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DNA as a Dietary Biomarker in Antarctic Krill, *Euphausia superba*

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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. This study examined an alternative approach utilizing DNA as a prey biomarker. Methods were developed for the preservation, extraction, and identification of prey DNA from krill collected in the field. Group-specific polymerase chain reaction (PCR) was used to amplify diatom prey (Phylum: Bacillariophyta) 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 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.

Keywords: diet analysis — foodweb — trophic

Introduction

The Antarctic krill (*Euphausia superba*) is central to one of the world's largest marine foodwebs. In the Southern Ocean, krill are the critical link between primary productivity and most of the predators at higher trophic levels such as birds, fish, seals, squid, and whales (Quetin and Ross, 1991; Loeb et al., 1997). Krill are major grazers of phytoplankton to the extent that they have previously been described as predominately herbivorous (Hart, 1934; Quetin and Ross, 1991). While there is evidence that krill also consume a range of protozoa and small zooplankton, the significance of this heterotrophic material is unclear (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 shows 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).

Krill diet has traditionally been analyzed via microscopic examination of gut contents, a technique that is laborious and biased because it detects only prey with hard parts capable of surviving ingestion. Other diet analysis methods that have been applied to krill include antibody detection of prey (Haberman et al., 2002), analysis of dietary pigments (Perissinotto et al., 2000; Haberman et al., 2003), isotope analysis (Gurney et al., 2001; Schmidt et al., 2003), and lipid analysis (Hagen et al., 2001; Phleger et al., 2002; Stubing et al., 2003). While these techniques have extended our knowledge of krill diet, they also have limitations. Antibodies are laborious to develop and the results cannot be easily replicated by other laboratories (Symondson, 2002). Pigment analysis is restricted to pigmented prey, which generally means autotrophs. Pigment, lipid, and isotope analyses all suffer to varying degrees from poor "taxonomic resolution" that restricts their ability to distinguish between ecologically important prey groups. Lipid and isotope analyses are confounded by factors other than diet (Schmidt et al., 2003; Stubing et al., 2003).

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

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group of interest (Symondson, 2002; Jarman et al., 2004). DNA-based methods also offer potential for rapid, high-throughput screening of samples (Harper et al., 2005). To date, all DNA-based diet analysis has relied on polymerase chain reaction (PCR) to amplify small regions of prey genomic DNA from the gut or scat of the predator. Most assays have been designed to generate amplicons from a specific prey species (Agusti et al., 2003; Nejstgaard et al., 2003). Several assays have been developed that generate amplicons from multiple prey and use species- or group- specific variations in the amplicon sequence to differentiate between prey types (Kasper et al., 2004; Harper et al., 2005).

This study describes the development of methods for the preservation, extraction, and analysis of prey DNA from krill collected in the field. Once suitable techniques were established, three krill from each collection site (3×2) 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.

Materials and Methods

Krill Collection. Adult krill were taken from two open ocean sites in the Indian Ocean sector of the Southern Ocean. During the Antarctic summer of 2003/2004, krill were collected from site A (longitude 66.33.46S; latitude 64.04.05E) on the January 18, 2003 and from site B (longitude 66.35.36S; latitude 69.39.56E) on the February 12, 2003. The two sites are 134 nautical miles apart and both sites are approximately 65 nautical miles from the Antarctic coast. Krill were caught by trawling a rectangular midwater trawl net from the RSV *Aurora Australis*.

Krill Preservation. Two methods of preserving intact krill for DNA-based diet studies were followed: preservation in ethanol and preservation by freezing. The ethanol preservation method was developed based on the recommendations of previous field studies that had successfully preserved animal tissue for DNA analysis (Masner, 1994; Dawson et al., 1998). Live krill were dropped into 2-L plastic sample containers filled with 80% ethanol (v/v). To maintain a high concentration of ethanol, the volume of krill was not allowed to exceed a third of the container volume. Samples were further dehydrated by draining and replacing the original 80% ethanol twice, once 15 min after the initial preservation and a second time 24 h later. Samples were subsequently stored at −20°C in the dark. 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.

Krill Dissection. The large size of adult krill makes DNA extraction from whole animals impractical. Krill stomachs were found to be relatively large and easy to isolate compared to other organs of the krill digestive tract and were subsequently targeted for analysis. 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 towels before dissection in a dry sterile Petri dish. Forceps were used to remove the carapace and expose the animal's stomach on the dorsal side. Stomachs were then removed with forceps, taking care not to squeeze out the stomach contents. To prevent the transfer of any contamination 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 then flame sterilized before removal of the stomach and between each krill dissection.

