Molecular approach (PCR-DGGE) to diet analysis in young Antarctic krill *Euphausia superba*

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ABSTRACT: Antarctic krill *Euphausia superba* Dana comprise a key component of the Southern Ocean food web, yet despite decades of research, questions concerning the regional, seasonal and ontogenetic differences in their diet remain. All current methods used to characterize krill diet have limitations for identifying the full complement of the diet. Using DNA as a marker molecule, our goal in this study has been to evaluate the efficacy of a PCR-DGGE (denaturing gradient gel electrophoresis) approach targeting the 18S rDNA gene to discriminate among diet constituents in gut and fecal pellet samples from young Antarctic krill relative to their feeding environment—the seawater and sea ice microbial community. We conducted 2 laboratory-based feeding experiments with known food items and 3 field samplings of both the krill and their feeding environment. Sequenced PCR-DGGE phylotypes from laboratory trials clearly distinguished diatom and copepod prey, while in situ feeding analyses revealed that a broad diversity of taxa were ingested, including diatoms (Bacillariophyta, the most prevalent group detected), dinoflagellates, cryptomonads, prasinophytes, ciliates, cercozoans, choanozoaflagellates, turbellarians and (possibly) sponge larvae. Band image analyses allowed environmental and diet phylotypes to be matched. On average, 32% of those from the environment were present in the diet; conversely, of the phylotypes detected in the diet, an average of 59% were in common with the environment. Changes in environmental phylotypes among sampling dates were reflected by similar changes in the krill diet as potential prey diversity (richness) decreased during a phytoplankton bloom.

KEY WORDS: Antarctic krill · *Euphausia superba* · Diet · DGGE · 18S rDNA · Sea ice microbial community · Omnivory

INTRODUCTION

Antarctic krill *Euphausia superba* Dana serve as both an important grazer (Walsh et al. 2001) and a critical prey item for the reproductive success of seals, seabirds and whales in the Southern Ocean (Croxall et al. 1999). Although generally characterized as herbivorous, *E. superba* is clearly omnivorous. Around South Georgia, one of the South Shetland Islands northeast of the Antarctic Peninsula, Antarctic krill are dependent on heterotrophic food sources, including small (1 to 3 mm) copepods (Atkinson & Snýder 1997). However, west of the Antarctic Peninsula, where the phytoplankton biomass is higher than around South Georgia and large copepods dominate (Ross et al. 1996), growth (secondary production) in krill is correlated with phytoplankton food sources (Ross et al. 2000). For larval krill, in which the feeding ecology is less well known than that of the adults, overwinter feeding and survival is suspected to rely on the complex assemblage of autotrophs (and possibly heterotrophs) comprising the sea ice microbial community (Frazer et al. 2002 and references therein).

All the current methods used to characterize the krill diet have limitations for identifying the taxonomic complexity of the diet, varying in both degree of specificity and sensitivity. These methods have various constraints; for example microscopy is limited to
undigested hard parts such as copepod mandibles, diatom frustules, or tintinnid loricae (Hopkins & Torres 1989), gut fluorescence detects only algal pigments (Ross et al. 2004), and monoclonal antibodies target specific species of interest (Haberman et al. 2002). Biomarker methods analyzing fatty acid and stable isotope composition of krill have been found to target a wider variety of diet components, but are limited in the taxonomic resolution they provide (Frazier 1996, Alonzo et al. 2003). PCR-based molecular methods for detecting DNA offer promising new avenues for diet analysis (Symondson 2002), and are currently being used in marine systems (Jarman et al. 2002, Nejstgaard et al. 2003). A distinct advantage of using a molecule such as DNA to detect diet diversity is that all potential prey items have DNA, and thus detection of a dietary item will depend more on sensitivity to the molecular signal than on such group-specific biases as those mentioned above. Although a number of studies have utilized some form of DNA detection of prey in predator diets (e.g. Zaidi et al. 1999, Rosel & Koche 2002, Jarman et al. 2004), molecular approaches have seldom been applied to marine crustaceans (Nejstgaard et al. 2003).

