DNA diet analysis is dependent on two factors: the amount of sequence divergence between species within the amplified DNA region and the amount of sequence data available across the targeted prey group. As more sequence information becomes available, it will be possible to have greater confidence in the identity and taxonomic resolution of prey amplicons. Currently, SSU is the only gene that provides reasonable taxonomic coverage for krill prey groups and coverage of protist groups remains poor. Based on the available sequences the 143 base region targeted in this study contains enough sequence variation to resolve diatoms to the level of family or genus. This level of resolution is close to that of high-powered light microscopy and superior to pigment, isotope and lipid analysis. Amplifying larger DNA fragments would provide more taxonomic resolution but it is unclear what size range of prey DNA fragments can be recovered from krill stomachs. Identification of species using DNA has been gaining popularity and broader issues related to identification are undergoing active debate (Meyer and Paulay, 2005; Steinke et al., 2005, Rubinoff et al., 2006).

2.5.4 Comparing DNA and microscopy

Verification of the DNA diet methods has previously been achieved using controlled feeding trials (e.g. Foltan et al., 2005; Juen and Traugott, 2005; Sheppard et al., 2005). This study is the first attempt to verify DNA diet analysis results using an independent method of diet analysis.

DNA diet analysis detected the same diatom groups identified by microscopy plus two additional groups, the diatoms genus *Chaetoceros* and clones that were probably derived from Chrysophyceae (Golden algae). *Chaetoceros* has fragile, lightly silicified valves that are unlikely to survive ingestion by krill (Marr, 1962). Chrysophyceae species generally lack hard parts that can be detected by light microscopy (F. Scott, pers. comm.). This probably accounts for their absence in the microscopic analysis. The ability to detect prey that cannot be identified by other methods is one of the strengths of DNA diet analysis.

The comparison between DNA and microscopy suggested that one of the techniques was biased in terms of reporting the relative abundance of the diatom genera *Fragilariopsis* and *Thalassiosira*. In general diatoms provide a good hard part signature for microscopic analysis but differences in size and fragility of valve structures can make some diatom

species easier to detect than others. This may account for a proportion of the observed differences between DNA and microscopy. However, *Fragilariopsis* and *Thalassiosira* generally have large and robust valves that are easily detected with microscopy. A more likely explanation is that the DNA diet analysis via clone libraries suffers from biases that alter the ratio of clones from the true ratio of consumed prey. The potential reasons for this bias are many and varied. The large *Thalassiosira* DNA signal at Site B could be a reflection of the krill feeding behaviour. Krill at Site B may have consumed *Thalassiosira* diatoms just prior to capture and the DNA signal may not have degraded to the same extent as the signal from other prey. Alternatively there could be issues with the methodology such as differences in SSU gene copy number between *Thalassiosira* and *Fragilariopsis* species, PCR bias (Polz and Cavanaugh, 1998; Suzuki et al., 1998; Becker et al., 2000) or cloning bias.

Now that DNA assays that detect multiple prey species are beginning to emerge the issue of detection bias needs further consideration. DNA diet studies have observed differences in the length of time that prey species are detectable (Harper et al., 2005). Clone libraries derived from scats have been shown to give biased estimates of diet (Deagle et al., 2005) similar to the results in this study. As a general rule it is unlikely that the proportions of prey amplicons in clone libraries will be an accurate representation of the ratios of consumed prey. An alternative approach that may be more suited to DNA and its potential for high throughput is to determine the presence or absence of prey in a large number of predator individuals and use this information to assess the importance of particular prey groups. This approach has recently been applied to the diet of spiders (Agustí et al., 2003a) and beetles (Harper et al., 2005). Chapter 4 of this thesis explores the approach in more detail.

2.5.5 Quantifying prey DNA extracted from krill stomachs

A rarely acknowledged problem with DNA diet analysis is that PCR reactions are often performed on very small amounts of poor quality template DNA. Given sufficient rounds of PCR amplification, PCR products and clone libraries can be generated from exceedingly small amounts of starting template but the results are generally difficult to reproduce (Chandler et al., 1997) and prone to contamination issues (Cooper and Poinar, 2000). This study was the first attempt to quantify the amount of prey DNA template in diet samples.

Subsequent studies have extended the application of quantitative PCR in DNA diet analysis (Deagle and Tollit, 2006; Troedsson et al., 2007) and the approach is explored further in Chapter 5.

The results from the current study demonstrate that a large amount of prey DNA can be extracted from ethanol preserved krill. This allows PCR amplification reactions to be carried out without excessive rounds of amplification. The results provide additional evidence that the application of DNA diet analysis to Antarctic krill is likely to be a robust approach. Future DNA diet studies should consider quantifying the amount of prey DNA extracted from predators to assist with assessing the reproducibility of their work.

2.6 Conclusions

This study demonstrates that DNA diet analysis can be applied to Antarctic krill collected in the field. The study identifies several issues with DNA diet analysis that need to be addressed before the approach can provide reliable quantitative data about krill diet. Overall the results suggest that the application of DNA diet analysis to krill warrants further investigation, particularly for prey that are difficult to study using other methods.

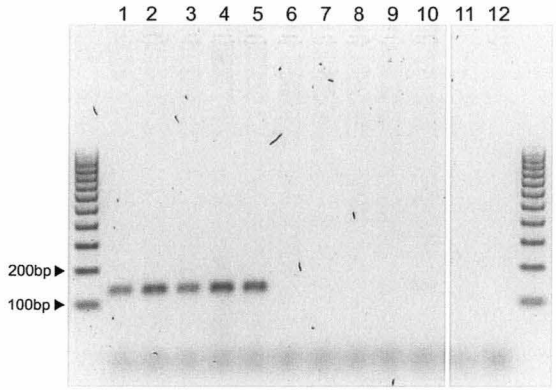

Figure 2-1 Effect of krill preservation on the PCR amplification of prey. Agarose gel showing prey specific PCR products derived from krill stomach DNA samples. Lanes 1 - 5, krill preserved in 80% ethanol. Lanes 6-10, krill preserved by freezing at – 80 °C. Lane 11 negative control for DNA extraction. Lane 12 no template PCR control.

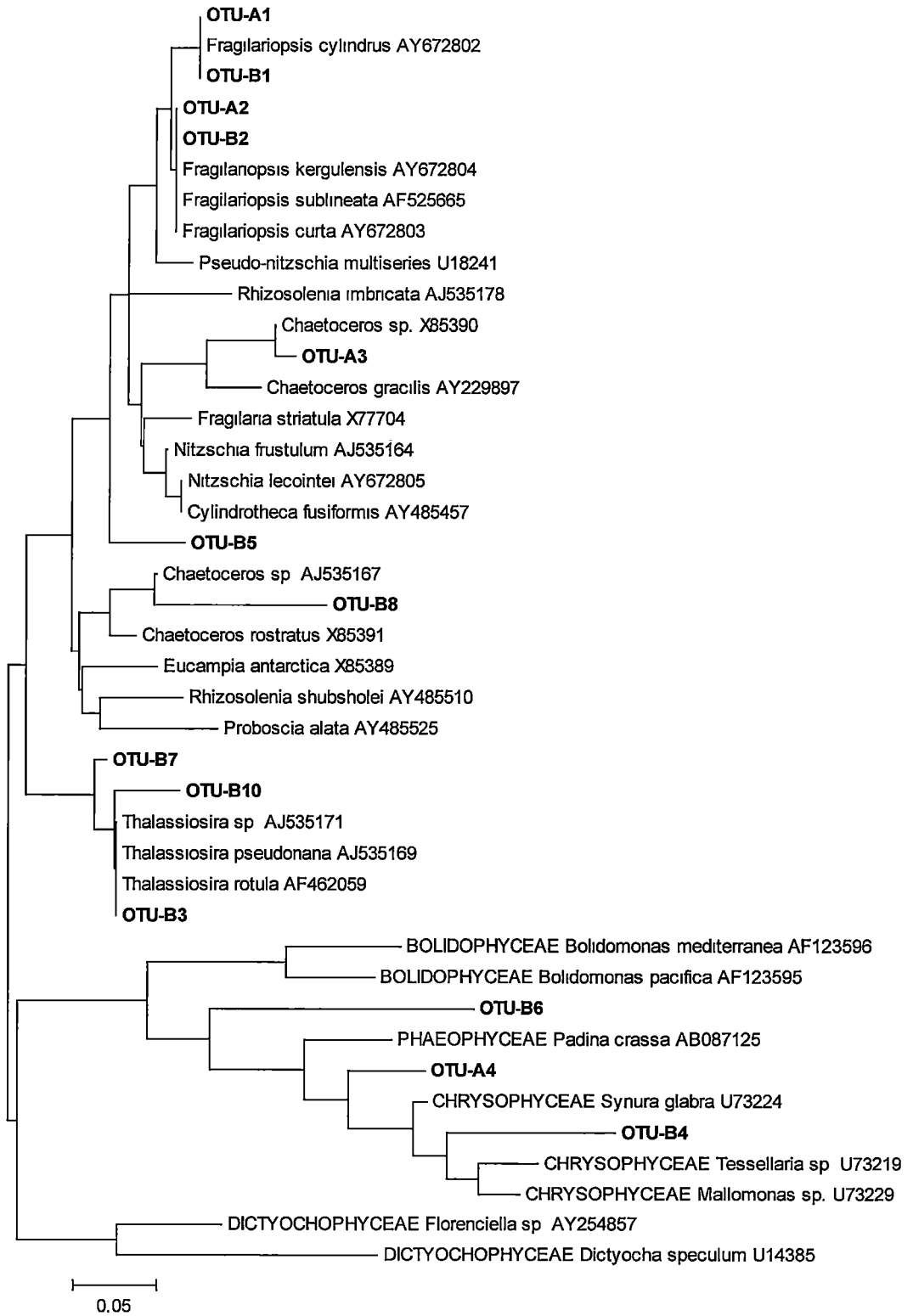

Figure 2-2 Sequence similarity tree of krill stomach OTUs and related sequences. Note. OTU-B9 does not appear on the tree because it is derived from the 16S chloroplast gene.

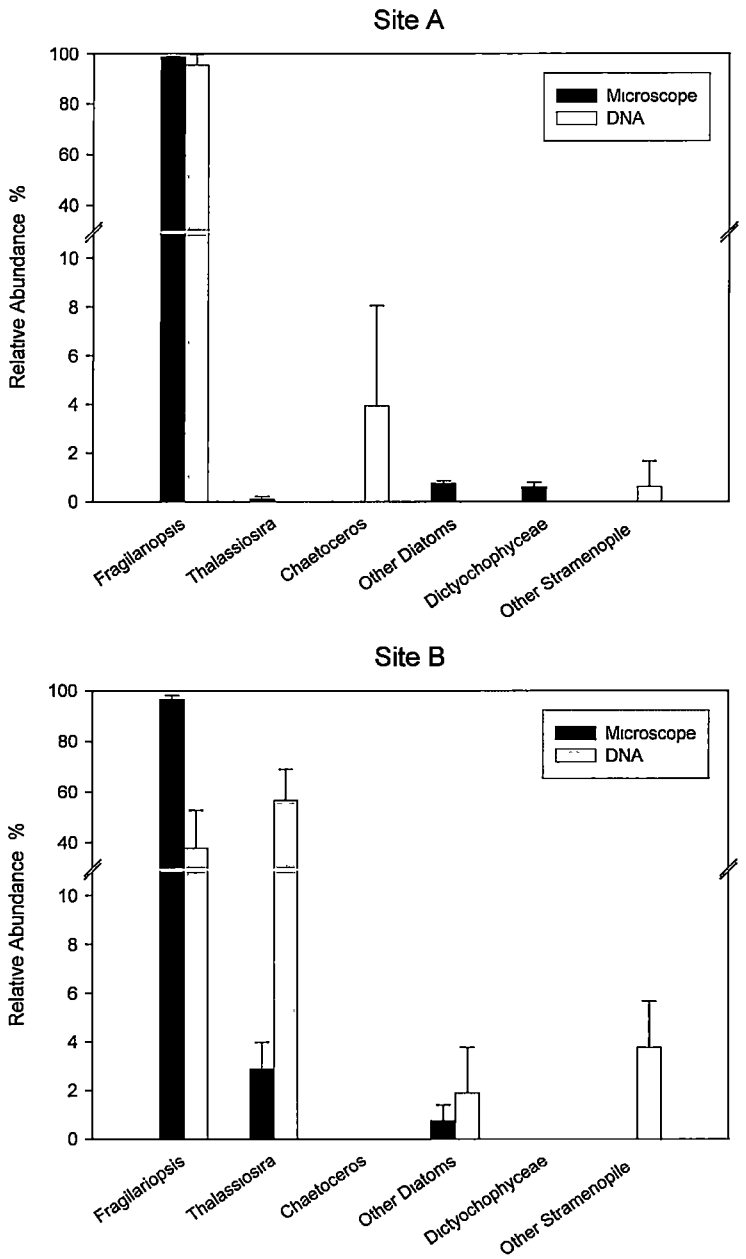

Figure 2-3 Comparison of microscope and DNA diet methods at Sites A and B. Columns represent site averaged estimates of consumed prey relative abundance. Error bars are SD (n = 3).

Table 2-1 Microscopic diet analysis of krill stomachs showing species identified and their relative abundance.

	Site A						Site B					
	Krill A-1		Krill A-2		Krill A-3		Krill B-1		Krill B-2		Krill B-3	
Species/Group Identified	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
BACILLARIOPHYTA - Diatoms												
Fragilariopsis cylindrus	2,017	68.9	1,368	71.9	580	77.4	9	12.0	162	11.7	267	11.6
Fragilariopsis curta	807	27.6	490	25.8	145	19 4	648	83.4	1,119	80.9	1,938	84.2
Fragilariopsis sublineata	24	0.8	4	0.2	6	0.8	3	0.4	5	0.4	2	0.1
Fragilariopsis angulata	10	0.3	3	0.2	1	0 1	8	1.0	18	1.3	32	1.4
Fragilariopsis ritscheri	4	0.1	1	0.1	0	0.0	0	0.0	3	0.2	1	0.0
Fragilariopsis obliquecostata	2	0.1	1	0.1	0	0.0	0	0.0	0	0.0	3	0.1
Fragilariopsis pseudonana	12	0.4	9	0.5	8	101	0	0.0	0	0.0	13	0.6
Thalassiosira gracilis	4	0.1	2	0.1	0	0.0	16	2.1	57	4.1	46	2.0
Thalassiosira antarctica	0	0.0	1	0.1	0	0.0	0	0.0	0	0.0	0	0.0
Thalassiosira lentiginosa	1	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Thalassiosira oliverana	0	0.0	0	0.0	0	0.0	3	0.4	0	0.0	0	0.0
Asteromphalas hookeri	0	0.0	0	0.0	0	0.0	1	0.1	0	0.0	0	0.0
Asteromphalas sp.	0	0.0	0	0.0	0	0.0	1	0.1	0	0 0	0	0.0
Navicula sp.	0	0.0	0	0.0	0	0.0	0	0.0	1	0.1	0	0.0
Nitzschia pseudonana	0	0.0	0	0.0	0	0.0	4	0.5	7	0.5	0	0.0
Nitzschia sp	16	0.5	10	0.5	6	0.8	0	0.0	0	0.0	0	0 0
Rhizosolenia sp.	7	0 2	2	0.1	0	0.0	0	0.0	11	0.8	1	0.0
DICTYOCHOPHYCEAE - Silicoflagellates												
Dictyocha speculum	23	0.8	11	0.6	3	0.4	0	0.0	0	0.0	0	0 0
Total hard part count	2,927		1,902		749		777		1,383		2,303	

Table 2-2 Description of OTUs isolated from krill stomachs and their closest match in the GenBank.

Name	Length (base pairs)	OTU Accession No.	Nearest MEGABLAST Match	% Match	Match Accession No.	Identified as
OTU-A1	143	AY672806	*Fragilariopsis cylindrus*	100%	AY672802	Fragilariopsis
OTU-A2	143	AY672807	*Fragilariopsis curta/sublineata/kergulensis*	100%	AY672803/AF525665/AY672804	Fragilariopsis
OTU-A3	142	AY672808	*Chaetoceros* sp.	99%	X85390	Chaetoceros
OTU-A4	143	AY672809	*Tessellaria volvocina*	91%	U7321	Other Stramenopile
OTU-B1	143	AY672810	*Fragilariopsis cylindrus*	100%	AY672802	Fragilariopsis
OTU-B2	143	AY672811	*Fragilariopsis curta/sublineata/kergulensis*	100%	AY672803/AF525665/AY672804	Fragilariopsis
OTU-B3	143	AY672812	*Thalassiosira pseudonana*	100%	AJ535169	Thalassiosira
OTU-B4	143	AY672813	*Mallomonas akrokomos*	91%	U73229	Other Stramenopile
OTU-B5	143	AY672814	*Rhizosolenia imbricata*	94%	AJ535178	Other Diatom
OTU-B6	146	AY672815	*Florenciella parvula*	86%	AY254857	Other Stramenopile
OTU-B7	143	AY672816	*Thalassiosira pseudonana*	98%	AJ535169	Thalassiosira
OTU-B8	143	AY672817	*Chaetoceros* sp.	93%	AJ535167	Other Diatom
OTU-B9[1]	119	AY672818	*Rhizosolenia setigera*	96%	AJ536461	Other Diatom
OTU-B10	143	AY672819	*Thalassiosira pseudonana*	97%	AJ535169	Thalassiosira

[1] Sequence is from 16S chloroplast gene not SSU

Table 2-3 Summary of DNA clone library analysis from individual krill stomachs showing the OTUs isolated and their relative abundance.

		Site A					
		Krill A-1		Krill A-2		Krill A-3	
Name	**Identification**	**No.**	**%**	**No.**	**%**	**No.**	**%**
OTU-A1	*Fragilariopsis*	48	87.3	53	86.9	57	98.3
OTU-A2	*Fragilariopsis*	4	7.3	3	4.9	1	1.7
OTU-A3	*Chaetoceros*	2	3.6	5	8.2	0	0.0
OTU-A4	Other Stramenopile	1	1.8	0	0 0	0	0.0
Total		55		61		58	

		Site B					
		Krill B-1		Krill B-2		Krill B-3	
Name	**Identification**	**No.**	**%**	**No.**	**%**	**No.**	**%**
OTU-B1	*Fragilariopsis*	25	47 2	10	18.9	12	22.6
OTU-B2	*Fragilariopsis*	4	7.5	4	7.5	5	9.4
OTU-B3	*Thalassiosira*	23	43.4	35	66.0	30	56.6
OTU-B4	Other Stramenopile	1	1.9	1	1.9	3	5.7
OTU-B5	Other Diatom	0	0.0	1	1.9	0	0.0
OTU-B6	Other Stramenopile	0	0.0	1	1.9	0	0.0
OTU-B7	*Thalassiosira*	0	0.0	1	1.9	0	0 0
OTU-B8	Other Diatom	0	0.0	0	0 0	1	1.9
OTU-B9	Other Diatom	0	0.0	0	0 0	1	1.9
BTU-B10	*Thalassiosira*	0	0.0	0	0.0	1	1 9
Total		53		53		53	

Table 2-4 Estimate of prey SSU molecules extracted from individual krill stomachs.

Krill ID	Concentration of DNA Extracted from krill stomach ng µl $^{-1}$	Copies of prey SSU molecules per PCR reaction[1]	SD (n = 3)	Estimate of the number of prey SSU molecules extracted from stomach[2]	SD (n = 3)	Number of diatom valves counted microscopically[4]	Fraction of 'hard part' sample counted	Estimate of the number of diatoms in the stomach from 'hard' part' analysis	Ratio of prey SSU molecules per diatom consumed
A-1	43	8,319	± 1,485	1,787,987	± 319,168	2,927	1/250	36,5875	4.9
A-2	42	4,366	± 368	916,886	± 77,282	1,902	1/250	237,750	3.9
A-3	25	2,713	± 294	339,172	± 36,755	749	1/250	93,625	3.6
B-1	44	1,142	± 80	251,304	± 17,604	777	1/250	97,125	2.6
B-2	47	1,194	± 61	280,535	± 14,332	1,383	1/125	86,438	3.2
B-3	28	1,006	± 133	140,898	± 18,627	2,303	1/125	287,875	1.0

[1] Calculated with quantitative PCR using 20 ng of template DNA per PCR reaction.
[2] Copies of target DNA per µL of PCR template DNA X dilution factor from original DNA extraction to PCR template solution X total amount of original DNA extraction (100 µL).
[3] Number of valves counted X (1/ fraction of sample counted) X 1/2 (two valves per diatom)
[4].These estimates should be considered tentative because no replicate counting was performed.