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 both the DNA and "hard part" components of a single krill stomach. The following method gave the best prey specific PCR amplification results (data not shown).

DNA extraction was performed on individual krill stomachs using the DNeasy 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. After overnight digestion, the DNA and hard parts were separated by centrifuging the samples at 2500 *g*. The supernatant containing the DNA was transferred into a fresh tube and DNA extraction was continued on this fraction as per instructions. The hard part pellet was processed separately as described later. At the end of the DNA extraction

procedure DNA samples were eluted in 100 μL of AE buffer and stored at 4°C.

After 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 *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.

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 the manufacturer's instructions. Stomach DNA samples were diluted to 10 ng/μL in AE buffer for use in PCR.

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 gene or DNA region. *SSU* is also a multicopy gene usually present in tens to hundreds of copies per nuclear genome (Prokopowich et al., 2003). 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 we developed a pair of group-specific primers (Jarman et al., 2004) to target diatoms. 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* sequence (GenBank accession no. AY672801). Primers were checked for hybridization to nontarget 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′-CGGAAACCTTGTT ACGACTTCA-3′.

The primers were tested empirically on DNA samples derived from seven cultures of diatoms (*Fragilariopsis curta*, *Fragilariopsis kergulensis*, *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).

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 μM of each primer (Geneworks), 1 × bovine serum albumin (BSA; NEB), 0.5 U of Amplitaq Gold (Gibco), 1 × manufacturers' 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, 72°C for 15 s, and a final extension at 72°C for 5 min. No-template and DNA extraction–negative controls were always run alongside krill stomach samples.

Testing Krill Preservation Methods. To determine which krill preservation method was most suitable for DNA diet studies, PCR amplification of prey DNA in frozen and ethanol preserved krill was compared. PCR was performed on individual stomach DNA samples as described in the preceding text and then 5 μL of the PCR reactions where run on a 2% agarose gel stained with GelStar (BMA). All subsequent analysis was performed on krill that had been preserved in 80% ethanol.

Microscopic Analysis of Krill Stomachs. Light microscopy was used to count and identify diatoms extracted in the "hard part" component of krill stomachs. Diatoms are amenable to microscopic diet analysis because their hard silica exoskeletons often survive ingestion relatively intact. The exoskeleton is composed of two overlapping valves, and Antarctic diatom valves or valve fragments can often be identified to genus or species level.

The hard parts 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× or 600× 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.

Generating Clone Libraries from Krill Stomachs. Prey DNA diversity was examined by generating clone libraries of PCR amplicons from each krill previously analyzed with the microscope. Amplicons were generated using the group specific primers ASF1630/18 and ASR1775/22. Amplicons were checked by gel electrophoresis (data not shown) and then used to generate a clone library for each krill with the TOPO-TA cloning kit (Invitrogen). At least 50 clones from each krill were purified and sequenced using the Mini Plasmid Prep kit (Mo Bio Laboratories) and CEQ2000 Genetic Analysis System (Beckman Coulter) as per the manufacturer's instructions.

Clone Identification. Clone sequences were grouped into "Operational Taxonomic Units" (OTUs) for each geographic location where individual clone sequences were considered to be a single OTU if they had less than 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 verify 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 the 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. After 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 phylum); and Other Stramenopiles (sequences that matched to other stramenopile sequences). For many OTU sequences,