The choice of indicator molecule or gene fragment is a central issue to consider when molecular signatures are used to describe prey. Although there are a few molecules to choose from that would be informative in terms of phylogenetic accuracy in prey identification (e.g. organellar 16S rDNA or Cytochrome C, nuclear ribosomal internal transcribed spacer, 28S rRNA), the largest amount of eukaryotic diversity data exists for the small subunit ribosomal RNA gene (18S rDNA). Surveys of picoplankton 18S rDNA in marine environments in recent years have revealed extensive phylogenetic diversity and novel lineages (López-Garcia et al. 2001, Moon-van der Staay et al. 2001). In addition to constructing and sequencing clone libraries to access 18S rRNA sequence diversity, denaturing gradient gel electrophoresis (DGGE), a method that separates PCR amplicons based on their melting points along a denaturing gradient gel matrix, can be used to profile sequence heterogeneity in a complex mixture. DGGE has long been used in studies of diversity in natural microbial assemblages (e.g. Muyzer et al. 1993, Murray et al. 1996), to characterize gut flora (Simpson et al. 1999, Regensbogenova et al. 2004), and recently to characterize eukaryote community diversity (van Hannen et al. 1998, Diez et al. 2001, Gast et al. 2004). Detecting molecular diversity in a zooplankter’s diet is similar to investigating diversity of the micro- or nanoplankton itself, although the potential for PCR inhibition, or signal masking by the host which could confound the diet signal, are issues that remain to be investigated.

In this study we conducted 2 laboratory-based feeding experiments with known food items, and 3 field samplings of a euphausiid grazer and its possible food sources. Our goal was to evaluate the efficacy of a PCR-DGGE approach to discriminate between diet constituents in gut and fecal pellet samples from young Antarctic krill Euphausia superba relative to the eukaryal diversity present in the feeding environment (seawater and the sea ice microbial community). As part of this effort, we sequenced a number of bands (phylotypes) separated by PCR-DGGE from both the environment and the diet; in addition, the sequence of the 18S rDNA gene was determined for this ephausiid species.

**MATERIALS AND METHODS**

**Study site.** This study was conducted with Euphausia superba collected from coastal waters (Hero Inlet or Arthur Harbor) south of Anvers Island (64° S, 64° W), west of the Antarctic Peninsula. Laboratory-based feeding experiments were conducted at Palmer Station (USA), located on Anvers Island. In situ feeding (ISF) experiments were conducted in the coastal waters on 3 occasions in 2002 (ISF-1 on 3 November, ISF-2 on 14 November and ISF-3 on 12 December) as the environmental conditions changed rapidly during the austral spring of 2002. Sea ice in the area, consisting mostly of ‘old’ floes colored by the sea ice-associated microorganisms and melting in the springtime waters, was transient between ISF-1 and ISF-2, and absent from the region by December. Water column chlorophyll a concentrations integrated above the 1% light level were about 75 mg m⁻² in early November, rapidly increased to a peak (bloom) value near 200 mg m⁻² by mid November, and then decreased to relatively low values (<50 mg m⁻²) from late November through mid December (M. Vernet pers. comm.).

**Laboratory feeding.** We ran 2 experiments to validate the use of DGGE in distinguishing diet items in krill with different feeding histories. An Antarctic diatom culture and a mix of freshly collected copepods were used as the 2 food sources. Krill for both experiments were collected by SCUBA divers during the austral spring of 2002 from under ice floes alongshore at Palmer Station using small (10 cm × 10 cm) hand nets and held in a flow-through seawater system under ambient conditions of food and temperature until used. In the first experiment, 15 to 20 late larval and early juvenile krill (size = 12 to 19 mm total length, TL) were starved 24 h in 0.2 µm filtered seawater and then allowed to feed for 3 h on the diatom Fragilariaopsis curta (Van Heurck) Hustad: inoculum purchased from Bigelow Laboratory under the synonym Nitzschia
and sub-adult krill were collected on 12 December in
scribed for the algal cultures above. In ISF-3, juvenile
experiment (1 l each) were filtered and frozen as de-
over 24 h) and seawater samples accompanying each
sources of prey items. Sea ice (allowed to melt at 4°C
the sea ice, were collected by divers as potential
larval (Furcilia 6) and early juvenile stage krill were
ice-dominated waters in 2 experiments (ISF-1 and 2),
potential food items were collected mid-day from sea
prey under different field conditions. Krill and their
samples to investigate DGGE as a method for examin-
tion protocol was used in the second experiment in
once above from aggregations of individuals
processed as described above.