Chapter 3

Overcoming predator DNA abundance in dietary samples

3.1 Abstract

DNA diet samples are a mixture of DNA derived from prey species and the predator that consumed them. Predator DNA is often the largest and best preserved component of diet samples which can cause significant problems for PCR based DNA diet analysis. This study examined methods that remove predator DNA from diet samples prior to PCR amplification. The study applied PCR clamping and restriction digests to krill stomach diet samples. Both approaches led to some reduction in the amount of krill DNA that was PCR amplified but neither method proved completely satisfactory.

3.2 Introduction

Samples used in DNA diet analysis contain DNA from prey species but often predator DNA is the largest and best preserved component (Chapter 2, Blankenship and Yayanos, 2005; Martin et al., 2006; Passmore et al., 2006). Predator DNA in diet samples creates significant problems for DNA-based diet analysis. In studies that have used 'universal' primers where predator DNA is also amplified, high levels of predator DNA can out-compete prey DNA for either PCR amplification or subsequent amplicon detection (Martin et al., 2006; Passmore et al., 2006). Most DNA diet studies circumvent the problem by developing group or species specific PCR primers that do not amplify predator DNA. However, this approach generally leads to compromises in prey species coverage becuase a subset of prey has the same DNA sequence as the predator at priming sites that are conserved across a broad range of species. Clearly it would be useful to develop methods that systematically remove predator DNA or block predator DNA amplification during PCR.

Two methods that deal directly with non-target DNA are PCR clamping and restriction digests. PCR clamping is a method that utilises a blocking probe to prevent the amplification of a specific non-target DNA template during PCR. Three PCR clamping strategies have been defined· (1) Primer exclusion, where the blocking probe competes with one of the PCR primers for the same binding site on the non-target DNA template, (2) Strand initiation interference, where the blocking probe binds specifically to the non-target template directly adjacent to the 3' end of one of the primers, thus preventing *Taq* polymerase from initiating strand elongation from the primer, and, (3) Elongation arrest, where the blocking probe binds

at a location some distance from the primer site and prevents *Taq* polymerase from progressing along the template strand (Figure 3-1, Orum et al., 1993; Orum, 2000).

Blocking probes cannot be constructed from unmodified DNA because (1) they must not act as primers for PCR that can be extended by *Taq* polymerase, and, (2) for the strand initiation interference and elongation arrest strategies the blocking probes must be resistant to the 5'-3' exonuclease activity of *Taq* polymerase (for descriptions of *Taq*'s 5'-3' exonuclease see Longley et al., 1990; Ceska and Sayers, 1998; Lyamichev et al., 1999). In addition, it is useful for blocking probes to have strong and specific binding affinity for the non-target template. To meet these criteria blocking probes have generally been constructed from a DNA analog known as a peptide nucleic acid (PNA) (Orum et al., 1993; Orum, 2000; von Wintzingerode et al., 2000; Iwamoto and Sonobe, 2004, Takiya et al., 2004). The chemical structure of PNA nucleotides is resistant to modification by *Taq* and the binding affinity and specificity of PNA probes is superior to their DNA equivalent. However, PNA probes are expensive and they lack flexibility with regard to designing probes that have the desired binding affinity because it is impossible to synthesize probes that are a mixture of PNA and DNA nucleotides. An alternative DNA analog with attractive properties is the locked nucleic acid (LNA). LNA nucleotides confer increased binding affinity similar to PNA but LNA probes are cheaper and hybrid probes that are a mixture of LNA and DNA nucleotides can be synthesised (Braasch and Corey, 2001; Petersen and Wengel, 2003). LNA probes have recently been trialled as PCR blocking probes (Hummelshoj et al., 2005; Senescau et al., 2005; Thiede et al., 2006).

Restriction digests have recently been applied to DNA diet samples to remove predator DNA in a study on the diet of molluscs and amphipods (Blankenship and Yayanos, 2005). The diet samples were PCR amplified with 'universal' primers. PCR amplicons were then subjected to a restriction digest using an enzyme that cuts near the centre of predator amplicons. Cut predator amplicons were then size-separated from uncut amplicons and the uncut amplicons were cloned and sequenced.

The aim of this study was to apply PCR clamping with LNA blocking probes and restriction digest strategies to krill stomach samples to determine whether either approach could improve the yield of prey amplicons in PCR reactions that utilise 'universal' primers.

3.3 Material and Methods

3.3.1 Developing a detection assay

To facilitate the rapid and cost effective assessment of amplicons generated in mixed template PCR reactions, an assay was developed that utilised capillary gel electrophoresis to size separate PCR amplicons.

A region near the centre of the SSU gene was targeted for PCR amplification because sequence alignments demonstrated that euphausiid species had a DNA sequence insertion (14 base pairs) in this region that was absent from krill prey species. This allowed for easy separation of predator and prey peaks on electrophoresis chromatograms. Peak heights provided a semi-quantitative estimate of the amount of different sized PCR amplicons present in the mix. The targeted region also contained a high level of sequence variation between prey species, including other small deletions and insertions that allow some additional separation of krill prey groups based on amplicon size.

PCR primers were developed that bound either side of the 14 bp insertion region and were capable of generating amplicons from a broad range of species, including krill (Table 3-1). Forward primer ASF1194/20 was labelled with Beckman sequencing dye D4 to facilitate amplicon detection during capillary gel electrophoresis. Optimized PCR reactions contained: 2 µL of template DNA, 2.5 mM $MgCl_2$ (Gibco), 0.1 mM dNTPs (Gibco), 0.1 µM of each primer (Geneworks), 1 X Bovine Serum Albumin (NEB), 0.5 units of Amplitaq Gold (Gibco), 1 X manufacturer's PCR buffer (Gibco) and made up to 20 µL with dH_20. Samples were amplified in a Tetrad thermal cycler (MJ Research) using the following cycling parameters: preheat at 95 °C for 10 min followed by 35 cycles of 95 °C for 10 s, 62 °C for 20 s and 72 °C for 40 s.

PCR reactions were analysed on a CEQ2000 96 well capillary sequencer (Beckman). PCR reactions were diluted 1 in 10 with mH_2O. Samples prepared for capillary electrophoresis contained: 2 µl of diluted PCR reaction, 25 µl of sample loading solution (Beckman) and 0.15 µl of 70 - 420 bp DNA size standard (Beckman). Samples were analysed with standard fragment analysis run conditions (Frag1).

3.3.2 LNA blocking probe

The LNA blocking probe was designed in conjunction with the detection assay PCR primers. The primer blocking PCR clamp strategy was selected because previous research suggested this was the most reliable blocking method (Orum, 2000) and the approach avoided concerns that LNA probes might be susceptible to degradation by the 5' - 3' exonuclease of *Taq* polymerase. The LNA probe was designed to bind specifically to krill DNA template and to block the binding site of the reverse PCR primer. Specificity and strong binding affinity were achieved via strategic placement of the probe and positioning LNA bases within the blocking probe to target bases unique to krill DNA (Table 3-1). Thermodynamic models predicted the melting temperature of the LNA probe bound to krill DNA would be 67 °C (Exiqon website http://www.exiqon.com/). This was well above the 60 °C predicted melting temperate for the reverse primer (Exiqon website http://www.exiqon.com/). Binding of the LNA probe to non-krill templates were well below the 60 °C melt temperature of the reverse primer (data not shown). The 3' end of the blocking probe was phosphorylated to prevent primer extension by *Taq* polymerase (probe supplied by Pro-oligo).

3.3.3 Plasmid test system

A plasmid test system was developed to allow an examination of predator DNA blocking methods under controlled conditions. The full length SSU genes from krill and the diatom *Fragilariopsis cylindrus* were separately cloned into the plasmid vector TOPO pCR2.1. Large stocks of the two plasmids were generated using a plasmid purification kit (Mo Bio). To remove any effect from plasmid supercoiling, both plasmids were linearised with restriction enzyme *Not*I (NEB). Plasmid DNA concentration was determined using a Picofluor fluorometer (Turner Designs) and Picogreen dsDNA quantification reagent (Molecular Probes) as per manufacturer's instructions. Plasmid DNA copy number per µl was estimated using the formula:

> Copy number per µl = Avogadro's number (6.022×10^{23}) X Concentration (kg L^{-1}) / Molecular Weight (Daltons)

where the weight of each base pair (bp) in the plasmid was assumed to be 650 Daltons. Plasmid sizes were 5737 bp and 5723 bp for krill and diatom, respectively. From these

stocks, a set of solutions was made that contained various ratios of diatoms and krill plasmid. All ratio solutions were made up to a final concentration of 10,000 copies of plasmid per µl (diatom : krill ratios 1 :0, 0 : 1, 1 : 1, 1 . 5, 1 · 10, 1 : 100, 1 : 1000, and 1 : 10,000). Ratio solutions were used as template DNA in PCR reactions and as controls when experiments on krill stomach samples were conducted.

3.3.4 Krill Stomach Samples

Five krill stomach samples were used from each of the four field collection sites (A-D) described in Chapter 4 (Figure 4.1 and Table 4.1). Samples were selected that had previously tested positive for either diatom or dinoflagellate DNA using group specific primers (Chapters 2 and 4). DNA concentration of the diet samples ranged from 11.6 – 41.0 ng μl^{-1}. Dilutions of stomach samples prior to PCR (1 : 1, 1 : 5, 1 : 10, 1 : 50, 1 : 100) were made using AE buffer (Qiagen).

3.3.5 Restriction enzyme digests

Restriction enzymes capable of cutting krill DNA within the amplified region were examined to determine whether they would also cut the DNA from potential prey groups. A sequence alignment containing sequences from 1508 species from a diverse range of potential prey groups was generated using sequences derived from the European Ribosomal RNA database (http://www.psb.ugent.be/rRNA/index.html). Restriction site analysis was performed using Bioedit software (Hall, 1999). *Bam*HI was found to be the most krill specific restriction enzyme, cutting only 10 of 1508 prey sequences (cut sequences were derived from isopod and amphipod sequences). The next best enzymes were: *Eag*I (22 / 1508), *Sac*I (824 / 1508) and *Sac*II (22 / 1508).

Previous attempts to apply predator specific restriction digests to diet samples applied the digestion step after PCR amplification (Blankenship and Yayanos, 2005). This approach raised concerns that predator DNA would have already out competed prey DNA for PCR amplification. Therefore the decision was taken to apply restriction digests to stomach samples prior to PCR amplification. Restriction digests contained 1X restriction enzyme buffer, 1X Bovine Serum Albumin (NEB), 5-40 units of restriction enzyme (not more than 10% final volume, *Bam*HI, *Eag*I or *Sac*II (NEB)), 10 µl of template DNA and were made up to either 30 or 50 µl final volume with dH_20. Digests were carried out at 37 °C with trials of both

4 hour and overnight incubations. Serial dilutions of krill stomach samples were tested in restriction digests (1 / 5, 1 / 10, 1 / 50, 1 / 100, 1 / 250, 1 / 500) and dilutions were made using AE buffer (Qiagen). Following incubation of the restriction digests, 2 µl of each digest was used directly as template in PCR reactions.

3.4 Results

3.4.1 LNA blocking probe titration

The plasmid test system was used to examine whether the LNA blocking probe was capable of suppressing krill DNA amplification during PCR. For the diatom : krill 1 : 1 plasmid ratio mix (D . K 1 : 1), the blocking probe was capable of completely suppressing krill DNA amplification across the full range of probe concentrations tested (0.5 - 8.0 µM) (Figure 3-2). Suppression was found to be krill DNA specific for low concentrations of LNA blocking probe (0.5 & 1.0 µM) but non-specific suppression of diatom DNA amplification occurred at higher concentrations (2.0 - 8.0 µM). Similar results occurred with the other diatom : krill plasmid ratio mixes (D : K 1 : 1 – D : K 1 : 10,000) (data not shown). This suggested that the blocking probe was functioning as expected at lower concentrations and further experiments were conducted with the LNA probe concentration held at 0.5 or 1.0 µM.

3.4.2 Suppression of krill DNA amplification in the plasmid test system

Plasmid ratio solutions were used to examine the effects of the LNA probe when different ratios of predator and prey DNA were present in the PCR template mix. Figure 3-3 shows the results obtained using 1 µM of LNA blocking probe. The blocking probe was capable of specifically suppressing krill DNA amplification across the full range of plasmid ratio mixes (D : K 1 . 1 – D : K 1 : 10,000). This caused a dramatic increase in the relative proportion of diatom amplicons in the amplified mix. However, suppression of krill DNA amplification did not give rise to an increase in the absolute number of diatom DNA amplicons. For the 1 : 1000 and 1 : 10,000 ratio mixes no diatom peak was evident in the samples that contained the LNA probe or the control. In these PCR reactions the amount of diatom template added was extremely low (~ 20 copies and ~ 2 copies respectively) and the diatom peaks probably fell below the detection threshold of the machine.

3.4.3 Krill stomach field samples

The LNA probe was applied to krill stomach field samples to determine whether this approach was capable of generating prey peaks in real samples. Figure 3-4 shows the typical results obtained from various dilutions of a krill stomach sample. The 1 · 1 dilution in AE buffer provides PCR conditions similar to those used in Chapters 2 & 3, where both diatom and dinoflagellate DNA were regularly amplified using group specific primers. Under these conditions 1 µM of LNA blocking probe only partially suppressed krill DNA amplification. In a subset of krill stomach samples diluted 1 : 1 very small peaks were observed down in the 260 - 270 bp range, where prey peaks were expected to be observed. Increasing the dilution of the krill stomach template resulted in complete suppression of krill DNA amplification (dilutions range 1 : 5 – 1 : 100). However, there was no evidence of prey DNA amplification in these samples. Increasing the concentration of LNA blocking probe (2 - 8 µM) applied to 1 : 1 template dilutions resulted in complete or almost complete suppression of krill DNA amplification but again no significant prey peaks were observed (data not shown).

3.4.4 Restriction enzyme digests of predator DNA

The application of restriction enzyme digests to plasmid ratio mixes prior to PCR amplification eliminated krill DNA amplification as expected (data not shown). The application of restriction digests to krill stomach field samples prior to PCR amplification did not eliminate krill DNA amplification or produce prey DNA peaks for any of the field samples assayed (data not shown). The efficiency of the restriction digests was subsequently examined by agarose gel electrophoresis. These gels suggested that the failure to remove krill DNA was due to the partial or complete failure of the restriction enzyme *Bam*HI to digest the stomach sample DNA (data not shown). Attempts to fix this problem included: (1) dilution of stomach sample DNA prior to digestion (1 / 5, 1 / 10, 1 / 50, 1 / 100, 1 / 250, 1 / 500), (2) cleaning up the DNA using phenol extraction and ethanol precipitation, (3) using alternative restriction enzymes (*Eag*I, *Sac*I), (4) increasing the amount of restriction enzyme (5 - 40 units), and (5) increasing the length of time for digestion from 4 hours to overnight. While some of these modifications provided minor improvements in digest results, only the 1 / 250 and 1 / 500 dilutions resulted in the elimination of krill amplicon peaks (data not shown).

None of the modifications produced prey peaks from any of the field samples tested (data not shown).

3.5 Discussion

3.5.1 Blocking predator DNA with LNA blocking probes

3.5.1.1 Blocking probe design

PCR clamping is an attractive method of dealing with predator DNA in diet samples. One of the main advantages is the ability to design probes that are highly specific for predator DNA. It is feasible to design blocking probes without using DNA analogs but such probes raise concerns about specificity because DNA : DNA hybrids are to some degree tolerant of base pair mismatches (Palumbi, 1990). DNA analogs provide superior specificity and binding affinity compared to DNA probes. Blocking probes constructed with DNA analogs are expensive but are probably highly cost effective because they save downstream processing of predator DNA amplicons. Another significant advantage of blocking probes is that they are simply added to the PCR amplification reaction and therefore do not require additional sample processing steps. This makes blocking probes amenable to high sample throughput without increasing the chances of contamination.

Although PCR clamping was pioneered more than a decade ago the design requirements for blocking probes are not fully understood and developing new probes is still a process of trial and error. The current study utilised the primer exclusion strategy which is probably the most design-constrained PCR clamp assay because it requires: (1) a suitable predator specific DNA sequence adjacent to the primer binding site, and, (2) careful control of primer and probe binding affinity to ensure that the primer out-competes the blocking probe for binding to prey DNA template but the probe out-competes the primer for binding to predator DNA. It should be remembered that these requirements are in addition to the normal requirements for prey detection assays that include primer binding sites that are conserved across prey species and primer binding sites that span a region of sequence variation to allow prey differentiation. The primer exclusion strategy was chosen because at the time there was no published work on LNA blocking probes and it was unclear whether LNA probes could function as internal blocking probes that were capable of resisting the 5' - 3' exonuclease activity of *Taq* polymerase.

The 5' - 3' exonuclease activity of *Taq* polymerase is not fully understood but current models suggest the 5' end of the DNA strand ahead of the polymerase is actively displaced by *Taq* or that *Taq* invades the space when the two strands melt (Ceska and Sayers, 1998). The displaced 5' flap structure then feeds into the exonuclease domain of the polymerase and cutting occurs at the point where the two strands bifurcate (Lyamichev et al., 1999). Cutting has been shown to occur on 5' flap structures up to 188 nucleotides in length (Lyamichev et al., 1993). This has important ramifications for internal blocking probes (used in strand initiation interference or elongation arrest) because 5' end modifications to blocking probes are probably insufficient for exonuclease resistance when the strand can be further displaced and cut beyond the modification. This suggests that successful internal probes probably function purely by resisting strand displacement, which relies on strong binding affinity.

Previous experience with internal blocking probes has shown mixed success. Early work with an internal probe constructed of unmodified DNA substituted a shorter amplicon extending from the probe to a primer for the full length amplicon sequence (Lewis et al., 1994). This strategy is really substitution of a long amplicon for a short amplicon rather than true blocking. Identical probes that were modified at the 3' end to prevent *Taq* extension were incapable of blocking amplification of the full length amplicon suggesting that they were easily displaced. The successful 'blocking' results in the study of Lewis et al. (1994) are difficult to reconcile with the 5' - 3' exonuclease activity of *Taq* and the study has not been repeated or cited since publication. Although PNA probes have much stronger binding affinities the results with internal probes have also been mixed, with some internal PNA probes failing to confer blocking (Orum et al., 1993; von Wintzingerode et al., 2000). Increasing the length of the probe improved blocking results (Orum et al., 1993), consistent with the theory that high binding affinity is important for internal blocking probes.

Since this work was completed three studies have been published that have used LNA blocking probes to screen human patients for genetic mutations (Hummelshoj et al., 2005; Senescau et al., 2005; Thiede et al., 2006). One study used a probe bound adjacent to the primer site to prevent primer extension (Senescau et al., 2005), the other two used internal probes to arrested strand elongation (Hummelshoj et al., 2005; Thiede et al., 2006). Similar to the current study, two of the studies used a phosphate group on the 3' end of the probe to

prevent primer extension (Senescau et al., 2005, Thiede et al., 2006). However, Hummelshoj and colleagues relied on evidence that LNA probes were resistant to primer extension (Di Giusto and King, 2004) to design LNA probes without additional chemical modifications. In all three studies the internal probes were able to resist the 5' - 3' exonuclease activity of *Taq* polymerase and cause partial or complete blocking of non-target template amplification (Hummelshoj et al., 2005; Senescau et al., 2005; Thiede et al., 2006). The freedom to place internal blocking probes anywhere along the predator amplicon sequence reduces constraints during assay design. In summary, designing internal blocking probes with a high percentage of LNA nucleotides will provide the most flexibility during assay design and the highest chance of successful PCR blocking.

3.5.1.2 Application of the LNA blocking probe to the plasmid test system

Applying the blocking probe to the plasmid test system demonstrated that the probe was capable of specifically inhibiting the amplification of krill DNA. At low concentrations of the blocking probe the blocking was specific to krill DNA. At higher concentrations there was some non-specific inhibition of diatom amplification similar to inhibition observed elsewhere (Senescau et al., 2005). Blocking probe concentration must therefore be optimised to find the best compromise between preventing the amplification of predator DNA and inhibiting the amplification of prey DNA. Inhibition could be caused by non-specific competition between primer and probe for binding to prey DNA template even though LNA bases confer high levels of specificity (Petersen and Wengel, 2003). Alternatively, high concentrations of blocking probe may sequester $MgCl_2$ within the PCR reaction. In this case increasing levels of $MgCl_2$ may allow higher blocking probe concentrations without causing non-specific inhibition.