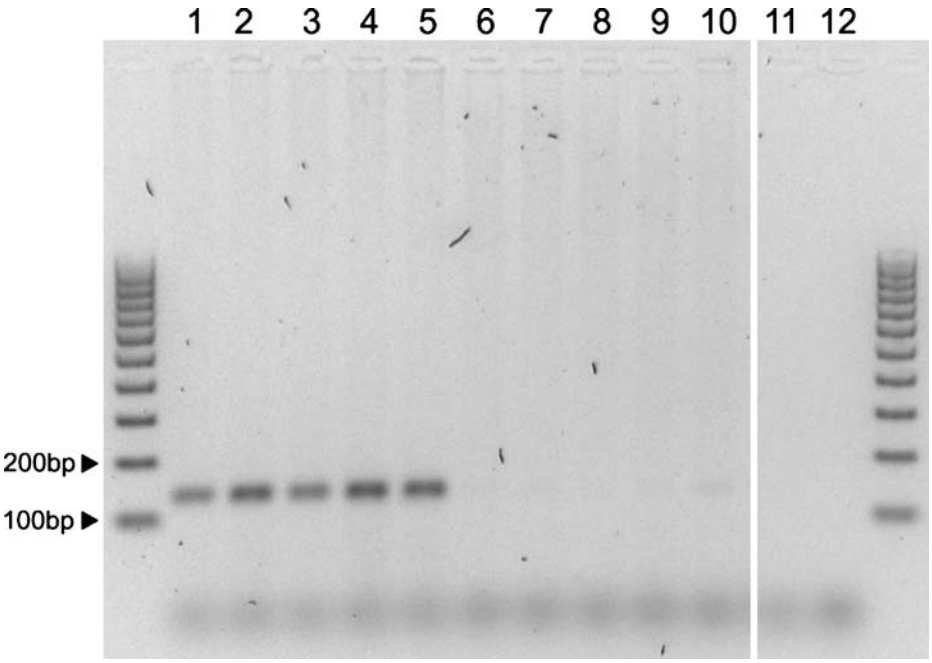

Fig. 1. Effect of krill preservation on the PCR amplification of prey. Agarose gel showing prey specific PCR products derived from krill stomach DNA samples. *Lanes 1–5*, krill preserved in 80% ethanol. *Lanes 6–10*, krill preserved by freezing at −80°C. *Lane 11*, negative control for DNA extraction.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