In situ feeding. We collected 3 sets of in situ feeding
samples to investigate DGGE as a method for examin-
differences between available prey and consumed
prefer to the experiment. Gut and fecal pellet samples were
frozen immediately for subsequent gut sampling or
frozen in aliquots of SLB at –70°C for DNA extraction. The same feeding and sample collec-
protocol was used in the second experiment in
krill and subadult krill were fed a mixture
beads (0.1 mm diameter) were added to the samples
and then bead-beaten 1 min to disrupt cells. Cells in all
samples were then lysed by the addition of (at final
concentration) sodium dodecyl sulfate (1%), and pro-
teinase K (5 mg ml–1) and incubating at 55°C for 1.5 h.
DNA was purified following standard phenol/chloro-
form purification using (1) ethanol precipitation fol-
lowed by suspension in TE (10 mM Tris-HCl pH 8.0,
0.1 mM EDTA pH 8.0), or (2) centrifugal concentration
(Centricon-100, Millipore) followed by 2 × 1 ml washes
in TE, and final concentration to ~20 µl. The DNA yield
was quantified using PicoGreen (Molecular Probes, as
per manufacturer’s instructions) on a fluorometer (Lab-
systems Fluoroskan Ascent), then stored at –20 or –70°C
until analyzed.

PCR. The extracted DNA was used as a template to
amplify a ~240 bp region coding for the eukaryal 18S
rRNA gene using eukaryote-specific 960bF-GC and
universal 1200R (Gast et al. 2004) oligonucleotide
primers. Most reactions were performed in 100 µl vol-
umes with the addition of (at final concentration)
deoxynucleoside triphosphates (0.2 mM each), MgCl2
(3.5 mM), primers (1.0 µM each), Taq DNA polymerase
(0.5 U) (Eppendorf or AmplitaqGold, ABI), PCR buffer
(1x) supplied with the Taq, and ~10 ng of template
DNA. Samples were amplified (ThermoHybaid PCR
Express; 28 cycles) following Gast et al. (2004). Suc-
cessful amplification products were quantified using the Pico-Green method following DNA precipitation in
ethanol. Amplifications of samples with DNA concentra-
tions below picogreen detection limits (some gut
samples) were run for a total of 35 cycles.

DGGE. Purified nucleic acid samples were separated
by electrophoresis using a BioRad DCode DGGE appar-
atus. Briefly, 8% polyacrylimide (acrylamide:bisacryl-
amide 37:5:1), 1 mm thick gels with a linear denaturant
gradient of 35 to 45% (where 100% denaturant = 40%
deionized formamide and 7 M urea), were run for 10 h at 90 V in a 60°C bath of 1x TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA, pH 7.4). Gels were then stained 15 min in SybrGold (Molecular Probes), UV-illuminated, and photographed. Optimal denaturing gradient and DNA amounts for comparative analysis were determined empirically; DNA amount varied by sample type and complexity (100 to 600 ng per lane for gut, fecal pellet, sea ice or seawater).

**DNA sequencing.** Bands with unique migration points (and several with identical melting points in adjacent lanes or different gels) were excised from the denaturing gradient gels for DNA sequence analysis. Acrylamide slices excised with a sterile scalpel were suspended in 200 µl of sterile water with an aliquot of glass beads, and bead-beaten for 1 min to disrupt the gel fragment. This solution was stored overnight at 4°C before using a 0.5 µl aliquot to re-amplify (PCR) the isolated product with the original primer set (less the primer GC-clamp sequence) and the same amplification conditions described previously. PCR products were verified by agarose gel electrophoresis, then purified with Qiaquick columns (Qiagen). DNA was sequenced bidirectionally with the 960F/1200R primer set on an ABI 3730 genetic analyzer, then sequences were assembled (Sequencher, GeneCodes) and compared to the ‘nt’ and ‘env_nt’ nucleotide databases provided by the NCBI using BLAST (Altschul et al. 1997) run locally. Taxonomic information was accessed through the NCBI taxonomy database. The trophic status of phylotypes (18S rRNA sequence types) with high sequence identity to characterized organisms were designated as either autotrophic or heterotrophic based on the predominant trophic mode for that taxa.

In some cases (16 of 35), co-migrating bands (with putatively identical sequences) were sequenced from different sample types and experiments. In nearly all cases (15 of 16), identical sequences were found, and 1 sequence was chosen as the ‘type’ sequence for that band. Despite this high degree of reproducibility, not all bands excised resulted in high quality sequences.

The 18S rRNA gene from the abdominal muscle of an adult *Euphausia superba* sample was PCR-amplified with Euk1A (Sogin & Gunderson 1987) and Univ1492 (Kane et al. 1993), then bidirectionally sequenced with Euk1A, Euk326F (reverse and complement from Eu309R, Lim et al. 1993), Univ533F (modification from Weisburg et al. 1991), Euk960F and Univ1200R (Gast et al. 2004), Euk516R (Amann et al. 1995) and Univ1492R. The GenBank Accession No. for the *E. superba* 18S rDNA sequence is DQ201509 (see Table 1 for accession numbers of unique DGGE phylotypes).