At low concentrations (0.5 - 1.0 µM) the blocking probe functioned well in the plasmid test system by completely blocking krill DNA. However, this did not result in the expected increase in the number of diatom amplicons produced. The apparent lack of competition in the plasmid test system raised an interesting question about whether competition for amplification occurs in krill stomach field samples. It is possible that the total number of prey amplicons produced in PCR reactions is unaffected by the large excess of krill template, but the large proportion of krill amplicons at the end of the reaction make it difficult to detect prey

amplicons, which makes it appear that competition has occurred. The question about whether competition is occurring has important ramifications for choosing methods that remove predator DNA. If competition has a negative impact on the number of prey amplicons produced during PCR, then methods that remove krill DNA prior to PCR amplification would be preferred. In the absence of competition, methods that remove krill DNA after PCR amplification would be equally valid.

3.5.1.3 Application of LNA blocking probe to field samples

The application of the blocking probe to krill stomach field samples did not produce strong amplification of prey DNA. The chromatograms demonstrated that the probe could partially or completely block the amplification of krill genomic DNA (as opposed to plasmid template), depending on the sample dilution. However, this was not sufficient to generate prey peaks in any of the field samples that were assayed. The fact that prey DNA was successfully amplified from these samples with group specific primers (Chapters 2 & 4) suggests the undiluted or weakly diluted samples contained sufficient prey DNA for PCR amplification. The most likely explanation for these results is that the prey to predator ratio was too low for the blocking probe to have a useful effect. In PCR reactions that contained a large amount of mixed template the blocking probe could not completely suppress krill DNA amplification (e.g. Figure 3-4, 1 : 1 dilution). In PCR reactions where the mixed template was heavily diluted, the amount of prey DNA in the sample fell below detection limits (e.g. Figure 3-4, 1 : 5 – 1 : 100 dilutions). Direct comparison with other LNA blocking probe studies is complicated by the use of different methods, assay systems and DNA templates. However, in these studies target amplification and non-target blocking tended to be poor when target to non-target template ratios were less than ~ 1 : 100 (Hummelshoj et al., 2005; Senescau et al., 2005). Thiede and colleagues suggested their assay was suitable for target to non-target ratios up to 1 : 1000. It is also worth noting that in these studies complete suppression of the non-target template does not always occur even in the 1 : 100 ratio range (Senescau et al., 2005). If LNA blocking probe systems are restricted to situations where the prey : predator ratio is greater than 1 · 100 or 1 . 1000 then this represents a significant limitation of the approach.

3.5.1.4 Summary of LNA blocking probe

It is possible that further experimentation with blocking probe concentration, template concentration, PCR reaction conditions and stomach dissection methods may have resulted in the successful detection of prey peaks in krill stomach field samples using the probe from the current study. Experiments that spiked prey DNA into real samples may also help to explain the current results. In combination with this work it would be valuable to determine the typical range of prey : predator DNA ratios that occur in krill stomach samples. This would assist in developing test systems for the blocking probe that are a more accurate representation of the PCR reaction conditions that occur with real samples.

3.5.2 Restriction digest of predator DNA

To date, only one study has utilised restriction enzymes to remove predator DNA (Blankenship and Yayanos, 2005). The approach was to PCR amplify the diet samples using universal primers and then apply a restriction digest to the PCR reactions with an enzyme that targets amplicons derived from the predator. Incorporating the restriction digest step after PCR amplification is problematic if the predator DNA is present in vast excess and competes with prey DNA for PCR amplification. For this reason the current study attempted to apply the restriction digest to the diet sample prior to PCR amplification. This approach was unsuccessful because of the failure of the restriction enzymes to digest the krill genomic DNA in the diet samples. Subsequent discussions with the authors of the previous study revealed that they had attempted the same approach and suffered similar problems (L. Blankenship, *pers comm*). The most likely explanation for this problem is that the restriction digests were affected by an unidentified contaminant that was co-extracted with the DNA. As a general rule restriction digests are more sensitive to the presence of contaminants than PCR. While it might be possible to develop a post-PCR restriction digest protocol similar to the work of Blakenship and Yayanos (2005), this does not address the issue of krill DNA competing with prey DNA during PCR amplification. If the restriction digest approach is to be pursued, it would be better to focus on developing a DNA extraction protocol that allows restriction digests to be carried out on diet samples prior to PCR amplification. Another less likely possibility is a DNA modification that makes crustacean DNA insensitive to restriction enzymes. The DNA modification is not the typical DNA methylation since both *Bam*HI and *Eag*I are methylase insensitive and would therefore cut methlated DNA. However, it is

possible that crustaceans have a novel DNA modification akin to methylation that makes their DNA insensitive to restriction digests.

There are several other concerns with the restriction digest approach Results from the previous study were somewhat mixed Although samples were treated with restriction enzymes 57 % and 10 % of the diet sample amplicons were derived from the mollusc and amphipod predators, respectively (Blankenship and Yayanos, 2005). This suggests that the approach was not fully optimised to remove predator DNA and that there are effects specific to the predator that confound this approach. Another issue is that restriction enzymes generally have a recognition sequence of only 6 -8 nucleotides, which limits their specificity for predator DNA (Blankenship and Yayanos, 2005). The restriction site analysis in the current study showed that *Bam*HI had the best specificity for krill DNA, cutting only 11 out of 1507 prey sequences that were surveyed. However, the prey species that were cut belonged to crustacean groups, which is unfortunate because krill predation on other crustaceans is of particular interest (Chapter 1). Since sequence similarity is higher among related species this is likely to be a consistent problem. Restriction digests and gel-based fragment separation require additional sample processing steps that are not conducive to high throughput. Additional processing steps also provide additional opportunities for contamination.

3.5.3 Other approaches to the problem of predator DNA

There are at least four other approaches that could be applied to removing krill DNA for diet samples. Subsequent to the work in this study a method was published that dissected open krill stomachs and removed the internal contents (Martin et al., 2006). This meant that the stomach lining was not included in the sample which reduced, but did not eliminate, krill DNA from the diet sample. This approach is more complex and time consuming than the method used throughout this thesis. There are also questions about the consistency of this approach. Removing intact krill stomachs is a simple and consistent technique. Whether it is possible to consistently collect all of the internal material from krill stomachs is unclear. However, at this point it appears that removing the stomach lining from krill diet samples is an approach that is worth pursuing.

The second approach to removing predator DNA is by affinity purification. In this approach predator specific probes are bound within columns or attached to beads. Samples are either

run through the columns or mixed with the beads and then centrifuged to pellet the beads and remove the predator DNA from the sample. Affinity purification with PNA probes has been used in a study on bacteria derived from field samples (Chandler et al., 2000).

The third approach is headloop PCR (Rand et al., 2005), where one of the primers has an additional 5' end tail that specifically recognises predator sequence downstream of the primer binding site. Once the predator strand is synthesised the 5' tail loops around and binds to the downstream site preventing further rounds of amplification from the strand. Headloop PCR is attractive because it does not require DNA analogs or chemical modifications. However, similar to the blocking probe in this study, the approach requires predator specific sequence close to the primer binding site.

The forth approach uses a pilot probe (Lyamichev et al., 1993) and utilises the 5'-3' exonuclease activity of *Taq*. Probes are designed to form a short hairpin loop at the 3' end while the 5' end of the probe binds specifically to predator DNA sequence. When these probes bind to predator template they form a bifurcated 5' flap structure recognised by *Taq*'s exonuclease, which then cuts the predator template preventing further exponential amplification. This elegant option is attractive because pilot probes can be placed anywhere along the predator template. However, the idea has only been was described theoretically and to date there is no study that has used this approach in a PCR reaction.

3.6 Conclusions

This study examined approaches for removing predator DNA or preventing predator DNA amplification during PCR but did not provide a simple robust method for removing predator DNA from krill stomach samples. Both the LNA blocking probe and restriction digest approaches had problems that made them difficult to implement in practice. However, the results provide a useful starting point for developing these approaches further. The success with the plasmid test system but subsequent failure with field samples highlights the need for robust model systems that accurately simulate real samples. Of the two approaches tested, the LNA blocking probe approach has a number of advantages that should make this the preferred choice for future development. Alternative approaches including an alteration to the krill stomach dissection method should also be considered.

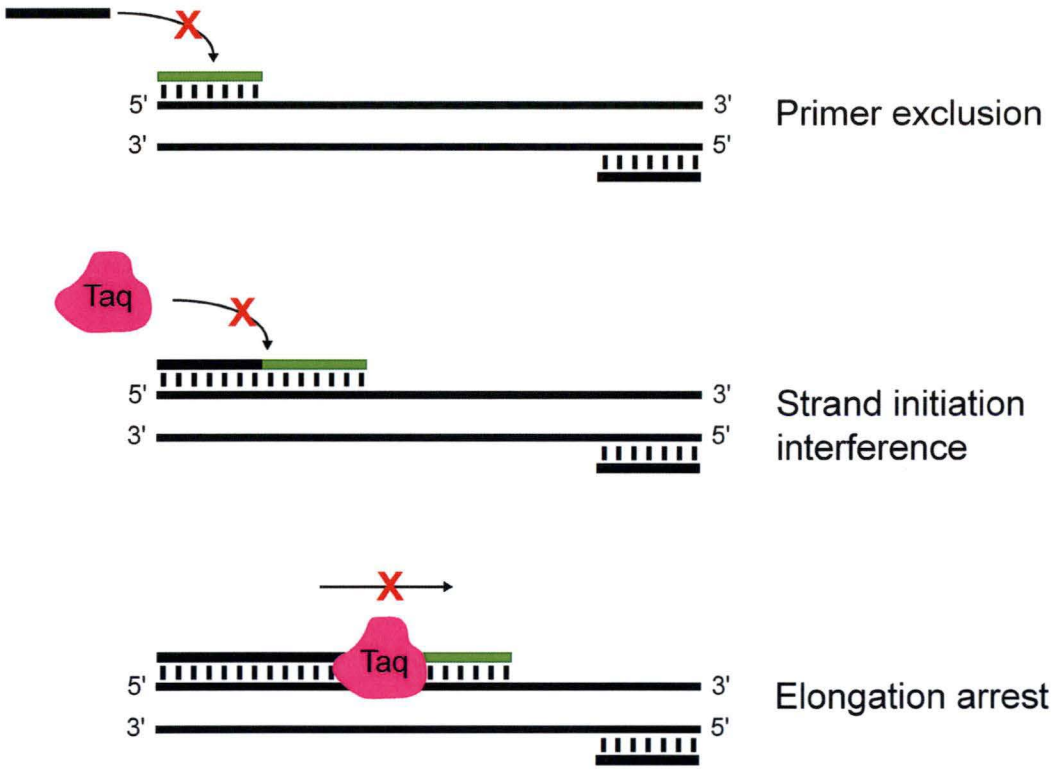

Figure 3-1 Three PCR clamp strategies. Blocking probe (green bar). Non-target template (long black bars). Forward and reverse primers (short black bars).

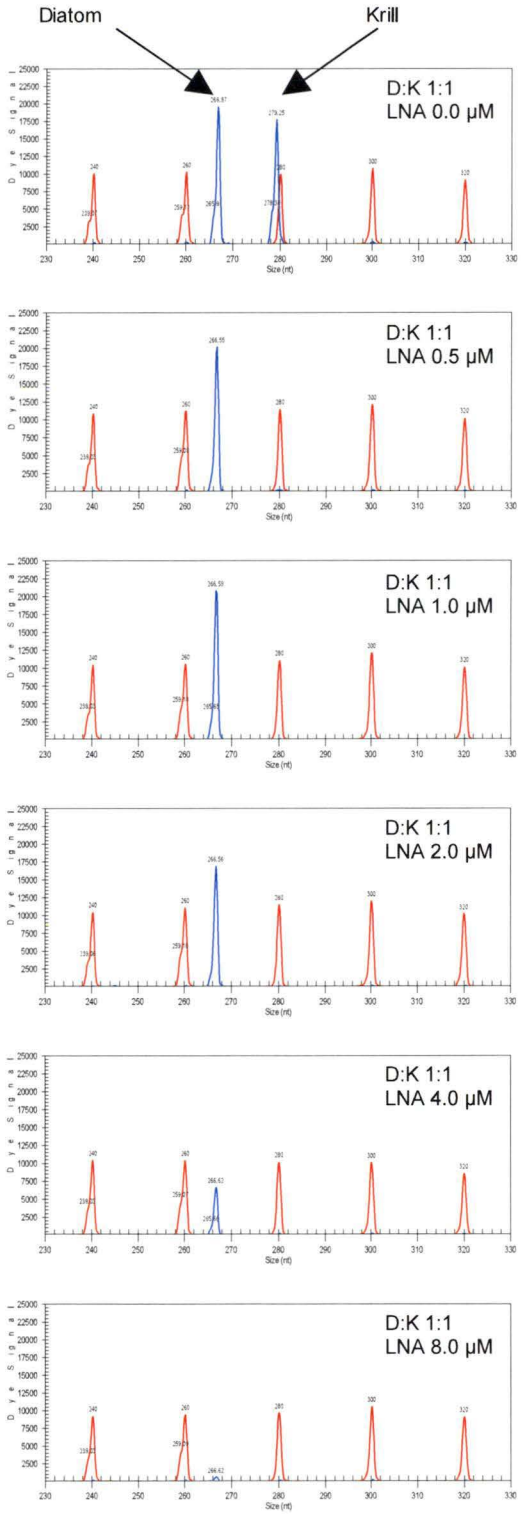

Figure 3-2 Capillary electrophoresis results showing the effect of various concentrations of LNA blocking probe on the PCR amplification of the 1:1 Diatom: Krill plasmid ratio mix. Red peaks are size standards. Blue peaks are PCR amplicons. Note: The initial blocking probe titration experiments were conducted using 25 cycles of PCR amplification, whereas subsequent experiments used 35 cycles to increase the number of PCR amplicons. This accounts for the small size of the peaks observed in Figure 3-2 compared to Figure 3-3 and Figure 3-4.

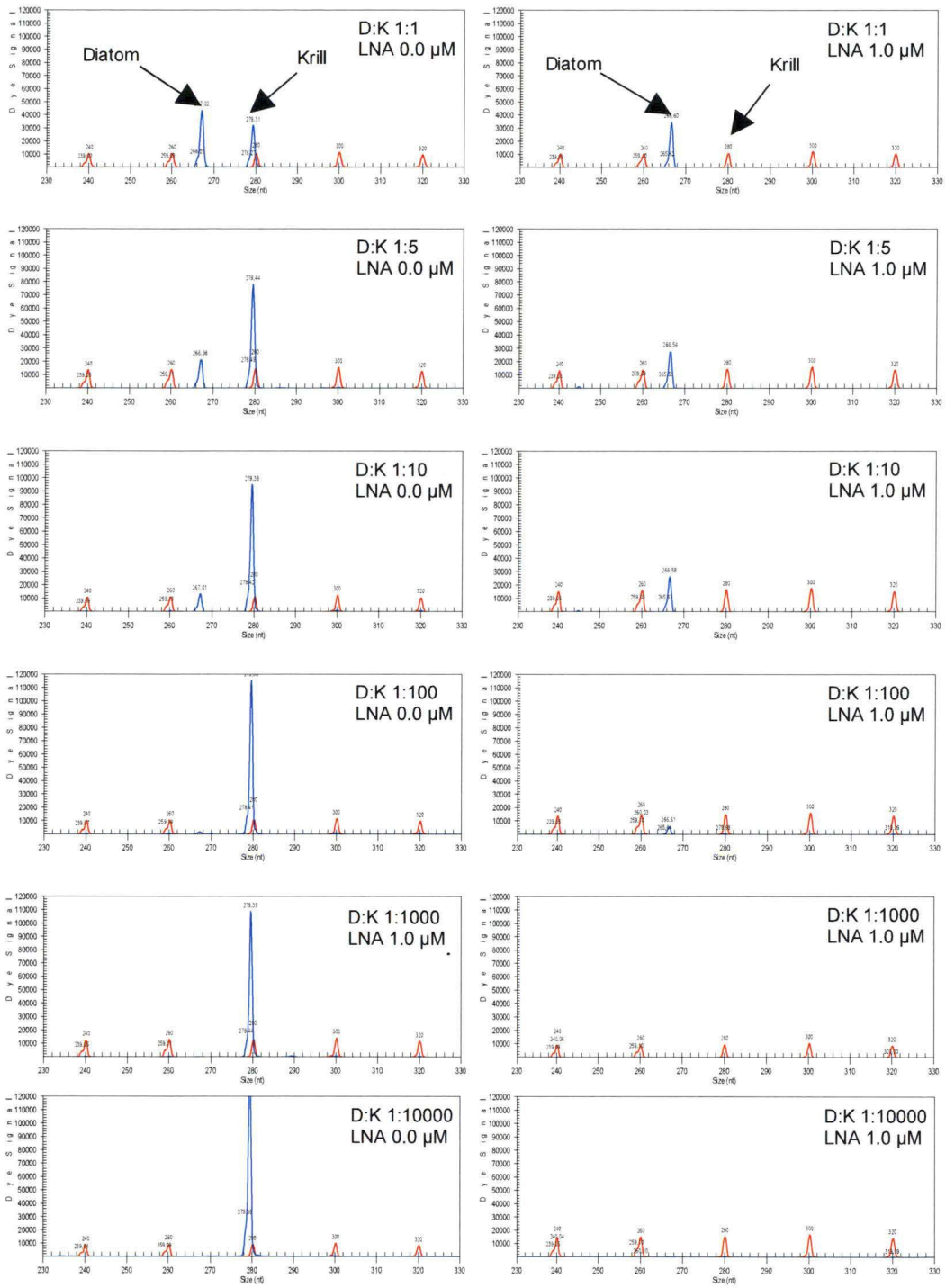

Figure 3-3 Capillary electrophoresis results showing the effect of the LNA blocking probe on various plasmid ratio mixes. Red peaks are size standards. Blue peaks are PCR amplicons.

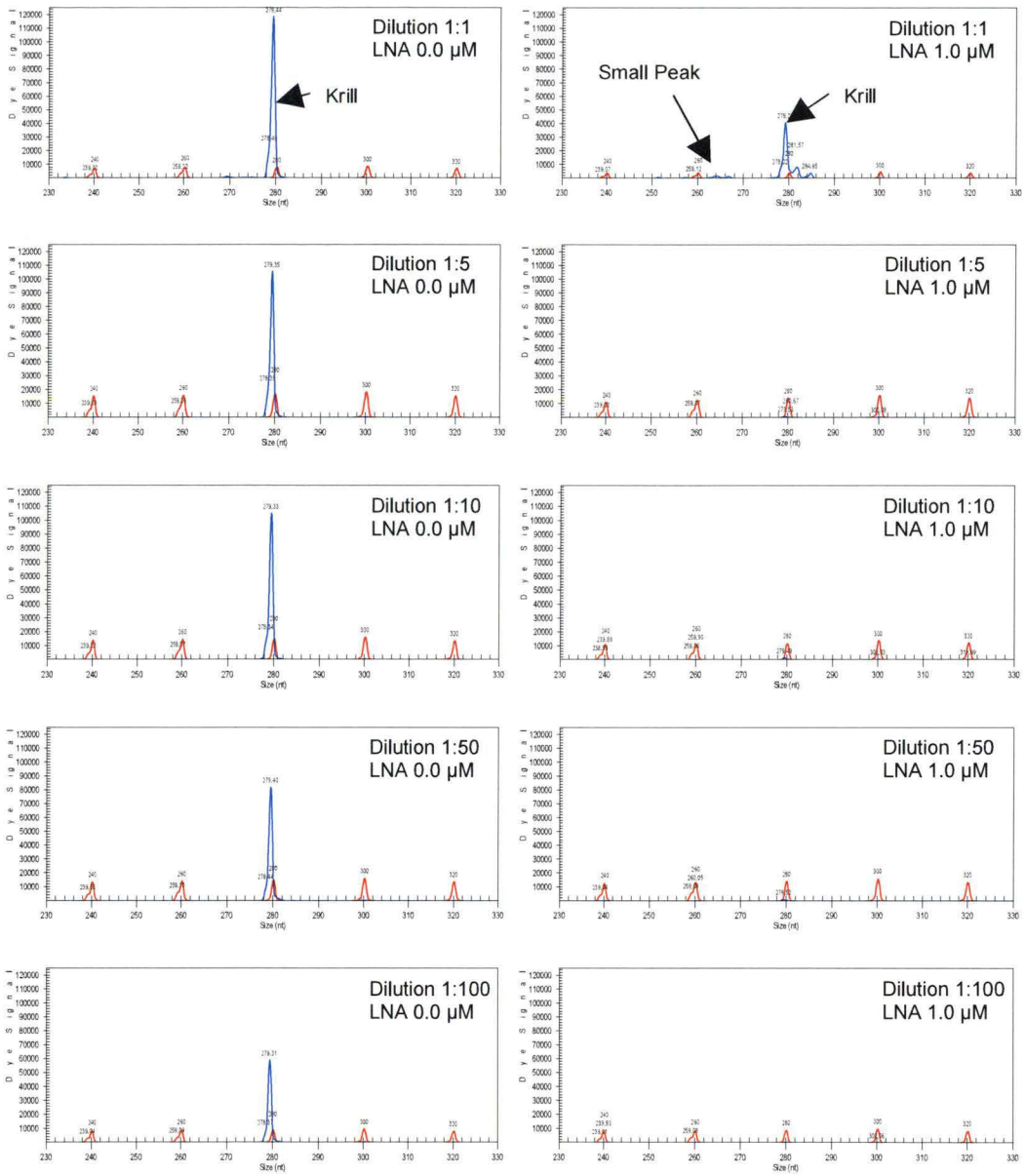

Figure 3-4 Typical capillary electrophoresis results from a krill stomach field sample amplified with and without LNA blocking probe. Red peaks are size standards. Blue peaks are PCR amplicons.