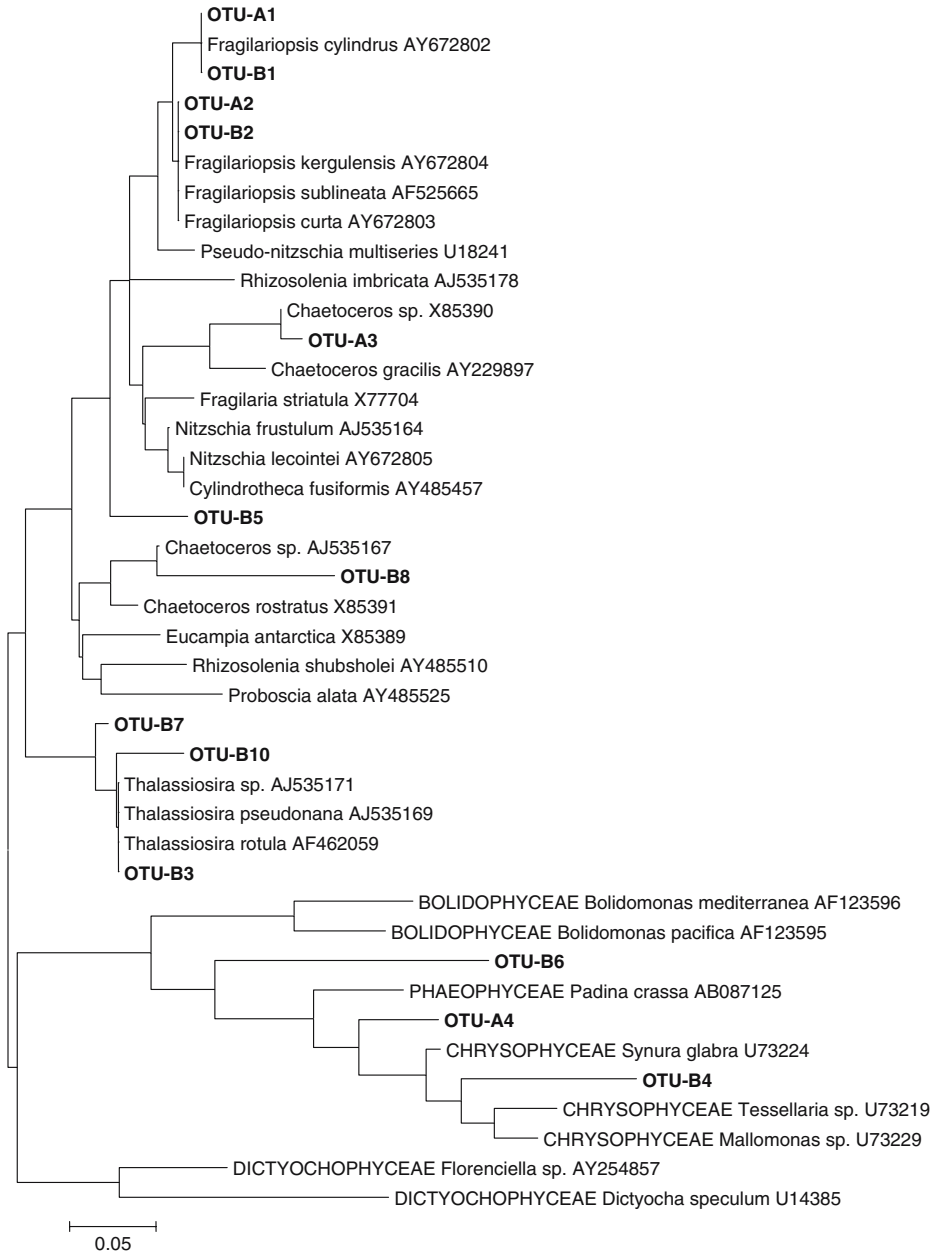

Fig. 2. Sequence similarity tree of krill stomach OTUs and related sequences. Note: OTU-B9 does not appear on the tree because it is derived from the 16S chloroplast gene.

there was complete agreement between MEGABLAST and tree-based identification but for OTUs with GenBank match scores less than 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.

Comparing Relative Abundance Estimates from DNA and Microscope. To compare the results of the two diet analysis methods, the microscopy data were grouped into the same categories defined previously for OTUs. For both DNA and microscopy data the relative abundance of each prey group was calculated for individual krill and then averaged across krill within a site to estimate the relative abundance of consumed prey and standard deviation ($n = 3$).

Quantifying Prey DNA Extracted from Krill Stomachs. Quantitative PCR was used to determine how many copies of prey *SSU* DNA were extracted from each krill stomach. The standard used to estimate prey DNA copy number was a plasmid that contained the 143-base amplicon of *SSU sequence from the diatom Fragilariopsis curta*. The plasmid was linearized with *Nco*I (NEB) to remove supercoiling and then purified with the Qiaquick

Table 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	2017	68.9	1368	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	1119	80.9	1938	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—Silico-flagellates												
Dictyocha speculum	23	0.8	11	0.6	3	0.4	0	0.0	0	0.0	0	0.0
Total hard part count	2927		1902		749		777		1383		2303	

PCR purification kit (Qiagen). A series of 10-fold dilutions from 10^{-1} ng μl^{-1} to 10^{-7} ng μl^{-1} (approximately 2.2×10^7 to 2.2×10 copies of the plasmid) was used to generate a standard curve for quantification of the krill stomach samples ($r^2 > 0.999$). 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 $MgCl_2$, 1 × BSA (NEB), 0.25 μM of each primer (ASF1630/18 and ASR1775/22), 1 × 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 and standard deviation ($n = 3$).

Results and Discussion

Preservation of Prey DNA. Ship-based sample preservation needs to be fast, simple, and safe. In addition, the ideal method preserves prey DNA within intact krill avoiding the need for krill dissection immediately after sample collection. Two methods of preservation 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 1, lanes 1–5). In contrast, krill preserved by freezing gave poor PCR amplification results (Figure 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.

Previous DNA diet studies on invertebrates have used ethanol (Hoogendoorn and Heimpel, 2001) for preservation but freezing (Zaidi et al., 1999; Agusti et al., 2003) has been the dominant preservation method. The poor result with frozen krill was therefore unexpected and it is unclear why frozen krill gave such poor results. A potential issue is that freezing does not destroy DNA nucleases. Successful DNA extraction from frozen tissue relies on rapid inactivation of nucleases prior to significant defrosting of the sample (Strauss, 1998). 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. Preservation in ethanol

Table 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[a]	119	AY672818	*Rhizosolenia setigera*	96	AJ536461	Other diatom
OTU-B10	143	AY672819	*Thalassiosira pseudonana*	97	AJ535169	Thalassiosira

[a]Sequence is from 16S chloroplast gene not *SSU*.

avoids this problem because it permeates the tissue and denatures nuclease enzymes (Flournoy et al., 1996). If this is the problem then preserving krill by freezing may be incompatible with dissection.