**DGGE band image analysis.** Images of DGGE gels were processed using GelComparII (Applied Maths) to facilitate band (phylotype) detection and alignment within each gel, allowing us to then determine the number of bands with unique melting points (i.e. phylotype richness). Phylotype profiles (phylotypes in each lane) were compared by pairwise similarity using Sörensen’s index \( C_S = 2 \times j / (a + b) \), where \( j \) is the number of common phylotypes, \( a \) is the number of phylotypes in Lane a, and \( b \) is the number of phylotypes in Lane b (Murray et al. 1996, adopted from Magurran 1988). In DGGE lanes where the krill phylotype appeared, this phylotype was not counted in richness or similarity measures. Also, in addition to considering pairwise similarity between individual DGGE lanes, the sum of phylotypes with unique melting points in gut plus fecal pellets profiles, and in sea ice plus seawater profiles were calculated to represent the complete diet and complete environment food sources, respectively.

**RESULTS**

**Laboratory feeding experiments.**

***Starved krill fed *Fragilariopsis curta.**** The PCR-DGGE results indicated that algal and krill rDNA phylotypes migrated to different positions in a 35 to 45% denaturing gradient gel, and that solely algal and krill rDNA sequences were detected in krill diet samples. In the cultured algal sample (food source), 2 bands were present that co-migrated with 2 bands in the fecal pellet sample (Fig. 1a); all 4 were sequenced and found to be identical over the 234 base region (AntEuk13-24, Table 1). There were also 2 bands present in the whole foragut sample that matched a region of the Antarctic krill 18S rDNA gene we sequenced from abdominal muscle (100% match over 250 bases; AntEuk13-5: Table 1).

***Starved krill fed a mix of wild copepods.*** A total of 16 bands were found in the wild copepod mixture (Fig. 1b), 5 of which had co-migrating bands in gut and fecal pellet profiles. Additionally, a large copepod removed prior to the feeding trial and processed separately resulted in a single band (Fig. 1b, Lane 2), indicating that different crustacean 18S rDNA sequences can be detected with this approach, and that they can be detected in krill gut and fecal pellet samples. Of the 13 bands sequenced from this experiment, 7 produced unique sequences. Several bands with the same sequence, yet varying in length at the 5’- and 3’-ends as a result of varying sequence quality (210 to 237 bases), matched AntEuk16-8, an environmental copepod clone, but migrated to different positions in the gel (Fig. 1b, Lane 1, lower 4 numbered bands). Image analysis revealed that 5 of 8 bands in the diet samples
were in common with those from the copepod mixture; 2 other phylotypes (AntEuk17-13 and AntEuk17-7; Table 1) matched a gastropod (96% identity over 212 bases) and an apicomplexan (99% over 131 bases) sequence, respectively.

**In situ feeding and environmental sampling**

**ISF-1: Under sea ice, early bloom conditions.** Phylo-
type profiles from the 3 November field samples were the richest in terms of diversity, with 41 phylotypes from the environment and 49 overall (Table 2, Fig. 2a). Sea ice and seawater phylotype profiles were quite different ($C_s = 0.33$). Of the 41 environment phylotypes, 11 (27%) were in common with the diet samples. The gut sample was more diverse and potentially more informative than the fecal sample, although the majority of detected phylotypes were different ($C_s = 0.35$). Of the 19 bands detected in the fecal and gut samples, those in common with sea ice and seawater phylotypes (11) represented 58% of the diet. Phylotype sequence analyses revealed a diversity of autotrophs dominated numerically by diatom-related phylotypes in both the environment and diet, although several heterotrophs (e.g. Ciliophora, Cercozoa, Turbellaria) were also present in these samples (Table 1, Fig. 2a).

**ISF-2: Under sea ice, peak bloom conditions.** Timing of the second experiment on 14 November was coincident with a significant algal bloom comprised in part of diatoms *Thalassiosira* spp. and *Chaetoceros* sp., and the prymnesiophyte *Phaeocystis* sp. (identified by light microscopy, W. Kozlowski pers. comm.). Phylo-
type richness was the lowest of the 3 periods exam-
ined, with a combined total of 14 bands with different melting points appearing in sea ice and seawater (Fig. 2b), and a higher phylotype profile $C_s$ of 0.60 (Table 2) than ISF-1. The reduced richness in the envi-
ronment was reflected in the diet, with only 7 phylo-
types detected in the combined fecal and gut samples. In this case, 5 of the 7 diet phylotypes (71%) were in common with bands in the environmental samples. The same 5 bands represented 36% of the environ-
mental pool of phylotypes. The gut profile was again more diverse and not very similar ($C_s = 0.44$) to the fecal profile. Sequence analysis of excised bands revealed that diatom-affiliated sequences dominated the phylotype diversity in both the diet and the envi-
ronment, although heterotroph-related taxa were found in each phylotype profile (e.g. Ciliophora, Turbellaria: Table 1, Fig. 2b).