Table 3-1 Primers and probe used in the prey detection and LNA blocking experiments.

Phylum	Class	Genus & Species	Accession Number	Forward primer region (5'-3')	Reverse primer and blocking probe region (5'-3')	Product Size
ASF1194/20				CTTAATTTGACTCAACACGG		
ASR1473/20					GCATCACAGACCTGTTATTG	
Krill probe					ATTGCTCAGTCTCTTGCG	
Arthropoda	Malacostraca	*Euphausia superba* (Krill)	AY672801	----------	----------	280
		Euphausia pacifica (Krill)	AY141010	----------	----------	280
	Maxillopoda	*Calanus finmarchicus* (Copepod)	AF367719	----------	----------A---TG----	260
		Rhincalanus gigas (Copepod)	AY335855	----------	----------A----G---A	260
Bacillariophyta	Bacillariophyceae	*Fragilariopsis cylindrus* (Diatom)	AY485467	----------	----------C-CTATCT-CCT-	264
	Coscinodiscophyceae	*Chaetoceros gracilis* (Diatom)	AY625895	----------	----------C-CAATCT-CCT-	263
		Thalassiosira pseudonana (Diatom)	AJ535169	----------	----------CGCCATCT-CCTT	264
	Fragilariophyceae	*Fragilaria striatula* (Diatom)	X77704	----------	----------T----C-GTATCT-CCT-	264
Dinoflagellata	Dinophyceae	*Gymnodinium catenatum* (Dinoflagellate)	AF022193	----------	----------CTCAAACT-CCTT	262
		Peridinium sp. (Dinoflagellate)	AF022202	----------	----------CTCAAACT-CCTT	262
		Prorocentrum micans (Dinoflagellate)	AJ415519	----------	----------CTCAAACT-CCTT	262
		Protoperidinium abei (Dinoflagellate)	AB181882	----------	----------CTCAGGCT-CCGT	262
Haptophyta	Prymnesiophyceae	*Phaeocystis antarctica* (Haptophyte)	X77480	----------	----------CGCAAACT-CCAC	261
Mollusca	Gastropoda	*Aplysia californica* (Sea Hare)	AY039804	----------	----------A----G--T-	261
Foraminifera	Granuloreticulosea	*Neogloboquadrina pachyderma* (Foram)	AY453128	----------G---	----------CGCAA-CT-CCTC	519

Red base denotes LNA nucleotide base. – denotes nucleotides that were the same as the krill sequence. Bold sequences are those used in plasmid test system.

Chapter 4

Using DNA to rapidly screen Antarctic krill (*Euphausia superba*) for protist and metazoan prey

4.1 Abstract

The Antarctic krill (*Euphausia superba*) is a key species in one of the largest marine ecosystems on the planet, the Southern Ocean. Understanding krill diet is important for developing accurate models of krill populations and for understanding the flow of carbon and other nutrients through the Southern Ocean foodweb.

DNA has the potential to provide new insights into the diet of Antarctic krill (*Euphausia superba*) by providing better prey coverage and higher sample throughput than previous methods of diet analysis. This study developed a new DNA-based assay system that was capable of screening a large number of krill stomachs for the presence of specific metazoan and protist prey groups. The assay system was trialled on 216 krill collected from four field sites in the Indian sector of the Southern Ocean. The assay identified dinoflagellates as an important prey item at all four collection sites (detected in 191 of 216 krill, 88 %). Diatoms were prevalent at the three summer collection sites but virtually absent at the early spring collection site (163 of 216, 75 %). Metazoan prey groups were only detected in a few stomachs. Gastropods were the most frequently detected metazoan group (10 of 216, 4.6 %). Copepod DNA was only detected in one krill (1 of 216, 0.5 %). Microscopic diet analysis verified that the DNA diet assay was correctly detecting important prey groups and similarities and differences in diet between field collection sites. However, DNA and microscopic data differed on the relative importance of dinoflagellates and diatoms which may indicate bias in DNA diet analysis.

4.2 Introduction

Increasingly sophisticated models suggest that the krill population is constrained by a complex array of factors (e.g. Fach et al., 2002; Constable et al., 2003, Nicol, 2006), and food availability is one of these potential constraints. To determine the importance of food as a population constraint, models must estimate the strength of trophic links between krill and potential prey groups. These estimates need to be supported by empirical diet data derived from krill collected in the field.

Gathering empirical data about krill diet is challenging because there are limited opportunities for sampling and significant scope for local, regional and seasonal variation in diet (Atkinson et al., 1999; Nicol, 2006). Three broad categories of krill 'prey' are autotrophic

protists, heterotrophic protists and metazoans. A variety of diet analysis techniques have demonstrated that krill consume a diverse range of prey (e.g. Haberman et al., 2002; Martin et al., 2006; Schmidt et al., 2006). However, due to problems with current diet analysis techniques the role of heterotrophic protists and metazoans in krill diet remains unclear (Chapter 1).

Krill diet analysis would benefit from new techniques that can detect and differentiate trophic links to a suitable level of taxonomic resolution. Improvements in sample throughput would help to provide data on scales that are ecologically relevant. Two recent studies have explored the feasibility of applying DNA diet analysis to krill (Martin et al., 2006; Passmore et al., 2006). Both studies used clone libraries to examine the diet of a small number of krill but due to variations in target gene copy number between prey species both approaches probably suffered from problems with bias. In one of these studies where DNA results were independently verified by microscopy, evidence for bias was found (Passmore et al., 2006).

Recent DNA diet studies on terrestrial insects have used an alternative approach where trophic links were examined by gathering prey incidence data from a large number of animals (Agustí et al., 2003a; Harper et al., 2005; Juen and Traugott, 2007; Birkhofer et al., 2008). The aim of the current study was to trial an incidence based approach on a large number of krill. Group specific PCR primers were developed for a range of protists and metazoans groups and individual krill were screened for the presence or absence of these groups. The results provided DNA based prey incidence profiles from krill collected at four field sites. The results from the DNA analysis were then compared to results obtained from microscopic diet analysis. The study highlights a range of methodological issues that need to be considered in future applications of DNA diet analysis.

4.3 Material and Methods

4.3.1 Sample collection

Antarctic krill were collected in the Indian sector of the Southern Ocean on voyages with the RSV *Aurora Australis*. Krill were obtained from four sites over a two year period spanning Jan 2003 to Feb 2005 (Table 4-1). Krill were collected with rectangular midwater trawls (RMT8, nominal mouth area of 8 m^2 and mesh size of 4.5 mm) towed at ~ 1 knot. For the summer collection sites (A, B, D), schools of krill were detected with sonar and collected in

targeted trawls. For the spring site (C), krill were collected in a non-targeted trawl of surface waters in the marginal ice zone. Immediately after capture krill were preserved in 80 % ethanol which was subsequently drained and replaced 15 minutes and 24 hours after capture as described previously (Passmore et al., 2006). The time taken from the start of the trawls to initial sample preservation was less than 30 minutes for all collection sites. Samples were then stored at 4 °C until the ship returned to Australia.

4.3.2 Satellite Data

Estimates of daily surface chlorophyll concentrations were obtained from a merged Aqua-MODIS and SeaWiFS data set (Feldman and McClain, 2007). A composite image for each collection site was generated by merging daily data for 10 days up to and including the day of collection using Matlab software (Mathworks, Natick MA, 2007).

4.3.3 Krill dissection and stomach DNA extraction

Krill were dissected to obtain intact stomachs for DNA extraction. Dissections were performed using a dissecting microscope and two pairs of forceps. Individual krill were rinsed in fresh ethanol and gently dried on paper towel prior to dissection in a dry sterile Petri dish. Forceps were used to remove the carapace and expose the animal's stomach on the dorsal surface. Intact stomachs were then removed with forceps taking care not to squeeze out the stomach contents. To prevent the transfer of any contaminants from the external surface of the krill to the stomach tissue, care was taken not to make contact with the stomach during the removal of the carapace and the forceps were flame sterilized prior to the removal of the stomach and between each krill dissection.

DNA extraction was performed on individual krill stomachs using a DNeasy Tissue kit in a 96 well plate format (Qiagen). Samples were processed according to the manufacturer's instructions except for the following modifications. Prior to overnight digestion in ATL buffer + Proteinase K, the stomachs were disrupted manually with a pipette tip to assist tissue digestion. DNA was eluted in 200 µL of AE buffer (Qiagen) and stored at 4 °C.

4.3.4 Determining DNA concentration

The concentration of DNA in stomach extracts was determined using a Genios microplate multireader (Tecan) set to read fluorescence. Picogreen reagent (Molecular Probes) was

diluted 1 : 200 in TE buffer (10 mM Tris-HCl, 1 mM EDTA) and 195 µL of this mixture was pipetted into the wells of black flat-bottomed microtitre plates. Five µL of krill stomach extract was added per well and fluorescence was read in the plate reader as per the manufacturer's instructions. Standard curves to convert fluorescence readings to DNA concentrations were generated using a dilution series of the DNA standard supplied with the Picogreen reagent.

4.3.5 Pre-screening stomach extracts

All stomach extracts were prescreened for the presence of amplifiable DNA. PCR was performed using a set of 'universal' eukaryotic large ribosomal subunit primers (LSU) (Table 4-2) capable of amplifying a 500 - 600 bp amplicon from a diverse range of eukaryotes including krill. PCR reaction conditions were the same as for the prey screen (see below), except that the PCR cycle extension step was set to 150 s to account for the greater length of the PCR products. Only one sample failed to generate an amplicon with the universal LSU primer set and this sample was omitted from subsequent analysis.

4.3.6 Group specific primers

Nine sets of group specific primers were used to screen stomach extracts for the presence of prey. Six of the primer sets had previously been developed in our laboratory (Jarman et al., 2004; Bissett et al., 2005; Jarman et al., 2006) or were taken from the literature (Pawlowski et al., 2002)(Table 4-2). The other three primers sets were developed as part of this study to target Bacillariophyta, Tintinnida and Dinophyceae. Primers were designed manually by examining large sequence alignments of the nuclear small ribosomal subunit (SSU) and large ribosomal subunit (LSU) genes. Alignments were developed using the ARB software package (Ludwig et al., 2004) and were constructed by using the large alignments available from the ARB website (http://www.arb-home.de) and European Ribosomal RNA database (http://www.psb.ugent.be/rRNA/index.html) as backbone alignments. Backbone alignments were then augmented with additional sequences of relevant prey groups taken from NCBI GenBank (http://www.ncbi.nlm.nih.gov/).

Estimates of primer coverage were based on all available non-redundant GenBank sequences for targeted groups that extended across the region amplified by the primers. A Southern Ocean specific estimate of coverage for the dinoflagellate primer set was achieved by selecting the sub-set of sequences derived from all dinoflagellate families described in a

recent text on Antarctic protists (McMinn and Taylor, 2005). Sequences were considered matched when both primers exactly matched the sequence or when specific mismatches occurred that were allowed for during primer design. These estimates are only a rough guide to coverage because of the limited amount of data that are available for species in the Southern Ocean.

The annealing temperatures used for these primer sets were determined empirically by testing them on DNA extracted from species within the target group across a range of temperatures (data not shown). Limited empirical testing of the primers on a small number of target and non-target species indicated that the primers were group specific (data not shown). Comprehensive testing using this approach was not feasible given the size and diversity of the groups involved and the fact that many species within the target groups have not been isolated or cultured. Primer specificity was instead proven by verifying the identity of amplicons obtained from prey screening of stomach extracts (see below).

4.3.7 PCR-based prey screening of stomach extracts

Stomach extracts were screened for the presence of nine potential prey groups using a standardised PCR protocol. DNA extracted from krill stomachs was diluted 1 : 1 with AE buffer (Qiagen) to create a working stock for PCR. PCR reactions contained: 2 µL of diluted stomach extract, 2.5 mM $MgCl_2$ (Gibco), 0.125 mM dNTPs (Gibco), 0.25 µM of each primer (Geneworks), 1 X Bovine Serum Albumin (NEB), 0.5 units of Amplitaq Gold (Gibco), 1 X manufacturer's PCR buffer (Gibco) and made up to 20 µL with dH_20. Samples were amplified in a PTC-225 thermal cycler (MJ Research) using the following cycling parameters: preheat at 95 °C for 10 min, 35 cycles of 95 °C for 10s, primer specific annealing temperature (Table 4-2) for 20 s and 72 °C for 40 s. The final extension was at 72 °C for 5 min. PCR reactions were visualized by running 5 µL of each PCR reaction on a 2% agarose gel stained with ethidium bromide. Presence or absence of PCR bands was scored by eye from examination of the agarose gels on a UV transilluminator. Positive controls were DNA extracts from cultures of prey organisms or prey individuals that belonged to targeted prey groups. Negative controls included no-template PCR and DNA extraction controls.

4.3.8 Post-screen verification of group primer specificity

To verify that the group specific primers used in this study were amplifying from the correct prey group, a subset of the PCR amplicons generated during the prey screen was cloned, sequenced and identified. All primer sets that produced positive results in the prey screen were tested for specificity. When a primer set produced amplicons in less than five stomach extracts, then at least one clone from each sample that produced amplicons was sequenced. When a primer set produced amplicons in more than five stomach extracts and where all products appeared to be the same size, five samples were selected at random and at least one clone from each sample was sequenced. Alternatively, if a primer set produced different sized amplicons within a site, at least one clone of each size class was sequenced with a minimum of five clones from five samples sequenced in total. Amplicons were cloned directly from the prey screen PCR reaction using the TOPO-TA cloning kit (Invitrogen). Plasmid DNA was prepared using the Mini Plasmid Prep kit (Mol Bio) and the plasmids were sequenced with the CEQ8000 Genetic Analysis System (Beckman Coulter) as per manufacturer's instructions. Sequences were grouped into 'Operational Taxonomic Units' (OTUs) for each site, where individual sequences were counted as a single OTU if they had < 1 % sequence divergence. All OTUs were 'identified' by comparing their sequence to sequences from the GenBank database using the BLAST algorithm (Altschul et al., 1990). OTUs were reported as identified if they had at least a 70 % match along their full length to a taxonomically identified GenBank sequence. The percentage match limit was deliberately set low to increase the chances of detecting false positives. Data were only allowed to contribute to the incidence results when all of the sequenced amplicons were identified as belonging to the prey group targeted by the primer set. Amplicon sequences were deposited in GenBank (Accession numbers EU249237 - 67).

4.3.9 Microscopic examination of krill stomachs

The stomach contents of four or five krill from each site were examined using light microscopy. Gut contents from each krill were rinsed into a Sedgewick-Rafter counting chamber and examined under a coverglass at 400 X magnification. Krill stomachs from the summer collection sites (A, B, D) contained thousands of prey items so a minimum of 400 items were examined per stomach. Krill stomachs from the spring site (C) contained less than 400 prey items per stomach so all items were examined. Items were identified to the

same taxonomic resolution as the PCR primers were designed to target, with one additional category for items that appeared to be derived from crustaceans but could not be identified further.

4.4 Results

4.4.1 Krill collection sites

The three summer collection sites (A, B and D) were from the Prydz Bay region, on the continental shelf slope, south of the southern boundary of the Antarctic circumpolar current (SBACC) (Figure 4-1, Table 4-1). Satellite data showed that there were strong and dynamic phytoplankton blooms in the Prydz Bay region during the 2002 / 03 and 2004 / 05 summer seasons but at the time of collection all three sites had low levels of surface chlorophyll (< 0.5 mg m^{-3}). The spring collection site (C) was more than 1000 nautical miles north east of the other sites and situated north of the SBACC. Satellite data at the time of collection was limited due to ice and cloud cover (Figure 4-1) but data from October to December 2004 suggested surface chlorophyll levels remained below 0.5 mg m^{-3} (data not shown).

4.4.2 Primer Design

Three sets of PCR primers were developed to target diatoms (Bacillariophyta), dinoflagellates (Dinophyceae) and tintinnids (Tintinnida). Developing group specific primers for protistian prey was difficult because: (1) most of these groups are large, ancient, diverse and often paraphyletic, (2) the abundance and diversity of species present in the Southern Ocean are only defined in broad terms, (3) DNA sequence data for protist groups tends to be sparse and unbalanced, with many of the sequences derived from a limited subset of the full taxonomic spectrum. It was therefore difficult to predict group specific DNA sequence motifs that would be shared by all or most of the potential prey species within a targeted taxonomic group, but not by species outside the group. To ensure a low level of false positives during prey screening, a conservative approach to primer design was employed with an emphasis on maintaining target group specificity. A consequence of this approach was that target group sequences were also excluded. A crude measure of group coverage suggested the Bacillariophyta primers matched 121 of the 148 (81 %) non-redundant diatom sequences that were available in GenBank. Coverage for the Tintinnida primers was similar but sequence data for the group were more limited (19 of 27, 70 %). The Dinophyceae primers

were only capable of amplifying 92 of 315 (29 %) of all dinoflagellate sequences. When the list of dinoflagellate sequences was restricted to dinoflagellate families that are known to occur in the Southern Ocean the estimate of coverage improved to 59 of 126 (46 %).

4.4.3 DNA-based prey incidence and diversity

Stomach extracts from 216 krill were assayed for the presence of DNA from nine potential prey groups. The three summer collection sites (A, B and D) showed strong similarity in their broad-scale prey incidence profiles (Figure 4-2, Table 4-3). Diatoms and dinoflagellates were both present in the majority of krill at each site. The incidence of dinoflagellates was consistently higher than diatom incidence across the three sites. Copepods, foraminiferans and gastropods were all detected at low frequencies. Most of the krill surveyed at the summer sites were positive for at least one prey group. Sequencing PCR amplicons derived from the prey screen revealed some differences in prey diversity between the three summer sites (Table 4-4). Site A had a more diverse mix of dinoflagellates than sites B and D.

The spring collection site (C) had a prey incidence profile that was different to the summer collection sites. Dinoflagellates were detected in approximately half of the animals but no diatom DNA was detected. Gastropods were again detected in a small number of krill. Almost half of the krill at site C had no detectable prey DNA.

4.4.4 Post-screen verification of primer specificity

Sequence data derived from a subset of PCR amplicons generated in the prey screen showed that the primer sets were amplifying from their designated target groups (Table 4-4). When prey incidence was high, all amplicons selected for identification were easily matched to taxonomically identified GenBank sequences and were derived from the correct target groups. When prey incidence was extremely low, several primer sets produced a small number of PCR amplicons that could not be identified (Table 4-4) despite the low percentage match threshold used for identification. Unidentified amplicons could have been due to a lack of sequence data about targeted groups. Alternatively this could indicate an increased propensity for false positives in the absence of target group DNA. Overall the results suggest that despite the limited sequence data for protists, it is feasible to design specific but conservative primers *in silico* that have low false positive rates during prey screening. Post

screen verification proved to be an efficient check of primer specificity when comprehensive empirical testing by other methods was not feasible.

4.4.5 Microscopic analysis of krill stomachs

Microscopic gut analysis produced results that were similar to the DNA diet analysis (Figure 4-2, Table 4-5). At the summer sites (A, B and D), there were thousands of prey items in each stomach, indicating strong and recent feeding activity. Gut contents were dominated by diatoms and dinoflagellates consistent with the DNA data, but, in contrast to the DNA results, diatoms were the dominant prey item. At the spring site (C), there were only a few prey items in each stomach suggesting that the animals were in an area of low food availability or were not actively engaged in feeding. Dinoflagellates dominated the microscopic prey proportions, which was consistent with the DNA results. However, in contrast to the DNA results, microscopy also detected a small number of diatom frustules. A small number of foraminiferans and gastropods were detected at all sites and the results showed a reasonable correspondence with the DNA data given the size of their contribution. Microscopy did not find any clear evidence of krill consuming copepods but did detect a small number of fragments that were thought to be derived from crustacean setae and exoskeletons (Table 4-5). Given the size of these structures it was likely that they were derived from copepods but definitive identification was not possible (K. Swadling, *pers comm*).