For all preservation and DNA extraction methods, contamination of diet samples is a serious concern. For aquatic predators, potential contamination can come from water carried over during collection or from contamination attached to the exterior of the animal, particularly food trapped among swimming and feeding appendages. Extracting digestive tissue must be carefully handled to prevent the transfer of external contamination to the stomach sample. Another potential issue with ethanol preservation is that krill dropped into ethanol may react by swallowing ethanol during the preservation process. Although we suspect the contamination risk from this source is small, it may be prudent to preserve and store krill individually to further minimize contamination risk. This is the first DNA-based diet study to compare preservation methods and more work in this area is required.

Microscopic Analysis of Krill Stomachs. Microscopic diet analysis presented a simple picture of krill diet at both sites A and B (Table 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 via microscopy. The diatom component of krill diet at both sites was dominated by a single genus (*Fragilariopsis*) and there was little variation in prey composition or relative abundance between the three individual krill analyzed within each site. Site A krill consumed mainly *Fragilariopsis cylindrus* (69% of total diet) and *F. curta* (26%), with the next highest contribution from a single species contributing less than 1% of the total diet. At site B, diet was also dominated by *F. cylindrus* (12%) and *F. curta* (85%). Site B krill had also consumed a small amount of the diatom genus *Thalassiosira* (3%). These results are consistent with previous microscopic diet analysis on krill collected during the Antarctic summer (Marr, 1962; Genhai, 1993; Pakhomov et al., 1997).

Detection and Identification of Prey DNA in Krill Stomachs. Prey DNA diversity was examined by generating clone libraries of PCR amplicons from each krill previously analyzed with the microscope. Sequencing confirmed that all OTUs generated using the group specific primers ASF1630/18 and ASR1775/22 were derived from organisms likely to

be krill prey (Table 2 and Figure 2). Most OTUs matched diatom *SSU* sequences, in accordance with the microscope-based diet analysis. Some of the rare OTUs had relatively poor percentage matches scores to Genbank sequences, reflecting the paucity of sequence information available for taxonomic groups likely to be krill prey.

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

DNA analysis also identified two groups that were not detected by microscopic analysis (compare Tables 1 and 2). 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). *Chaetoceros* has valves that are fragile and only lightly silicified, which means that they are unlikely to survive ingestion by krill (Marr, 1962). Chrysophyceae species have few hard parts that are likely to be detected using light microscopy (F. Scott, pers. comm.). This probably accounts for their absence in the microscopy-based analysis.

The ability to develop assays that detect a diverse range of prey is a major advantage that DNA diet analysis has over other methods. However, the development of prey detection assays still requires careful design. Predator DNA swamped initial universal primer-based assays despite the use of dissection to increase the relative concentration of prey DNA. Current assays must be designed to avoid amplification of the predator's DNA, a requirement that restricts the exploration of prey diversity. Recent work suggests there may be other ways of dealing with predator DNA (Blankenship and Yayanos, 2005) and such techniques might be applicable to krill.

The taxonomic resolution of DNA-based 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. 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 also provide more taxonomic resolution but it is unclear what size range of prey DNA fragments will be easily retrieved from krill stomachs. Identification of species using DNA has been gaining popularity and broader issues are related to identification are undergoing active debate (Meyer and Paulay, 2005; Steinke et al., 2005; Rubinoff et al., 2006).

Comparing Prey Relative Abundance Estimates from DNA Analysis and Microscopy. Prey relative abundance estimates obtained from DNA clone libraries and microscopy analysis differed significantly (Figure 3). Site B shows a large discrepancy in the relative abundance estimates for *Thalassiosira* between the two methods. The ratios