**ISF-3: Open water, post bloom conditions.** On 12 De-
cember, 18 environmental phylotypes were detected in the open water sample (Table 2, Fig. 2c), which in comparison to ISF-1 and ISF-2 suggests a moderately rich eukaryal assemblage. Of these 18, 6 (33%) were in common with those in the diet samples. Phylotype rich-
ness of the diet samples was high (13 bands in gut plus fecal samples) relative to the environment, with 46% of the diet phylotypes (6 bands) in common with the environ-
ment. Diet samples were derived from juvenile (ca. 19 mm TL) and small adult (ca. 33 mm TL) size classes.
collected at the same time, although gut composition was quite different between these individuals ($C_s = 0.29$). Phylotype profiles from the juveniles' (n = 2 individuals, pooled to partly compensate for the volume of the adult gut) foreguts more closely matched the seawater (5 of 6 bands) than the adult (n = 1 individual; 3 of 10 bands) profile. Sequenced phylotypes in this period, as in ISF-1 and 2, continued to show dominance of *Fragilariopsis curta*-related sequences (AntEuk13-24) in both the diet and environment. Heterotrophs were again common in the environment (e.g. choanoflagellates, Cercozoa, Porifera), 1 of which co-migrated with a band in the diet (AntEuk20-29; Table 1, Fig. 2c). The same phylotype was sequenced in ISF-1.

<table>
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<th>Band ID</th>
<th>NCBI Accession No.</th>
<th>Sequence length (bp)</th>
<th>% Sequence identity closest relative</th>
<th>Closest relative</th>
<th>Closest relative higher taxon</th>
<th>Trophic mode</th>
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*a'env.nt' database
bAccession No. is for nearly complete *E. superba* 18S rDNA sequence; the 250 bp AntEuk13-5 sequence matches bases 1202 to 1486 of *E. superba* sequence
cSequence is 100% identical to *Thalassiosira antarctica*, *Fragilariopsis cylindrus*, and *Stauroneis contricta*
dSequence is 100% identical to *T. punctigera*, *T. eccentrica*, *Thalassiosira* sp., and *Minidiscus trioculatus*
eSequence is 97% identical to *T. pseudonana*, which = A
DISCUSSION

The results suggest that PCR-DGGE is efficient in detecting and distinguishing many diverse diet items of *Euphausia superba*. At least 16 autotroph-related phylotypes and 10 heterotroph-related phylotypes were identified by DNA sequence analysis. The diet (foresgut plus fecal phylotypes) reflected the food source in laboratory feeding experiments, and when investigated in naturally feeding juvenile krill, an average of 59% (n = 3, SD = 12.6) of the diet phylotypes were detected in the environmental (sea ice plus seawater, or just seawater) samples. Diet phylotypes that were not detected in the environmental samples (8 in ISF-1, 2 in ISF-2, and 7 in ISF-3).

Fig. 2. DGGE images and sequenced phylotype results from *in situ* feeding (ISF) samples of *Euphausia superba* and their environment. Phylotype profiles reflect different environmental conditions from nearshore waters off Palmer Station, Antarctica, during austral spring 2002 on 3 occasions: (a) 3 November with sea ice cover (Lane 1: sea ice; 2: seawater; 3: krill fecal pellets; 4: krill gut contents), (b) 14 November with sea ice cover during peak spring bloom (Lane 1: sea ice; 2: seawater; 3: krill fecal pellets; 4: krill gut contents), and (c) 12 December in open water (Lane 1: seawater; 2: krill fecal pellets; 3: gut contents from 2 juvenile krill [total length = 19 mm]; 4: gut contents from 1 adult krill [total length = 33 mm]). Numbers are codes for bands excised and sequenced for phylotype analysis (see Table 1, Column 1). Dots indicate gel position of bands not sequenced.
ISF-3) probably represent organisms that were either below the detection limits (~1.5% abundance in sample), or were not present in those samples due to krill feeding in a patchy environment. Environmental richness was high (2-fold higher than diet samples), with an average of 32% (n = 3, SD = 4.6) of the phylotypes detected being found in dietary components.