4.5 Discussion

4.5.1 Ecological interpretation

The summer collection sites (A, B and D) were in the Prydz bay region on the continental shelf slope. This region experiences significant phytoplankton blooms during the summer but the three collection sites were not in areas with high levels of surface chlorophyll. Despite this the stomachs of krill at all three sites were generally full. This suggests that in these cases surface chlorophyll was not a good indicator of strong feeding activity in krill. Given that the animals at these sites were collected below a depth of 20 m it is possible that they were feeding on phytoplankton blooms that were below the depth detected by satellite. Deep phytoplankton blooms are known to occur in Antarctic waters but their prevalence is unclear (Holm-Hansen et al., 2005).

Previous surveys of the protist assemblage in Prydz Bay (Kopczynska et al., 1995; Fiala et al., 1998; Zhu et al., 2003) and across the Indian Ocean sector (Kopczynska et al., 1986; Waters et al., 2000) provide a reasonably consistent picture. The assemblage is typically dominated by diatoms both in terms of cell number (54 – 84 % of total cells) (Kopczynska et al., 1986; Kopczynska et al., 1995; Zhu et al., 2003) and biomass (42 – 80 % of total carbon) (Kopczynska et al., 1995; Fiala et al., 1998). Often the largest diatom contribution comes from the genus *Fragilariopsis* (Kopczynska et al., 1986; Kopczynska et al., 1995; Fiala et al., 1998, Waters et al., 2000; Zhu et al., 2003). Small flagellates (< 10 µm) are often important numerically (24 – 40 % of total cells) (Kopczynska et al., 1986; Kopczynska et al., 1995) but make a variable contribution to biomass (5 – 24 % of total carbon) (Kopczynska et al., 1995; Fiala et al., 1998). Commonly occurring flagellate groups include Cryptophytes, *Pyramimonas* and *Phaeocystis* (Kopczynska et al., 1986; Kopczynska et al., 1995; Fiala et al., 1998; Waters et al., 2000). Dinoflagellates make a small contribution in terms of abundance (3 – 6 % of total cells) (Kopczynska et al., 1986; Kopczynska et al., 1995; Zhu et al., 2003) but are significant in terms of biomass (14 – 33 % of total carbon) (Kopczynska et al., 1995, Fiala et al., 1998). Common dinoflagellate genera include *Gymnodinium*, *Gyrodinium*, *Protoperidinium* and *Prorocentrum* (Kopczynska et al., 1986, Kopczynska et al., 1995; Fiala et al., 1998; Waters et al., 2000).

In broad terms the DNA and microscopic diet analyses from the summer sites were consistent with previous studies of the Indian Ocean protist assemblage. The microscopic diet analysis was more consistent with the protist surveys because it suggested that diatoms were a more abundant prey item than dinoflagellates, whereas DNA analysis indicated that dinoflagellates were more prevalent. The reason for this discrepancy is unclear. It is possible that dinoflagellates are underrepresented in the microscopic analysis. Delicate athecate dinoflagellates are generally more abundant than thecate dinoflagellates but are often underrepresented in protozooplankton surveys due to poor preservation (Garrison and Gowing, 1993). For similar reasons athecate dinoflagellates are also unlikely to survive ingestion to be detected by microscopic diet analysis. The identification of DNA clones provides limited support for this explanation with the majority of clones from site A derived from the athecate genus *Gymnodinium*. However, the clones from site B and D are from thecate genera. Alternatively the difference between DNA and microscopy may indicate that

DNA analysis is overestimating the role of dinoflagellates in krill diet (further discussion on bias in DNA analysis below).

The spring collection site (C) was over deeper water north of the SBACC. Satellite data suggested that the collection site had low levels of surface chlorophyll, consistent with previous studies of satellite data that show this region of the Indian Ocean has low levels of surface chlorophyll throughout the year (Sullivan et al., 1993; Moore and Abbott, 2000; Meguro et al., 2004). Ship based surveys have also identified the waters north of the SBACC to be an area with low protist stocks (Chiba et al., 2000; Waters et al., 2000), where flagellates rather than diatoms are dominant (Waters et al., 2000). The microscope and DNA diet analyses of krill diet at site C were consistent with the view that this area had low stocks of protists. Microscopy revealed that there were only a small number of items that could be identified in krill stomachs from this site. In the krill examined less than 300 items were detected per krill stomach, compared to thousands of items in krill at the three summer sites. Furthermore, DNA diet analysis revealed that a large proportion of animals contained no detectable prey DNA. Not withstanding this low incidence of prey in general, both microscopy and DNA identified dinoflagellates as the major prey item for krill at site C. While microscopy detected a small number of diatom frustules, the DNA analysis did not detect the presence of any diatoms. Given the small number of diatom frustules that were detected by microscopy, it seems likely that these frustules were the remnants of a meal that was consumed a long time prior to capture and that diatom DNA was no longer present.

The Indian Ocean sector contains a diverse assemblage of metazoans including significant contributions from krill, copepods, amphipods, gastropods and tunicates (Hosie and Cochran, 1994, Hosie et al., 1997; Hosie et al., 2000). There is also evidence of spatial separation between krill and other metazoans (Hosie and Cochran, 1994, Hosie et al., 2000) which may indicate krill predation on other metazoan groups (Atkinson et al., 1999). While copepods have often been discussed as a food source for krill the field based evidence for this trophic link is not strong (Chapter 1, Schmidt et al., 2006). In the current study DNA diet analysis only detected copepod DNA in one of the 216 krill surveyed. Microscopy did not provide any definitive evidence of copepod consumption (e.g. easily identified mandibles). These results suggest that copepods were not an important component of the krill diet at any

of the four collection sites. While there is sa significant body of literature examining krill predation on copepods, there is only one study that has detected gastropods in krill diet (Hopkins and Torres, 1989) and they have not been described as an important food source for krill. The current study provides evidence of gastropod consumption at all four collection sites that was limited but well in excess of copepod consumption. This suggests that gastropods may be a more important food source for krill than previously recognised. It is difficult to decide what level of prey incidence should be considered significant and this is especially true for metazoans. Their size and high nutritional content mean they could occur at low incidence but still make an important contribution (Atkinson and Snyder, 1997). If these low incidence trophic links are important they will only be elucidated by examining large numbers of krill using methods that can detect and differentiate metazoans from other types of prey.

Overall the results of this study suggest that krill were mainly feeding on protists, and that metazoans were not a dominant component of krill diet. This is similar to the conclusions of another recent summer field study that used other methods to examine krill diet (Schmidt et al., 2006). Future DNA diet studies will need to integrate DNA analysis with sampling of the protist and zooplankton assemblages at collection sites to provide a more detailed assessment of krill feeding behaviour.

4.5.2 Critique of DNA methods

DNA diet analysis has been applied to a range of vertebrate and invertebrate predator-prey systems and this work has been reviewed elsewhere (Chapter 1, Symondson, 2002; Sheppard and Harwood, 2005). Two previous studies have explored the application of DNA diet analysis to Antarctic krill (Martin et al., 2006; Passmore et al., 2006). Passmore et al. (2006) used a group primer approach similar to the current study. Martin et al. (2006) advocated a universal primer approach as a method that provides better prey coverage and less bias than group specific primers. The following discussion compares universal and group primer approaches in terms of prey coverage, sample throughput and bias.

In terms of prey coverage, universal primers are attractive because they amplify sequences from a broad range of prey species in a single PCR reaction (Martin et al., 2006). However, it is important to recognise that even the best universal primers are not universal (Brunk et al.,

1996, Marchesi et al., 1998, Diez et al., 2001), and that targeting short DNA fragments, to counter the degraded nature of diet DNA, generally leads to further compromises in prey species coverage. Once this has been acknowledged, the distinction between universal and group specific primers becomes less clear since, either way, multiple sets of primers would be required to provide comprehensive prey coverage. Group specific primers are best viewed as a directed approach for examining specific trophic links, not as a method for providing comprehensive prey coverage. As with universal primers there is inevitably some discrepancy between desired and actual prey coverage that will vary for different prey groups and primer sets. Reporting species coverage for primer sets is uncommon because the process is not straightforward. There are no simple rules to define what sequences should be included or excluded in coverage estimates, particularly when sequence data and information about what prey species are present in the environment are limited. Ultimately, a move towards reporting coverage is important to allow proper evaluation of PCR-based methods and the data they produce.

In terms of sample throughput, the universal primer approach provides efficient amplification from a broad range of prey species. However, significant effort is then required to separate and identify the PCR amplicons. Traditionally this has been achieved using clone libraries or with gel-based separation techniques such as denaturing gradient gel electrophoresis (DGGE). Clone libraries are expensive which limits their application to a small number of samples. DGGE is more cost effective per sample. When the universal primer/DGGE approach has been applied to environmental samples it tends to produce complex banding patterns (e.g. Diez et al., 2001, Marie et al., 2006; Martin et al., 2006). Generally, only a subset of these bands are cloned, sequenced and identified because of the cost and effort involved. In most cases the strongest bands are selected for identification based on the assumption that these bands represent the most important species in the sample. Comparing the banding profiles from different samples and matching bands across samples does reduce the number of bands that need to be identified but studies that use DGGE to comprehensively examine hundreds of samples are rare.

For the group primer approach, significant effort is required to design high quality primers and to empirically test their specificity. However, once this has been achieved a large

number of samples can be efficiently screened for the presence of specific prey groups of interest. In the current study, sample throughput was sufficient to allow one person to screen 216 stomach samples for a prey group in a single day. This could easily be extended to thousands of samples per day, at low cost, by changing the detection method from agarose gels to quantitative PCR. These samples sizes are starting to reach the point where it is feasible to make statistically valid conclusions about krill diet.

In regards to bias, all methods of diet analysis are biased to some degree but how and to what extent DNA diet analyses are biased is not well understood. Universal primers are unlikely to have significant advantages over group specific primers in terms of bias. For krill diet the most likely source of bias is differences in target gene copy number and cell number between different prey items. Gene copy number and cell number combine to produce a specific amount of signal per prey item but how signal varies across different prey species and life stages is not known. Krill DNA diet analysis has focused on nuclear ribosomal genes primarily because this is the only region that provides significant amounts of sequence data for krill prey species. Also, ribosomal genes have several characteristics that make them ideal for PCR assays (Hillis and Dixon, 1991). The problem is that ribosomal gene copy number varies by five orders of magnitude across different eukaryotic species (Long and Dawid, 1980; Prokopowich et al., 2003; Zhu et al., 2005). This results in an unknown but potentially large distortion in the ratio of ribosomal genes away from true prey consumption ratios.

For the universal primer / DGGE approach targeting eukaryotes, variation in ribosomal gene copy number invalidates the assumption that strong DGGE bands represent important species in the sample. Furthermore, the combined effects of bias caused by rDNA copy number variation (Farrelly et al., 1995) and from other sources (Suzuki and Giovannoni, 1996; Chandler et al., 1997; Ishii and Fukui, 2001) call into question claims that these profiles represent important species in the sample. Studies that have examined the sensitivity of universal primer / DGGE using artificial mixtures of bacterial sequences suggest the approach is capable of detecting sequence that contribute > 1 % of the total sequence mix (Muyzer et al., 1993, Murray et al., 1996). However, this is inadequate to cope with the five orders of magnitude variation in eukaryotic rDNA copy number. When multiple sets of

universal eukaryotic primers have been applied to the same sample, the overlap between the species lists obtained with different primer sets has been low (Diez et al., 2001; Stoeck et al., 2006). This suggests that the results from universal primer sets are not an unbiased representation of sequence diversity and abundance (Stoeck et al., 2006). It seems likely large differences in rDNA copy number and competition for PCR amplification will significantly affect results derived from universal primers. This issue has been covered in studies on bacteria where ribosomal gene copy number varies between ~ 1 and 15 (Farrelly et al., 1995; Chandler et al., 1997) but is not strongly acknowledged in work on eukaryotes where the differences between species is much greater.

The group primer approach is to some extent protected from ribosomal gene copy number bias because group detection is independent of other prey groups that are present in the sample. However, variation in ribosomal gene copy number will affect group detection limits and comparisons between groups. In the current study this may explain the high DNA-based incidence of dinoflagellates compared to the results obtained via microscopy. Previous work has demonstrated that in some dinoflagellate species rDNA copy number is extremely high (Zhu et al., 2005). Despite these bias problems, the comparison of the DNA-based incidence results with microscopic prey proportions suggested the group primer / DNA incidence approach was providing a useful indication of important prey groups and accurately detecting changes in diet between collection sites.

4.6 Conclusions

Krill are a generalist predator that consumes a taxonomically diverse range of prey. This makes it extremely difficult to develop a diet analysis system that provides comprehensive prey coverage. While the goal of providing comprehensive prey coverage with a DNA based system is worthwhile and may be feasible in the future, it is clear that there are issues with current approaches that need to be addressed before robust ecological interpretations can be made. In particular, target gene copy number is likely to have a dramatic effect on results when targeting nuclear ribosomal genes. In the meantime, studies that focus on specific trophic links are easier to implement and can provide useful information. The current study highlights the potential of DNA to examine large numbers of samples and to detect and differentiate prey items that are difficult to examine using other methods. This study is also

the first attempt to provide a quantitative examination of krill diet for key prey groups using DNA. In assessing the relative merits of DNA diet methods it is important to keep in mind the final goal which is to provide quantitative diet analysis on ecologically relevant sample sizes.

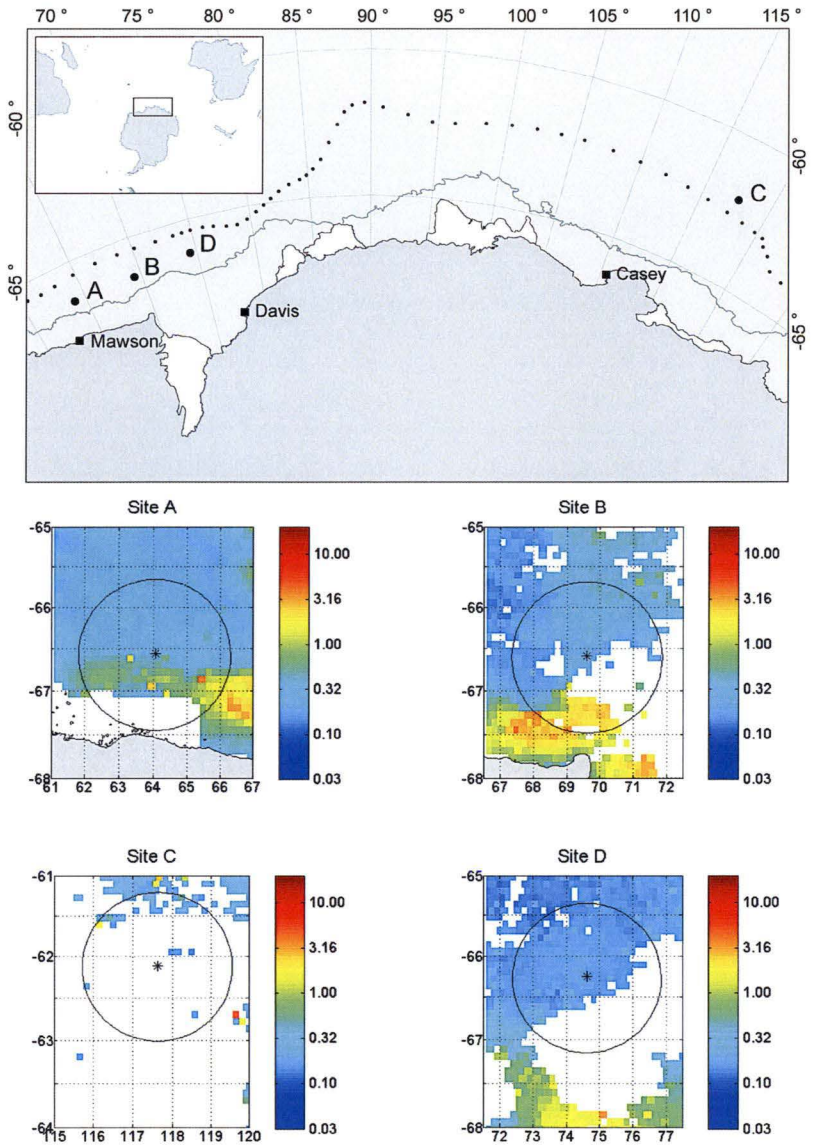

Figure 4-1 Krill collection sites. Upper panel shows the location of collection sites in relation to the Antarctic coastline, Australian Antarctic bases and oceanographic features. Continuous grey line represents continental shelf break (1000 m bathymetry line). Black dotted line shows the historical position of the Southern Boundary of the Antarctic Circumpolar Current (SBACC) (Orsi et al., 1995). Lower four panels show the surface chlorophyll levels (mg m^{-3}) derived from satellite data for each collection site. White areas represent missing data resulting from clouds or sea ice.

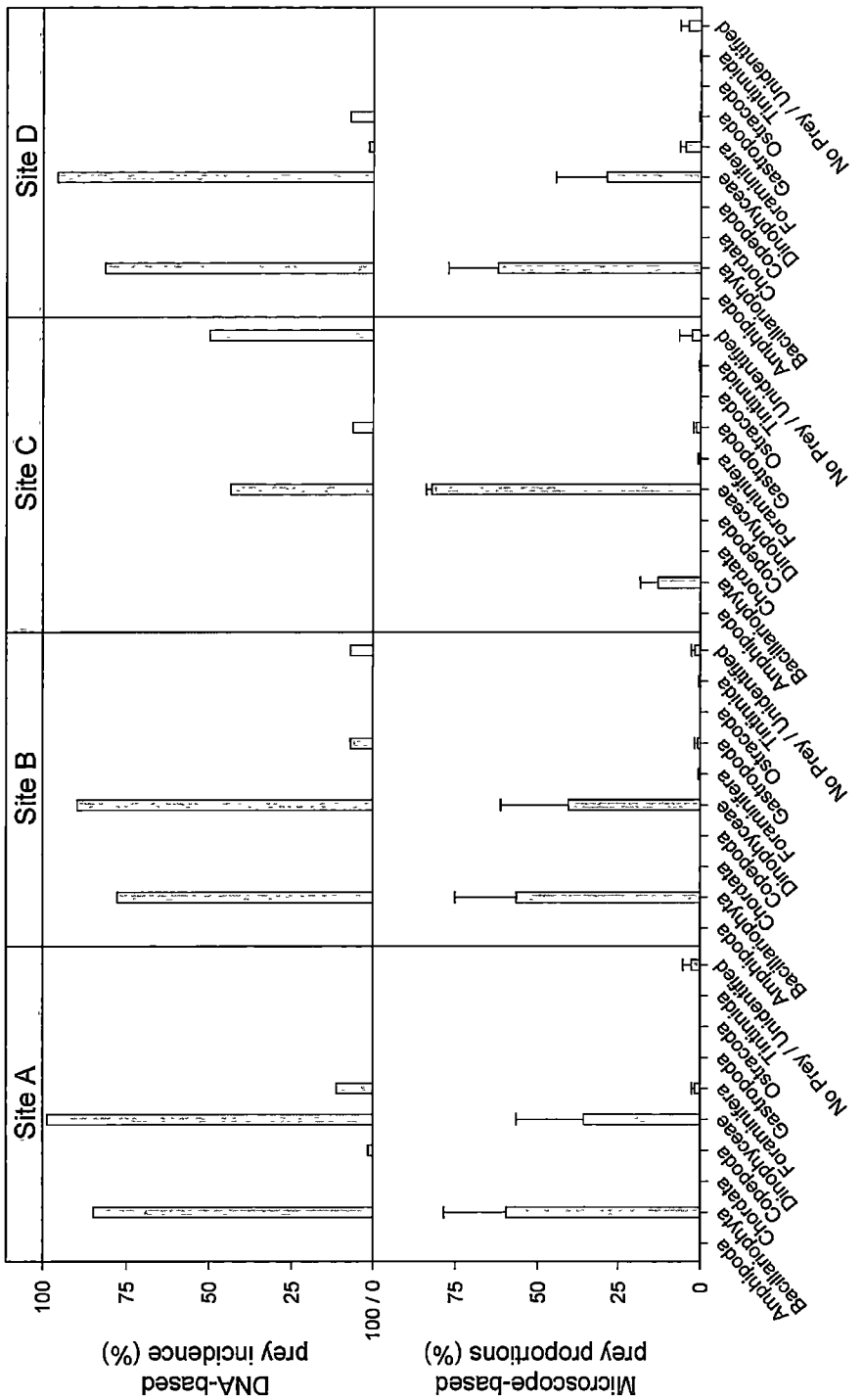

Figure 4-2 DNA prey incidence and microscopic prey proportion results for four field collection sites. The category 'No Prey' is specific to the DNA data and refers to individual krill that had no detectable prey DNA. The category 'Unidentified' is specific to the microscope data and refers to items detected but not identified. Note that the microscopic diet analysis category 'Unidentified crustacean remains' (Table 4-5) was included with the other unidentified items. Error bars are standard deviation (n = 4 or 5 see Table 4-5).