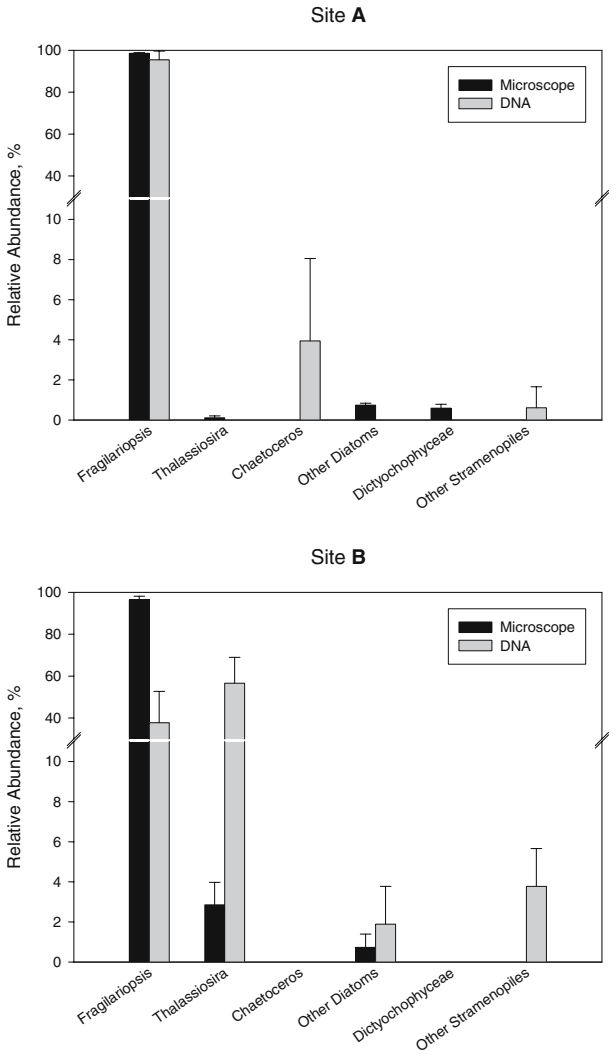

Fig. 3. Comparison of microscope and DNA diet methods at sites **A** and **B**. Columns represent site averaged estimates of consumed prey relative abundance. Error bars are SD (n = 3).

Table 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	
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
OTU-B10	Thalassiosira	0	0.0	0	0.0	1	1.9
Total		53		53		53	

of *F. cylindrus* and *F. curta* OTUs at both sites were also inconsistent with the ratios obtained by microscopic diet analysis (compare Tables 1 and 3). 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.

Diatoms have a good hard part signature for microscope analysis compared to many other prey groups, but because of differences in size and fragility some diatom species are easier to detect than others. While this may account for a proportion of the observed bias both *Fragilariopsis* and *Thalassiosira* generally have robust valves that are a reasonable size. A more likely explanation is that the DNA analysis in the current form suffers from biases that alter the ratio of clones from the true ratio of consumed prey. The potential reasons for DNA bias in this study are many and varied. The large *Thalassiosira* DNA signal at site B could be a reflection of the krill feeding behavior. Krill at site B may have consumed *Thalassiosira* diatoms just before 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.

The issue of prey detection bias needs further consideration now that systems to detect multiple prey are beginning to emerge. Differences have been observed in the length of time that prey species are detectable (Harper et. al, 2005) and clone libraries derived from scats gave biased estimates of diet

Table 4. Estimate of Prey DNA Molecules Extracted from Individual Krill Stomachs

Krill ID	*Concentration of extracted DNA ng/µl*	*Copies of prey SSU DNA per PCR reaction*	*SD (n = 3)*	*Copies of prey SSU DNA extracted from stomach*	*SD (n = 3)*
A-1	43	8319	± 1485	1,787,987	± 319,168
A-2	42	4366	± 368	916,886	± 77,282
A-3	25	2713	± 294	339,172	± 36,755
B-1	44	1142	± 80	251,304	± 17,604
B-2	47	1194	± 61	280,535	± 14,332
B-3	28	1006	± 133	140,898	± 18,627

(Deagle et al., 2005) similar to the results of this study. For the reasons outlined in the previous paragraph we suspect that accurate quantification of prey proportions within an individual krill will be difficult to achieve. An alternative approach that avoids some prey bias issues 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 (Agusti et al., 2003) and beetles (Harper et al., 2005).

Quantifying Prey DNA Extracted from Krill Stomachs. Quantitative PCR showed that all krill stomach DNA samples contained a large amount of prey DNA template that could be amplified with the prey specific PCR primers (Table 4). All stomachs analyzed 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 should be capable of providing robust PCR results.

Quantifying the amount of available DNA template is important for assessing the robustness of DNA diet analysis. Given sufficient PCR amplification cycles, 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). Quantifying the amount of starting template provides is an important check when analyzing environmental samples.

Overall the results of this study suggest that the application of DNA diet analysis to krill warrants further investigation, particularly for prey that are difficult to study using other methods.

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