Table 4-1 Krill collection sites.

Site	Collection date	Latitude (decimal degrees)	Longitude (decimal degrees)	Trawl depth (m)	Trawl duration (min)[1]	No. of krill assayed using DNA Total (Male/Female/Juvenile)	Average krill length (mm ± SD)	Average concentration of extracted DNA (ng μL^{-1} ± SD)	Site Description
A	18 Jan 2003	-66.5575	64.0670	35	3	72 (12/60/0)	46.1 ± 2.8	8.8 ± 4.1	Open water site. Daytime. Targeted horizontal trawl through a dense swarm of krill
B	12 Feb 2003	-66 5890	69.6087	23	6	58 (11/36/11)	43.0 ± 5.8	15 1 ± 7.4	Open water site. Daytime. Targeted horizontal trawl through a dense layer of krill
C	07 Nov 2004	-62.1120	117.6375	10	15	16 (9/7/0)	41.1 ± 6.5	31 1 ± 14.8	Retreating sea ice edge. Daytime. Untargeted horizontal trawl
D	07 Feb 2005	-66 2537	74.6202	30	16	70 (34/36/0)	40.0 ± 3.4	13.6 ± 7.6	Open water site. Daytime. Targeted oblique trawl through a dense swarm of krill.

[1] Trawl duration refers to the amount of time from when the mouth of the net was opened until the catch arrived on deck

Table 4-2 PCR Primers.

Target Taxon	Primer Names	Primer Sequence 5'-3'	Target Gene	Annealing Temperature	Amplicon Size (base pairs)	Taxonomic Resolution	Reference
Amphipoda	AmphNSSf1 AmphNSSr1	CTGCGGTTAAAAGGCTCGTAGTTGAA ACTGCTTTRAGCACTCTGATTTAC	SSU	51°C	204 - 375	genus / species	(Jarman et al., 2006)
Bacillariophyta (subset)	DiatomLSUf1 DiatomLSUr1	GGAAGCGAAGGAAACCAGTG AGACCGTTCTCCCGARGGAT	LSU	65°C	290-364	genus / species	this study
Chordata	ChordVf ChordVr	ACAYACCGCCCGTCAC CATRATGCAAAAGGTA	MSS/MLS	52°C	~370	genus / species	(Jarman et al., 2004)
Copepoda	CopF2 CopR1	TGTGTGGTGGTAAACGGAG CCGCCGACCTACTCG	LSU	61°C	~230	genus / species	(Bissett et al., 2005)
Dinophyceae (subset)	DinoSSUf1 DinoSSUr1	CCAGGACTTTTACTTTGAGG CCCCTAACTTTCRTTCTTGATC	SSU	57°C	~251	family / genus	this study
Foraminifera	Foram-S14F3 Foram-S17	ACGCAMGTGTGAAACTTG CGGTCACGTTCGTTGC	SSU	50°C	350 - 600	species?	(Pawlowski et al., 2002)
Gastropoda	GastNLSf1 GastNLSr1	GCGGYAACGCAAACGAAGT CGAAAWTMACACCGTCTCCG	LSU	52°C	188 - 198	genus	(Jarman et al., 2006)
Ostracoda	OstracodMSSf1 OstracodMSSr1	GTGACAAGAAGACCCTARGAG AATCCAACATCGAGGTCA	MSS	46°C	247 - 252	species	(Jarman et al., 2006)
Tintinnida (subset)	TintinnidSSUf2 TintinnidSSUr2	AGGGACTTTGCAAGCAACTG GATGTGGTTTACTCGACTTTCCAA	SSU	65°C	~343	genus?	this study
Universal Eukaryote	28Sf 28Sr	CCCTGTTGAGCTTGACTCTAGTCTGGC AAGAGCCGACATCGAAGGATC	LSU	55°C	500 - 600	variable	(Werren et al., 1995)

SSU=nuclear small ribosomal subunit. LSU=nuclear large ribosomal subunit. MSS=intragenic spacer between mitochondrial small and large ribosomal subunits. MLS=Mitrochondrial large ribosomal subunit

Table 4-3 DNA-based prey incident results.

Site	Amphipoda	Bacillariophyta	Chordata	Copepoda	Dinophyceae	Foraminifera	Gastropoda	Ostracoda	Tintinnida
A	0	61	0	1	71	8	0	0	0
B	0	45	0	0	52	0	4	0	0
C	0	0	0	0	7	0	1	0	0
D	0	57	0	0	61	1	5	0	0

Table 4-4 Nearest blast match results for diet amplicons.

Phylum Class Order Family Genus/Species	% Match (length)	Site A							Site B							Site C							Site D						
		A	B	C	D	F	G	O	A	B	C	D	F	G	O	A	B	C	D	F	G	O	A	B	C	D	F	G	O
Bacillariophyta (diatoms)																													
Bacillariophyceae																													
Bacillariaceae																													
Fragilariopsis cylindrus	100 (310)	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fragilariopsis curta	97 (311)	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-
Coscinodiscophyceae																													
Thalassiosiraceae																													
Thalassiosira rotula	93 (364)	-	-	-	-	-	-	-	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-	-	-	-	-
Fragilariophyceae																													
Thalassionemataceae																													
Thalassionema frauenfeldii	72 (284)	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Apicomplexa																													
Dinophyceae																													
Peridiniales																													
Protoperidiniaceae																													
Protoperidinium pellucidum	97 (248)	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Peridiniaceae																													
Peridinium polonicum	97 (252)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
Gymnodiniales																													
Gymnodiniaceae																													
Gymnodinium sp.	96 (252)	-	-	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Prorocentrales																													
Prorocentraceae																													
Prorocentrum micans	100 (252)	-	-	-	1	-	-	-	-	-	-	5	-	-	-	-	-	-	4	-	-	-	-	-	-	4	-	-	-
Prorocentrum gracile	88 (235)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-

Phylum Class Order Family Genus/Species	% Match (length)	Site A							Site B							Site C							Site D						
		A	B	C	D	F	G	O	A	B	C	D	F	G	O	A	B	C	D	F	G	O	A	B	C	D	F	G	O
Foraminifera Globigerinida *Neogloboquadrina pachyderma*	91 (413)	-	-	-	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
Mollusca Gastropoda Opisthobranchia Aplysiidae *Aplysia californica*	95 (195)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
Aplysia californica	93 (195)	-	-	-	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-
Arthropoda Maxillopoda Calanoida Clausocalanidae *Drepanopus bispinosus*	93 (303)	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Unidentified		-	-	-	-	-	-	1	7	-	-	-	2	-	-	-	-	-	-	-	-	1	5	-	-	-	-	-	-
False Positives		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Amplicon length is in DNA base pairs. Letters indicate primer set that generated the amplicons. A = Amphipoda, B = Bacillariophyta, C = Copepoda, D = Dinophyceae, F = Foraminifera, G = Gastropoda, O = Ostracoda.

Table 4-5 Microscopic diet analysis of krill from each of the four collection sites. Data displayed in Figure 4-1.

Krill ID	Amphipoda	Bacillariophyta	Chordata	Copepoda	Dinophyceae	Foraminifera	Gastropoda	Ostracoda	Tintinnida	Crustacean remains	Unidentified	Total items counted
A1	0	147	0	0	239	12	1	0	0	2	4	405
A2	0	263	0	0	129	7	1	0	0	0	0	400
A3	0	210	0	0	242	4	0	0	0	0	7	463
A4	0	378	0	0	34	9	0	0	0	2	25	448
A5	0	274	0	0	121	4	0	0	0	3	17	419
B1	0	371	0	0	25	2	10	0	3	0	13	424
B2	0	216	0	0	196	0	2	0	0	0	10	424
B3	0	183	0	0	217	0	0	0	0	2	3	405
B4	0	241	0	0	170	3	0	0	0	0	0	414
B5	0	145	0	0	206	1	4	0	0	1	5	362
C1	0	18	0	0	196	2	5	0	0	3	14	238
C2	0	37	0	0	163	0	1	0	0	0	2	203
C3	0	49	0	0	66	0	1		0	0	3	119
C4	0	26	0	0	165	1	3	0	1	1	0	197
D1	0	214	0	0	183	19	0	0	2	0	8	426
D2	0	242	0	0	147	23	0	0	0	0	16	428
D3	0	365	0	0	25	12	0	0	0	4	28	434
D4	0	238	0	0	133	27	3	0	0	0	11	412

Chapter 5

The stability of prey DNA in the stomachs of Antarctic krill (*Euphausia superba*)

5.1 Abstract

Using DNA as a dietary biomarker is a promising approach for studying the diet of Antarctic krill. PCR-based methods have proven successful at amplifying prey DNA extracted from krill collected in the field. However, the time period of feeding that this prey DNA represents is unclear. This study examined the stability of prey DNA in the stomachs of Antarctic krill under experimental feeding trial conditions. Krill were fed a pulse of the diatom *Thalassiosira antarctica* and the accumulation and decay of DNA signal from this prey item was tracked over time with quantitative PCR. Stomach evacuation was identified as an important factor in determining the longevity of prey DNA signal in the stomach. After feeding for 2 hours on *Thalassiosira antarctica*, prey DNA was detectable for a further 3 hours when krill continued to feed on an alternative food source, and for 7 hours after feeding when krill were moved into starvation conditions. This demonstrates that prey DNA is reasonably stable in krill stomachs and it is therefore likely that prey DNA is representative of recent feeding behaviour at field collection sites.

5.2 Introduction

Several studies have recently shown that it is feasible to extract DNA from the stomachs of Antarctic krill and use PCR-based methods to amplify and identify prey DNA (Chapter 4, Martin et al., 2006; Passmore et al., 2006). While these initial studies are encouraging, DNA has not been well characterised as a dietary biomarker for krill and there are several features of its performance in this role that warrant careful investigation. One of these is to determine the stability of prey DNA in the stomach. Previous studies have been conducted on krill collected in the field and as a consequence the feeding history of these krill was unknown. It is unclear whether prey DNA extracted from krill stomachs represents feeding behaviour from seconds, minutes or hours prior to capture. It is also unclear what factors are responsible for the decay of prey DNA signal in krill stomachs. Another aspect of using prey DNA as a biomarker that has not been well characterised is the sensitivity of PCR based methods for detecting prey, either in terms of DNA molecules detected, or the number of prey items that these molecules represent.

The aim of this study was to explore the dynamics and stability of prey DNA in krill stomachs under the controlled conditions provided by an experimental feeding trial. Krill were fed the

diatom *Thalassiosira antarctica* and the fate of the DNA signal from this prey item was then tracked over time with quantitative PCR. Following the initial 'feeding pulse' krill were moved into new tanks where they were either starved or fed another alga, *Isochrysis galbana,* to examine the effect of consuming additional prey on the longevity of the *T. antarctica* DNA signal.

5.3 Material and Methods

5.3.1 Quantitative PCR assay for detecting *Thalassiosira antarctica*

Thalassiosira antarctica was selected as a DNA diet tracer because. (1) it is an Antarctic diatom with a diameter that is in the optimal range for filter feeding by krill (Boyd et al., 1984); (2) cultures of *T. antarctica* were available and culturing on a small-scale had shown that *T. antarctica* was robust and fast growing for an Antarctic species; and (3) earlier work had suggested that nuclear ribosomal genes were present in high copy number in diatoms from the genus *Thalassiosira* (Armbrust et al., 2004; Zhu et al., 2005; Passmore et al., 2006), which is useful for providing a strong DNA signal.

The small ribosomal subunit gene (SSU) of *T. antarctica* was selected as the target of the DNA detection assay and was cloned and sequenced (GenBank Accession Number EF140621). The sequence was then aligned to the sequences of other species in the feeding trial (*Euphausia superba* AY672801; *Phaeodactylum tricornutum* EF140622, *Isochrysis galbana* DQ079859) and a Taqman assay specific for *T. antarctica* was developed. The PCR primers amplify a 132 base pair region near the 3' end of the nuclear small subunit ribosomal DNA (SSU). Forward primer CACCTACCGATTGAATGGTCC, Reverse primer CGGAAACCTTGTTACGACTTCA (Proligo), Taqman probe HEX-CGGGATTGTGGTTTGGCTCCTTCAT-BHQ-1 (Biosearch Technologies).

PCR reactions contained: 5 µL of template DNA, 3.0 mM $MgCl_2$ (Gibco), 0.125 mM dNTPs (Gibco), 4 µM of each primer, 2 µM of Taqman probe, 1 X Bovine Serum Albumin (NEB), 0.5 units of Amplitaq Gold (Gibco), 1 X manufacturer's PCR buffer (Gibco) and made up to 20 µL with dH_20. Samples were amplified in a Chromo4 thermal cycler (MJ Research) and cycling parameters were: preheat at 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s and 58 °C for 60 s.

5.3.2 Estimating SSU copy number in *Thalassiosira antarctica*

SSU copy number per *T. antarctica* cell was estimated from six samples of a *T. antarctica* culture that had recently reached the plateau phase of growth. The cell density of the *T. antarctica* culture was estimated from three separate counts of over 400 cells each, using an Improved Neubauer chamber. DNA was then extracted from three samples of approximately 100,000 cells and three samples of approximately 500,000 cells. Initial attempts to separate the cells from the culture media by centrifugation resulted in significant cell loss. Instead, samples were vacuum filtered onto 5 mm diameter discs of GFF-75 filter paper (Whatman). DNA was then extracted from the discs using a DNeasy tissue kit (Qiagen) and eluted in 200 µL of AE buffer (Qiagen). The standard used to estimate SSU copy number was linearised plasmid that contained the full length SSU sequence from *T. antarctica*. A series of five-fold dilutions from 1.0×10^{-3} ng μL^{-1} to 3.2×10^{-7} ng μL^{-1} (equivalent to 160,000 to 51 copies of plasmid per µL) was used to generate a standard curve for quantification of culture extract samples ($y = -0.29x + 11.56$, $r^2 > 0.998$). Quantitative PCR was performed in triplicate on 100-fold dilutions of culture extracts. An estimate of the number of copies of SSU per *T. antarctica* cell was obtained using the formula:

> SSU copies per cell = (Copies of SSU in PCR reaction x DNA extract elution volume X DNA extract dilution factor) / (Volume of DNA extract used in PCR x Number of cells from which DNA was extracted)

where: DNA extraction volume = 200 µL, DNA extract dilution factor = 100, Volume of DNA extract used in PCR = 5 µL, Number of cells from which DNA was extracted = 1×10^5 or 5×10^5 cells.

5.3.3 Supply and culturing of algae

Thalassiosira antarctica culture T1 (cell diameter 10 - 25 µm) was supplied by Andrew Pankowski, University of Tasmania, and was one of several strains isolated from the Barents Sea. *Phaeodactylum tricornutum* (cell diameter 1 - 3 µm) was originally sourced from the CSIRO Collection of Living Microalgae #CS-29 and was grown at the Australian Antarctic Division in bulk cultures as a food supply for krill. *Isochrysis galbana* (cell diameter 4 - 8 µm) was originally sourced from the Provasoli-Guillard National Center for Culturing Marine

Phytoplankton CCMP1324 and was supplied in a concentrated liquid form called Instant Algae (Reed Mariculture).

Thalassiosira antarctica and *P. tricornutum* were cultured in f2 medium at 4 °C under high light conditions with aeration. Small scale (50 mL) cultures of *T. antarctica* grew to densities above 1 x 10^6 cells mL^{-1}. Cultures of *T. antarctica* were successfully scaled up to 15 L plastic carboys but increasing the culture size to 120 L polyethylene bags was unsuccessful. This is not uncommon when culturing algae and is thought to be because many species do not tolerate the violent aeration that is required to prevent bag cultures from settling (Chris Bolch pers. comm.). Carboys inoculated with 500 mL of *T. antarctica* culture took approximately four weeks to reach maximum cell density, which was typically on the order of 50,000 to 100,000 cells mL^{-1}. The smaller and more robust *P. tricornutum* grew in 120 L bags to cell densities greater than 1 x 10^7 cells mL^{-1}.

5.3.4 Krill collection and maintenance

Krill were collected in the Indian sector of the Southern Ocean (-66.2537, 74.6202) on the 7th of February 2005 during a voyage with the RSV *Aurora australis*. When the ship returned to Australia krill were transferred to the Australian Antarctic Division's live krill facility (Hobart, Tasmania). Krill were kept in an 1860 L holding tank connected to a 5000 L chilled seawater recirculation system. Water temperature was maintained at 1 - 2 °C. Light conditions were 12 hours of light and 12 hours of darkness with a maximum midday lux of 45. Krill were maintained with a daily feeding regime where the diatom *P. tricornutum* and the haptophyte *I. galbana* were supplied at high concentrations during the daylight phase of the light cycle. Krill were kept under these conditions for approximately one month prior to the feeding trials. Krill used in the feeding trial were a mixture of males and females ranging in length from 28 to 45 mm, with an average length of 38 mm.

5.3.5 Feeding trial

Initial experimentation with 100 L tanks proved unsatisfactory for the feeding trial because the krill required long periods to acclimate to the small tanks and often collided with the walls of the tanks which disrupted their feeding behaviour. Feeding trials were therefore conducted in the large 1860 L holding tanks. The original plan for the feeding trial was to starve the krill for two days and then present krill with *T. antarctica* at low cell concentrations, similar to cell

concentrations observed in the field. However, following a month of daily feeding on high concentrations of algae, several days of starvation was insufficient to induce the krill to engage in active and consistent feeding behaviour on low concentrations of *T. antarctica*. The supply of *T. antarctica* was constrained by the diatom's poor growth in large scale cultures and, as an alternative to additional culturing, the feeding pulse phase of the trial was altered to a mix of *P. tricornutum* at high cell concentrations and *T. antarctica* at low cell concentrations. This feed mix successfully induced feeding behaviour in the majority of krill Visual observations suggested ~ 80 – 90 % of animals were feeding with the rest engaged in slow swimming activity. After the feeding trials were completed, attempts were made to develop a second quantitative PCR assay for the detection of *P. tricornutum* but they were unsuccessful.

The feeding trials were conducted during the daylight phase of the light cycle consistent with the feeding regime used during the maintenance period. Trials began with approximately 1200 krill in an 1860 L tank. Krill were initially starved for two days and then cultures of *P. tricornutum* and *T. antarctica* were added to the tank to a final concentration of 20,000 cells per mL^{-1} and 10 cells mL^{-1} respectively. Krill were then left to feed for a two hour 'feeding pulse'. Following the feeding pulse, two groups of approximately 50 krill were selected at random and transferred with aquarium nets to one of two conditions. The continuous feeding group was moved into an adjacent 1860 L tank that contained *I. galbana* at a concentration of 10,000 cells mL^{-1} (reduced concentration because *I. galbana* has larger cells than *P. tricornutum*). The starvation group were evenly distributed among three clear plastic 20 L fish tanks that were filled with filtered seawater and were floating almost fully submerged in the continuous feeding tank to maintain a constant temperature. The krill were left in these two conditions for another 7 hours to complete the trial. At the time points 0 (food pulse added), 1, 2 (food pulse terminated), 3, 4, 5, 7 and 9 hours, six animals per condition were selected at random and preserved in 500 mL of 80 % ethanol for DNA analysis. The entire feeding trial was conducted three times on three separate days in the same tanks.

5.3.6 Krill dissection and stomach DNA extraction

Dissections were performed using a dissecting microscope and two pairs of forceps. Individual krill were briefly rinsed in fresh ethanol and gently dried on paper towel prior to

dissection in a dry sterile Petri dish. Forceps were used to remove the carapace and expose the animal's stomach on the dorsal surface. Stomachs were then removed with forceps taking care not to squeeze out the stomach contents. To prevent the transfer of contaminants from the external surface of the krill to the stomach tissue, care was taken not to make contact with the stomach during the removal of the carapace and the forceps were flame sterilized prior to the removal of the stomach and between each krill dissection.

DNA extraction was performed on individual krill stomachs using a DNeasy Tissue kit in a 96 well plate format (Qiagen). Samples were processed according to the manufacturer's instructions except for the following modifications. Prior to overnight digestion in ATL buffer + Proteinase K, the stomachs were disrupted manually with a pipette tip to assist tissue digestion. DNA was eluted in 200 µL of AE buffer (Qiagen) and stored at 4 °C.

5.3.7 Determining DNA concentration of stomach extracts

DNA concentrations were determined using a Genios microplate multireader (Tecan). Picogreen reagent (Molecular Probes) was diluted 1 : 200 in TE buffer (10 mM Tris-HCl, 1 mM EDTA) and 195 µL aliquoted into the wells of black flat-bottomed microtitre plates. Five µL of krill stomach extract was added per well and fluorescence was measured in the plate reader as per the manufacturer's instructions. Standard curves to convert fluorescence readings to DNA concentrations were generated using a dilution series of the DNA standard supplied with the Picogreen reagent.

5.3.8 Quantitative PCR on krill stomach extracts

The DNA concentration of stomach extracts ranged from 3.3 to 47.8 ng μL^{-1}. Quantitative PCR was performed in triplicate on 5 fold dilutions of stomach extracts. PCR replicates were performed on separate plates with a separate PCR reaction master mix for each plate An eight point standard curve of SSU plasmid was included on each plate and ranged from 2.5 x 10^{-2} to 3.2 x 10^{-7} ng per PCR reaction (equates to ~ 4 x 10^{6} - 50 copies of SSU plasmid).

5.3.9 Quantitative PCR data analysis

Raw fluorescence data generated by the quantitative PCR reaction was analysed using quantitative PCR software that uses a standard curve based method (Larionov et al., 2005). All data were processed using a standard curve that was derived from the pooled data of all

replicates of the plasmid standards. The software's amplitude normalisation option was not used because the PCR reactions were terminated before the fluorescence accumulation curves of all samples had reached a clear plateau, which violated the conditions for amplitude normalisation stipulated by the software's author. The software provided cycle threshold (Ct) values for all PCR reactions which were the point at which the accumulated fluorescence within a PCR reaction reached a threshold that was defined by the software based on a statistical analysis of the results from the plasmid standards.

Data were screened to remove PCR replicates with aberrant fluorescence accumulation curves and samples where replicates had a Ct coefficient of variance (CV) greater than 1%. Samples with little or no detectable DNA were handled in a different way. A detection threshold was set at a Ct value of 31.4 PCR cycles, which was equivalent to 256 copies of plasmid standard per PCR reaction. Krill stomach samples where all PCR replicates produced less fluorescence signal than the 256 copy standards were considered valid data points with no detectable DNA regardless of the CV between replicates.

Ct values were converted to estimates of copy number per PCR reaction using the formula. copies = $10^{(\text{slope} \times Ct + \text{y-intercept})}$ (Larionov et al., 2005), where slope and intercept were derived from the standard curve of plasmid standards. Copies of SSU per stomach were calculated using the formula described above for estimating SSU copy number in *T. antarctica*, except. DNA extract dilution factor = 5.

5.4 Results

5.4.1 Characterisation of the *Thalassiosira antarctica* detection assay

The Taqman assay developed for detecting *T. antarctica* showed a log-linear relationship between PCR cycle number and SSU copy number when applied to plasmid over a range of 50 to 4 x 10^6 copies (Figure 5-1).

The limit of sensitivity for PCR assays is difficult to define in precise terms, but in samples that contained the lowest concentrations of plasmid, 50 or 256 copies, there was an increase in the amount of variation between PCR replicates (Figure 5-1). In addition, PCR reactions with 50 copies of plasmid sometimes failed to produce normal fluorescence accumulation curves. This was probably due to the extremely low concentration of template DNA causing

stochastic variation as in previous studies (Morrison et al., 1998; Stenman and Orpana, 2001, Stahlberg et al., 2004). The detection threshold of the assay was set at a more conservative limit that was equivalent to 256 copies of SSU per PCR reaction. When this detection limit was scaled up to an estimate of SSU copies per stomach it was equivalent to ~ 51,200 copies of SSU or ~ 12 cells of *T. antarctica*, based on the estimate of SSU copies per *T. antarctica* cell (see below).

Assay precision was assessed by examining the variation between three PCR replicates for each feeding trial sample. Replicates were performed on different plates with different PCR master mixes which captures most of the variation inherent in the assay system. Quality control removed 17 out of 234 samples for having a Ct coefficient of variation greater than 1%. This places an upper limit on error estimates for the remaining valid samples. When the variation in Ct values was propagated through to estimates of SSU copy number, the coefficient of variation was below 20 % for all valid samples, with an average of 9 %. Overall the results were consistent with previous studies that have examined linearity, sensitivity and inter-run precision of Taqman assays (Kleiber et al., 2000; Schmittgen et al., 2000; Weiss et al., 2004).

5.4.2 Estimating SSU copy number in *Thalassiosira antarctica*

Six samples of *T. antarctica* culture were used to estimate SSU copy number per cell (Figure 5-2). Variation between PCR replicates for a given sample was small compared to the variation between samples. This was consistent with the theory that most of the variation in PCR assays occurs in the sample processing steps prior to PCR amplification (Bustin, 2002; Zhu et al., 2005). Given that all samples were derived from a single culture, the sources of variation can be narrowed down to sampling error, cell filtration or DNA extraction. Using more sophisticated membranes and filtration methods (e.g. Zhu et al., 2005) may have improved the precision of rDNA copy number estimates. However, the fact that samples of 100,000 cells and 500,000 cells gave similar estimates of SSU copy number per cell provided increased confidence in the accuracy of the estimate. Based on an average of the six samples, the estimate of SSU copy number per cell for *T. antarctica* was 3766 with a 95% confidence interval of ± 888.

5.4.3 Feeding trial

Krill were fed a pulse of *T. antarctica* and the amount of DNA signal in krill stomachs was tracked through time using quantitative PCR (Figure 5-3). A comparison of the results obtained across the three separate feeding trial days showed some variation between days in the total amount of *T. antarctica* DNA signal that accumulated during the feeding pulse. However, the general trends in signal accumulation and decay were consistent. *T. antarctica* DNA signal peaked at the end of the 2 hour feeding pulse, with an average signal of over one million copies of *T. antarctica* SSU (2 hour time point). In the animals that were subsequently moved into continuous feeding conditions on *I. galbana*, the loss of *T. antarctica* signal was quite rapid with little or no detectable DNA five hours after the feeding pulse (seven hour time point). Animals that were moved into starvation conditions retained more DNA signal although there was some evidence of signal decay seven hours after the feeding pulse when the feeding trial was terminated (nine hour time point). The difference between fed and starved krill demonstrates that feeding activity affects the longevity of prey DNA signal in the stomach.

5.5 Discussion

5.5.1 *Thalassiosira antarctica* rDNA copy number

Estimates of rDNA copy number per cell or genome are limited to a handful of species for most taxonomic groups (Prokopowich et al., 2003). Quantitative PCR has been used to estimate rDNA copy number in three diatom species, *Thalassiosira* sp. ~ 400 copies, *Nitzschia closterium* ~ 80 copies and *Thalassiosira weissflogii* ~ 20 copies (Zhu et al., 2005). Genome sequencing of *Thalassiosira pseudonana* found ~ 35 copies (Armbrust et al., 2004). The estimate of ~ 3766 for *Thalassiosira antarctica* is an order of magnitude above the estimates for other diatom species. In eukaryotes, rDNA copy number varies across five orders of magnitude, and, significant variation between species from the same genus is not unprecedented (Long and Dawid, 1980; Prokopowich et al., 2003; Zhu et al., 2005). The mechanisms that determine rDNA copy number within species are not fully understood, but correlations between rDNA copy number and cell size have been observed (Zhu et al., 2005). *Thalassiosira antarctica* is the largest diatom species for which an rDNA copy number estimate has been obtained which may account for the high rDNA copy number estimate for *T. antarctica* compared to other *Thalassiosira* species.

5.5.2 Krill feeding trial

Previous DNA diet studies have developed PCR based presence/absence assays but have not explored the conditions that lead to success or failure to detect prey items in much depth. Several studies used feeding trials to determine how long after a feeding event that prey DNA could be detected (e.g. Asahida et al., 1997; Agustí et al., 1999; Zaidi et al., 1999). However, these studies did not determine the number of DNA molecules detected or consider the number of prey items that these molecules represent. Quantitative PCR has recently been used to determine the amount of prey DNA in DNA extracts (Deagle and Tollit, 2006; Deagle et al., 2006; Passmore et al., 2006). The first DNA diet study to use quantitative PCR to characterise prey items for target gene copy number and estimate the number of prey items consumed has recently been published (Troedsson et al., 2007). The current study is the first to use quantitative PCR to track the accumulation and decay of prey DNA signal through time and examine the affect of additional feeding on the decay of prey DNA signal.

5.5.3 Variation in prey signal between individual krill

The amount of prey DNA signal that accumulated showed significant levels of variation between individual krill including between animals collected at a single time point. Since by all indications the assay system was working, this was interpreted as variation in krill feeding behaviour. High levels of variation in the feeding behaviour between individual krill seems to be a consistent feature of krill feeding trials (e.g. Antezana et al., 1982; Perissinotto and Pakhomov, 1996; Perissinotto et al., 1997; Haberman et al., 2003). The variation is probably related to the fact that krill engage in a range of behaviours other than feeding that include swimming, moulting and escaping from a disturbance. In the current study, variation between individual krill was probably exacerbated by the short time period for the feeding pulse. The short pulse was necessary to avoid the complete depletion of the limited supply of *T. antarctica* cells during the feeding pulse phase of the trial. It is likely that more consistent results would be achieved by using higher concentrations of prey and a longer feeding pulse. In addition, future trials should consider incorporating an independent measure of individual feeding rates to help explain individual variation. Measuring individual feeding rates would also allow the amount of signal detected per prey item consumed to be calculated, similar to the recent work of Troedsson et al., 2007.

5.5.4 Accumulation of prey DNA signal

The feeding trial demonstrated that DNA is a dynamic dietary biomarker in krill. Prey DNA signal accumulated rapidly over the two hour feeding pulse and the maximum value from an individual krill was ~ 5 million copies of rDNA. The following calculations demonstrate that this figure is reasonable. Based on the estimate of 3766 copies of SSU per *T. antarctica* cell, 5 million copies equates to 1327 *T. antarctica* cells. Using the concentration of *T. antarctica* cells at the start of the feeding trial (10 cells mL^{-1}), 1327 cells could be accumulated by filtering ~ 140 mL of water during the two hour feeding pulse. This filtration rate is feasible given that the most recent estimates of maximum filtration rates for adult krill exceed one litre per hour (Price et al., 1988; Miller and Hampton, 1989; Quetin et al., 1994). Given that *T. antarctica* was a minor component of the diet during the feeding pulse, and that signal was still accumulating at the end of two hours, it is unlikely that this figure represents the maximum amount of prey DNA signal that can occur in krill stomachs. The results demonstrate that the amount of prey DNA signal can vary over at least six orders of magnitude. Clearly, *T. antarctica*'s high SSU copy number per cell was a significant factor in determining the amount of DNA signal that accumulated. As DNA diet analysis matures it will have to account for these large variations in prey DNA signal. For example, the sensitivity of the current PCR assay suggests that it is feasible to detect prey items at levels that are below what would be considered biologically relevant.

5.5.5 Decay of prey DNA signal

The decay of prey DNA signal in krill stomachs is likely to be the result of some combination of gut evacuation and DNA degradation within the stomach. There is a significant body of work examining gut evacuation in krill and most of the results suggest that this is not a simple process that occurs at a constant rate. Gut evacuation rates have been estimated by measuring the decay of field-ingested chlorophyll-a over time (Perissinotto and Pakhomov, 1996, Atkinson and Snyder, 1997, Pakhomov et al., 1997; Perissinotto et al., 1997; Pakhomov and Froneman, 2004b). Estimates have also been based on visual observations of stomach evacuation (Antezana et al., 1982) and tracking the appearance of radioactive isotopes in faecal pellets (Pond et al., 1995). Passage times for the entire gut vary over a broad range between field sites and studies, from 0.78 hours (Pond et al., 1995) to 9.9 hours (Perissinotto et al., 1997) for adult krill (> 40 mm). Several studies suggest that the gut

evacuation rate is affected by food type, food quality and the amount of feeding activity (Clarke et al., 1988; Pond et al., 1995; Perissinotto and Pakhomov, 1996). There is also some evidence for the alternative view, that gut evacuation rate is not affected by the animal's feeding activity (Atkinson and Snyder, 1997). Comparing the DNA signal derived from starved and feeding krill over time showed that prey DNA signal decayed faster in feeding krill. The most likely explanation for this result is that stomach evacuation rates were higher in feeding krill. It could be argued that starved krill were engaged in coprophagy, which extended the amount of time that prey signal was detected. However, the krill were under observation for most of the feeding trial and there was little evidence of faecal pellet egestion from starved krill and no evidence the krill were feeding at the bottom of the tanks on faecal pellets. An additional point that also makes this explanation unlikely is that prey DNA in krill faecal pellets is quite degraded when compared to stomach samples (Martin et al., 2006). The difference in DNA signal decay between fed and starved krill supports the view that krill gut evacuation rates, and therefore the decay of prey DNA signal, varies with feeding activity.

It is generally assumed that prey DNA is rapidly degraded in the digestive systems of living animals (Symondson, 2002). This view has been supported by evidence that PCR assays targeting short prey DNA fragments are more successful than assays targeting long DNA fragments, in a variety of invertebrate predator-prey systems (Agustí et al., 1999; Zaidi et al., 1999; Agustí et al., 2000; Chen et al., 2000; Hoogendoorn and Hempel, 2001; Sheppard et al., 2004). The physiology of the predator's digestive system probably plays a significant role in determining the amount of DNA degradation that occurs, and it should be expected that results will vary across different predator-prey systems. Short fragments of prey DNA were reasonably stable in the stomachs of krill. In starved krill, prey DNA was routinely detected at the end of the feeding trial, 7 hours after the feeding pulse. There was some evidence of a decline in the amount of prey DNA signal in starved krill over time. This may have been due to DNA degradation but may also have been the result of stomach evacuation. This study cannot say definitively that DNA degradation did not occur in krill stomachs because the necessary parameters were not measured; i.e., the amount of signal consumed by individual krill, and the stomach evacuation rates. However, it does seem clear that, for krill, stomach

evacuation is at least as important as DNA degradation in determining the rate of decay of prey DNA signal.

5.5.6 Time limits for prey DNA detection

The two feeding trial conditions, starvation and *I. galbana* at very high cell density (10,000 cells / mL), represent extreme ends of the food density spectrum that krill might encounter in the field. These conditions were chosen to provide an estimate of the detection limits for prey DNA in the stomach. The minimum detection limit was approximately 3 hours when krill continued to feed on *I. galbana*. The maximum limit was greater than 7 hours under starvation conditions. These results were comparable to previous estimates of gut evacuation rates obtained using chlorophyll-a (Perissinotto and Pakhomov, 1996, Atkinson and Snyder, 1997; Pakhomov et al., 1997; Perissinotto et al., 1997; Pakhomov and Froneman, 2004b), but they were at the higher end of the scale given that this study focused on the stomach not the entire digestive tract. The time limits for prey DNA detection should be considered tentative because prey ingestion rates were not measured, and it is unclear whether the krill were feeding at maximum rates when food was available. However, the results demonstrate that prey DNA is stable in krill stomachs for hours rather than seconds or minutes. This suggests that prey DNA isolated from krill collected in the field will be representative of feeding behaviour that has occurred several hours prior to capture. Further work is required to define these limits more clearly taking into account feeding rates, different food sources and variation in target gene copy number between prey species.

5.6 Conclusions

The results from this study are encouraging for the use of DNA to examine the diet of Antarctic krill. Prey DNA was stable on the stomachs of krill for several hours after ingestion which suggests that prey DNA extracted from krill stomachs will be representative of recent feeding activity at field collection sites. The study also highlights the fact that underlying simple PCR based presence / absence detection assays there is a complex interaction between target gene copy number, stomach evacuation, and DNA degradation. It is important that these factors are considered in the design of future DNA diet assays and when ecological interpretations of the data are undertaken.

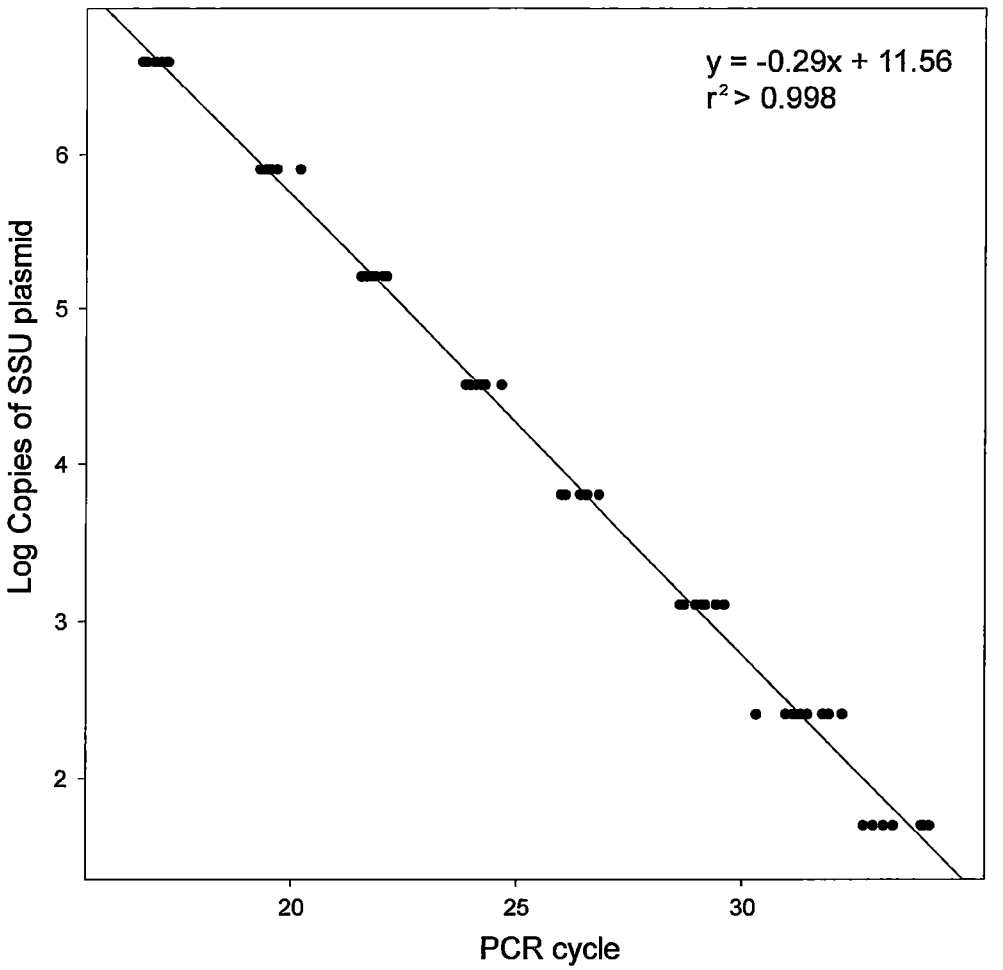

Figure 5-1 Standard curve of *Thalassiosira antarctica* detection assay showing log-linear relationship between SSU copy number and PCR cycle.

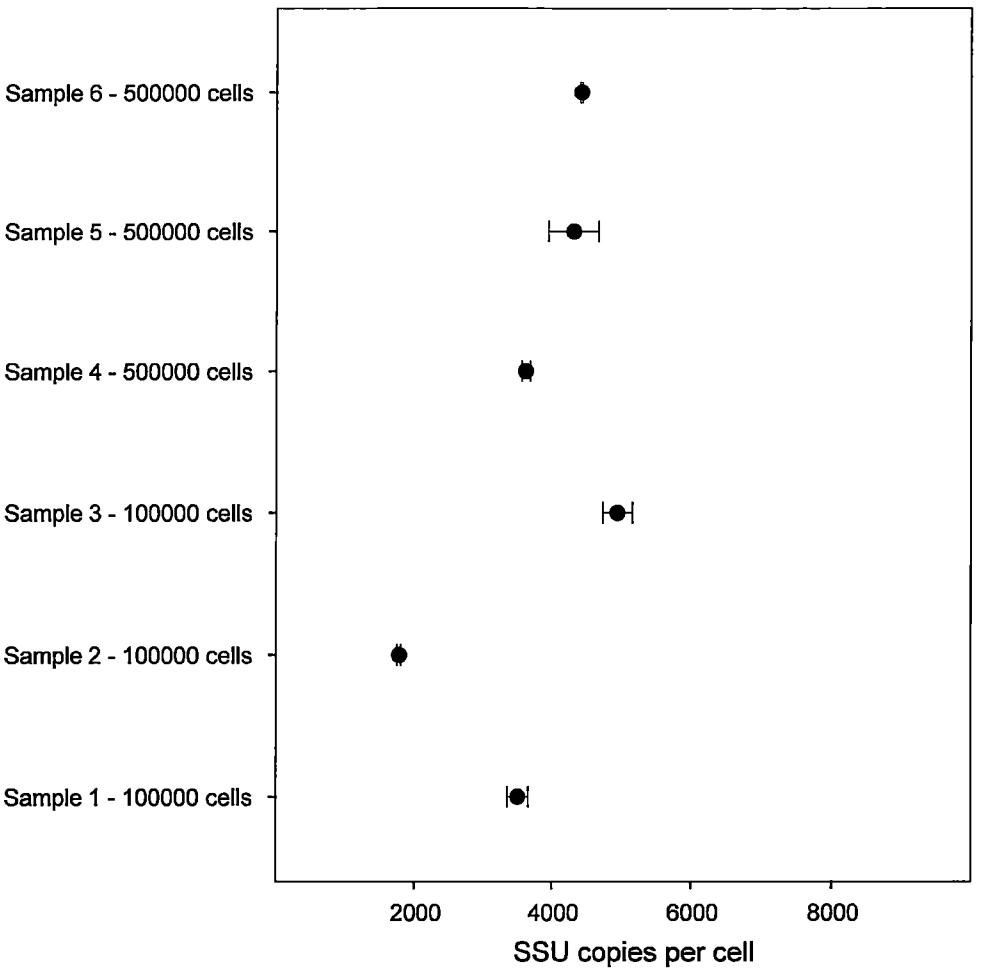

Figure 5-2 Estimates of SSU copy number in *Thalassiosira antarctica* based on six samples of cells. Error bars show 95% confidence intervals of each individual estimate based on three PCR replicates.

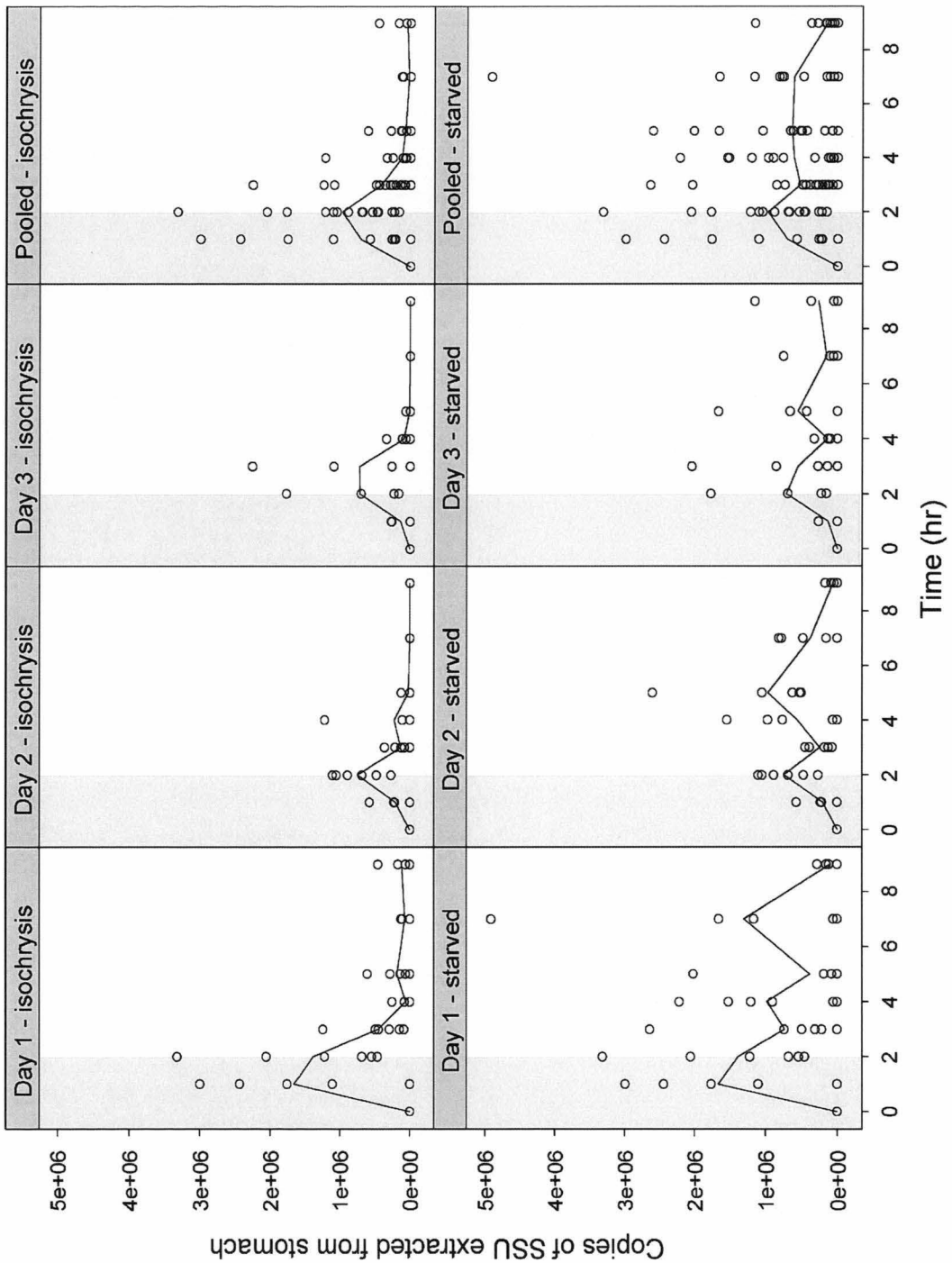

Figure 5-3 Feeding trial time series data showing the build up and decay of prey DNA signal in krill stomachs over time. Grey areas represent feeding pulse (0-2 hours). Data points in feeding pulse are replicated in isochrysis and starved panels for clarity. Line connects the mean value for each time point.

Chapter 6
Conclusions

6.1 Overview of findings

This thesis provides some foundational work in assessing whether DNA can provide useful information about krill diet. Prior to this work there were no studies on krill diet that had utilised DNA but during the course of this work one study from another laboratory was published (Martin et al., 2006). The thesis focused on developing DNA diet methods that are applicable to krill collected in the field. Strong emphasis was placed on examining whether DNA can provide quantitative data about krill diet, which was the key issue identified in Chapter 1. The major conclusions of this thesis are:

- A large amount of prey DNA can be successfully preserved and then extracted from the stomachs of Antarctic krill (Chapters 2 & 5).
- The choice of sample preservation method has a significant effect on the yield of prey DNA (Chapter 2).
- The majority of DNA extracted from krill stomachs is derived from krill. This is problematic because krill DNA competes with prey DNA for PCR amplification. However, this issue is not easily resolved by cutting krill DNA with restriction enzymes or by blocking the amplification of krill DNA during PCR (Chapter 3).
- Krill diet was mainly composed of a mix of autotrophic and heterotrophic protists with only a limited role for metazoan prey (Chapter 4).
- Current DNA diet approaches that target nuclear ribosomal genes with universal or group specific primers are not capable of providing reliable quantitative data about krill diet (Chapter 2 & Chapter 4).
- Prey DNA is stable within krill stomachs for several hours after ingestion (Chapter 5).
- The amount of prey DNA signal within krill stomachs varies over at least six orders of magnitude (Chapter 5).
- In krill, feeding activity increases the rate at which DNA signal is lost from the stomach (Chapter 5).

6.2 Problems with DNA diet analysis

Several issues need further exploration before DNA can provide a quantitative analysis of krill diet. Variation in nuclear ribosomal copy number between prey species is probably the most significant issue. The chapters in this thesis and the other published krill DNA diet study (Martin et al., 2006) have targeted nuclear ribosomal genes because, for krill prey species, there is only limited DNA sequence data available from other regions of the nuclear, chloroplast or mitochondrial genomes. Nuclear ribosomal genes are problematic however because their copy number varies by at least five orders of magnitude between eukaryotic species (Prokopowich et al., 2003). This level of variation in DNA signal between prey species cannot be ignored if the goal is to obtain quantitative data about diet.

The large variation in signal between species has ramifications for PCR assays that use universal primers to amplify DNA from multiple prey. Large variation in signal between species probably results in common DNA sequences out competing rare sequences for amplification, thereby giving a biased picture of diet. Evidence that universal primer methods detect sequences contributing more than 1% of the total sequence mix (Muyzer et al., 1993; Murray et al., 1996) suggest that the approach is not capable of dealing with the orders of magnitude variation in DNA signal that will occur. Prey species that are consumed in large numbers but have low nuclear ribosomal gene copy per genome will easily fall below the 1% detection threshold. Future studies that advocate a universal primer approach need to demonstrate that competition does not bias diet results.

There are two ways to tackle the problem of variation in ribosomal gene copy number between species. The first is to characterise and account for the variation in ribosomal gene copy number between species. At present the data on ribosomal gene copy number for eukaryotic species are sparse and have been gathered using a variety of methods (Prokopowich et al., 2003). In Chapter 5 ribosomal gene copy number was estimated for the diatom *Thalassiosira antarctica* using quantitative PCR, but this required multiple samples and accurate cell counts. Characterising all potential krill prey species for ribosomal gene copy number would be a large and difficult task, particularly when many krill prey species have not even been successfully grown in culture. There is a linear relationship between rDNA copy number and cell size (Zhu et al., 2005) or genome size (Prokopowich et al.,

2003), but there is significant variation around the mean and it is unlikely that these relationships will provide easy and reliable predictions of ribosomal gene copy number. For diet studies that focus on a small subset of prey it is feasible to characterise ribosomal gene copy number. Using this approach it should be feasible to develop an understanding of meaningful prey detection limits and correct for variation between species. Recent diet studies have quantified the amount of signal derived from prey items (Chapter 5, Troedsson et al., 2007, Durbin et al., 2008; Nejstgaard et al., 2008).

The second approach is to target an alternative region of the genome that has less variation in copy number between species. Nuclear ribosomal genes are one of the most variable regions in the genome in terms of copy number so in this regard most other regions would be an improvement. The main problem with this approach is the lack of available sequence data. These data are required for the design of PCR primers and for the identification of PCR amplicons derived from prey species. In recent years the barcode of life project (BOLD) has been attempting to characterise a unique DNA barcode for all species on the planet. This project has so far focused on the cytochrome oxidase I (COI) gene of the mitochondrial genome and most of the species that have been characterised are relatively large metazoan animals. The concept of DNA barcoding and the selection of COI as the target region has been controversial for a variety of reasons (Meyer and Paulay, 2005; Vences et al., 2005; Rubinoff et al., 2006a; b; Waugh, 2007). COI is capable of providing species specific identification for some animal and protist groups (Hebert et al., 2003a; Hebert et al., 2003b; Evans et al., 2007; Smith et al., 2008) but there are also examples where COI fails to correctly delineate between animal species (Meyer and Paulay, 2005; Vences et al., 2005), and, the region is unsuitable for plants (Rubinoff et al., 2006a; Pennisi, 2007). Work on these issues is ongoing and it seems likely that a mature DNA based identification system will incorporate multiple DNA regions (Kress et al., 2005; Rubinoff et al., 2006a). In terms of target gene copy number, there is variation in the number of mitochondria per cell and in the number of cells per animal (Waugh, 2007), but, the tight link between mitochondria and metabolism suggests that there will be less variation than occurs with nuclear ribosomal genes and that the variation will have a reasonably consistent relationship with the size of the organism. A recent DNA diet study has shown that for copepod nauplii there is a linear relationship between COI copy number and carbon biomass (Durbin et al., 2008).

Regardless of which DNA region is targeted, the effect of target gene copy number should be carefully considered. Overall it is recommended that COI and other DNA regions that emerge from large scale barcoding projects should be targeted in future DNA diet studies to overcome the paucity of available sequence data and potentially reduce the variation in DNA signal between prey species.

The amount of prey DNA signal in krill stomachs derived from a single prey species also shows significant levels of variation. Prey DNA signal varied by at least six orders of magnitude within krill stomachs as food was consumed and then processed (Chapter 5). DNA diet assays must develop the capacity to handle this level of signal variation. In light of this variation it is interesting to consider the presence/absence detection assays commonly used in DNA diet studies. Under what conditions does 'presence' occur and what does this mean biologically. The assay developed in Chapter 5 was sensitive enough to detect the equivalent of twelve *T. antarctica* cells. This was below what would be considered biologically relevant since krill stomachs routinely contain tens to hundreds of thousands of diatom cells (Chapter 2). On the other hand, the presence of a single large metazoan such as a pteropod would be a significant event. DNA based assays therefore need to develop biologically relevant detection thresholds. In broad terms the amount of DNA signal should correlate with the number of cells consumed. The number of cells consumed should relate to the amount of nutrition derived from the prey. Focusing on the amount of DNA signal should therefore be a valid approach. Within a single krill there will be a range of other factors that will influence the amount of signal detected. The only way to counter this problem and establish the importance of a trophic link is to observe significant levels of DNA signal in a large number of krill.

6.3 Future Research

In krill, feeding activity increases the rate at which DNA signal is lost from the stomach (Chapter 5). This issue needs to be explored in more depth to gain a better understanding of how feeding affects DNA residence time in krill stomachs. Future feeding trials with different types and concentrations of prey would help to further define DNA residence time in the stomach under a range of conditions. This work should be a high priority because it is important to establish how much variation in signal loss occurs under different conditions.

High levels of variation would make it difficult or impossible to provide quantitative data on krill diet. An important aspect of this work would be to differentiate between the roles of stomach evacuation and DNA degradation in determining how DNA signal is lost from the stomach. It has been argued that quantitative PCR cannot provide quantitative estimates of diet because the amount of signal detected is the total amount of signal consumed minus the signal lost due to DNA degradation since the item was consumed. Since, under field conditions, the time since the item was consumed cannot be determined, it is impossible to extrapolate back to total amount of signal consumed (King et al., 2008). However, in krill, DNA degradation in the stomach might be negligible (Chapter 5). Therefore the amount of signal detected would be close to the amount of signal consumed which would allow more accurate quantification of prey. If DNA degradation is negligible there is still the issue of stomach evacuation. For stomach evacuation rates it seems likely that a relatively simple relationship between food concentration/volume in the water column and DNA residence time in the stomach will emerge. This relationship could then be used to estimate DNA residence time for field samples. It is important to note that in individual krill some portion of the DNA signal may have been evacuated from the stomach leading to an underestimate of prey abundance. Again the only way around this problem is to observe significant amounts of signal in a large number of krill. Overall it is important to determine the amount of variation that occurs in rates of DNA degradation and stomach evacuation and then decide whether this variation is within acceptable limits for providing quantitative data.

An important aspect of future work will be to incorporate the analysis of water samples collected at field sites for comparison with krill diet samples. This will help to validate DNA diet results but may also provide evidence that krill feed selectively from the water column. However, comparisons between diet and water samples should be approached with caution. A recent krill DNA diet study compared the DNA from krill stomach samples with the DNA from one litre of water collected at the same site (Martin et al., 2006). The study then goes on to suggest that the absence of specific DNA bands in DGGE profiles from krill samples compared to water samples was evidence of selective feeding. However, the study does not provide a rationale for why comparing krill stomach samples with one litre of water is a valid comparison. Comparing diet and water samples on the basis of DNA requires additional work to determine what volumes of material represent a valid comparison. This work should

include replicate sampling to determine the amount of variation that occurs between samples. This would ensure that the observed differences between diet and water samples are due to selective feeding and not random variation. An approach that couples universal primer amplifications with DGGE gels generally shows significant levels of between sample variation (pers. obs, King et al., 2008) which would make it difficult to differentiate between selective feeding and random variation.

What ecological questions might be profitably examined with current PCR based technology? With over 700 species of protists and hundreds of species of metazoans, the task of providing a comprehensive analysis of krill diet is immense. Given the variation in ribosomal gene copy number and the paucity of sequence data for other DNA regions it is not currently feasible to quantitatively examine all or even a significant proportion of krill's diet. The most immediate gains will probably be made by developing single species assays to target specific trophic links of high interest. Given the developments in the BOLD project it seems wise to target COI and examine trophic links between krill and metazoan animal prey. One potentially interesting and little studied trophic link identified in Chapter 4 is krill feeding on pteropods. Pteropods are a significant component of the metazoan fauna and are one of the organisms most at risk from ocean acidification caused by global warming (Orr et al., 2005). If pteropods are a significant component of krill diet then global warming may have additional impacts on krill beyond warmer sea temperatures and loss of their sea ice nursery. There are only a few species of pteropods in the Southern Ocean so coverage of this group could be achieved with single species assays. The issue of coverage is more challenging for speciose metazoan groups like copepods. However, while copepod species diversity is high, most of the biomass resides in relatively few species. Identifying key indicator species for important groups may help to narrow the required coverage to manageable levels. There is also a range of soft bodied autotrophic and heterotrophic protists that are difficult to detect using other methods of diet analysis. DNA methods could provide information about these groups but significant background work is required to characterise DNA barcodes for these species. A single species approach should utilise quantitative PCR, rather than presence/absence assays, to gain an understanding of the amount of prey signal detected. Given the issues discussed above it is unlikely that such an assay system would be perfect, but, quantitative PCR provides additional information about the amount of signal detected

with little additional effort. PCR is capable of detecting extremely small amounts of prey DNA. If the amount of prey DNA detected was quantified, it seems likely that ecologically relevant detection thresholds would be set higher than the minimum detection limits that are achievable with PCR.

Looking further into the future it seems likely that DNA diet methods will change radically. The technology to characterise complex mixtures of DNA fragments has been evolving rapidly and non-PCR based approaches are starting to provide more comprehensive coverage without PCR-based artefacts (Giovannoni and Stingl, 2005). The last decade has seen extensive development of platform and bead based microarrays that detect and quantify thousands of DNA fragments simultaneously (Spiro et al., 2000, Cook and Sayler, 2003; Peplies et al., 2003, Ellison and Burton, 2005). More recently there has been a move towards direct sequencing approaches that are capable of characterising hundreds of thousands of short DNA fragments (Giovannoni and Stingl, 2005; Tringe and Rubin, 2005; Goldberg et al., 2006). At present these approaches are too expensive to apply to the large number of samples required in ecological studies of krill diet. However, the need to characterise complex mixtures of DNA extends across large and well funded fields of research including human medical research. Therefore the current trend of rapidly decreasing costs per sample seems set to continue.

6.4 Comparison with other methods of diet analysis

While DNA is yet to make a significant contribution to the quantitative analysis of krill diet the approach has advantages over previous diet methods. In terms of taxonomic resolution, isotopes, pigment analysis and lipids are all severely limited in their ability to differentiate between prey groups. Isotopes attempt to place animals into the very broad trophic categories of herbivore, omnivore or carnivore. Current pigment studies targeting chlorophyll-a cannot differentiate between autotrophic prey or detect heterotrophic prey. Lipids may distinguish between autotrophs and heterotrophs but not between heterotrophic protists and metazoans (Schmidt et al., 2006). On the other hand, microscopy offers excellent taxonomic resolution, in some cases down to species level, but fails to detect a broad range of soft bodied prey. In terms of taxonomic resolution, DNA is clearly superior to isotopes, pigments and lipids even when assays are based on highly conserved genes like

the nuclear ribosomal genes where there is limited variation between species. Protein coding genes with higher levels of sequence variation (e.g. COI) will rival microscopy by providing species or genus level identification of prey. In terms of species coverage, all living organisms have DNA and are therefore detectable. In this respect DNA has a significant advantage over previous methods. DNA can therefore extend our knowledge of krill's prey guild and provide direction for more targeted studies.

For all methods of diet analysis the diet signal represents feeding behaviour integrated over a period of time. Lipids and isotopes integrate signal over periods of days to weeks. This has been described as an advantage of these approaches because it provides a more holistic view of diet averaged over the long term (Dalsgaard et al., 2003). However, an alternative view is that this averaging makes it difficult to link krill diet data to the environmental conditions occurring at field collection sites. Microscopy, pigments and DNA are all similar because they detect recent feeding behaviour. Of these approaches pigments appear to be the most transient because of the rapid breakdown of Chl-a (Perissinotto and Pakhomov, 1996; Perissinotto et al., 1997). Microscopy and DNA are probably similar in terms of signal residence time in the stomach. Given this similarity, microscopy is probably a useful tool for verifying DNA results in cases where prey has identifiable hard parts.

6.5 Conclusion

At this point in time there is reason to be optimistic that DNA diet analysis will provide useful information about krill diet. The temptation is to rush into making ecological interpretations of the data without understanding the underlying mechanisms that determine how the data are generated. However, acknowledging and dealing with the issues that effect DNA diet analysis will be the only way to obtain meaningful ecological results.

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