The use of a primer set targeting all eukaryotes, the approach applied in this study, differs from most studies employing PCR-based diet analyses, which often utilize highly specific primers designed to identify single species (Jarman et al. 2002, Symondson 2002, Nejstgaard et al. 2003), or group-specific primers (Höss et al. 1992, Jarman et al. 2004). The use of primers targeting all eukaryotes allowed us to (1) develop an approach inclusive of all potential prey, (2) develop a comparatively broad picture of the diversity in *Euphausia superba*’s diet, and (3) avoid additional potential for PCR bias (see below) by conducting a single round of PCR. Efficiency, low cost and detailed phylogenetic resolution are desirable for comparative, multi-sample ecological studies, which is an advantage of this approach (Diez et al. 2001).

We identified 4 areas of consideration that are key to diet composition analysis: DNA extraction (recovery), uniform PCR amplification, sensitivity of detection, and unique sequence detection using DGGE. First, extraction or recovery of genomic DNA in proportion to amounts naturally occurring in ingested prey may be affected by differential digestion rates among prey types (e.g. faster in soft-bodied prey) or cellular differences influencing DNA extraction. In an attempt to minimize effects of digestion, we analyzed contents of the foregut, not the larger midgut of the krill, and targeted a relatively short (~240 base) segment of DNA (Symondson 2002). However, results from our fecal pellet analyses may be biased toward poorly digested taxa, since diversity in the fecal samples was consistently lower than in the foregut, and 2 of the 4 sequenced phylotypes were affiliated with diatoms (AntEuk13-24 and AntEuk13-107).

The second issue to consider is 18S rRNA gene copy number, which can be highly variable among species of eukaryotes (Long & Dawid 1980), resulting in unequal PCR amplification simply from differences in the relative copy numbers between different taxa rather than the mass of the organisms in the sample. Ribosomal gene copy number reflects the growth rates of the individual (Elser et al. 2000), and is often proportional to genome size (Prokopowich et al. 2003). This issue is a major challenge for PCR-based studies striving to quantify proportions of constituents in a mixed diet. In our study, the ribosomal gene copy numbers are unknown for most of the organisms that were detected, although they are inevitably different. Indeed, a recent study reported that rDNA copy numbers in phytoplankton can vary from 1 to >12,000 copies (Zhu et al. 2005). Zhu et al. (2005) reported rDNA copy numbers for a few species related to phylotypes detected in our study (at least at the genus level: *Thalassiosira* sp., ~400 copies; *Nitzschia closterium*, ~100 copies; *Bathycoccus prasinos*, ~10 copies). We are not aware that our survey was compromised by variation in the 18S rDNA gene copy number; however, we cannot rule this out at this time, and must emphasize that intensities of bands can vary for 2 reasons: (1) differences in rDNA copy number and (2) differences in organism abundance. We did detect a high number of different phylotypes, including 10 different phytoplankton in a single spring seawater sample (Fig. 2a, Lane 2), suggesting a relatively high sensitivity of detection.

Thirdly, as with any environmental PCR application, sensitivity of detection is influenced by the complexity of the target sequence population, primer design, and method of detection. Our results indicate that the eukaryote primer set was sensitive over a broad range of sample complexity (Figs. 1 & 2) using a basic ethidium bromide stain for band detection. Previous studies have shown that sequences representing 1 to 1.5% of the population can be detected (Muyzer et al. 1993, Murray et al. 1996), limiting detection of rare sequences. This may explain the discrepancy observed of phylotypes detected in the diet that were not evident in the environment (17 of 39 diet phylotypes), and also...
suggests that krill selectively grazed these items. Although there is always the chance that we have missed elements of the prey population as a result of primer specificity, the wide range of taxonomic diversity detected is encouraging.

Lastly, DGGE has its limits when analyzing heterogeneous sequences found in natural populations. Originally developed for mutation detection in human chromosomal DNA (Fischer & Lerman 1979), the complexity of natural populations can challenge the ability to discriminate between all different sequences. Confounding factors in which maximum resolution of different sequences is not achieved (reviewed by Muyzer & Smalla 1998) can result in co-migrations of different sequences, causing difficulties in re-amplification and direct sequencing of the DNA. Also, sequences having more than 1 equally stable secondary structure, interspecies variation of rRNA genes (Dahllöf et al. 2000), double bands resulting from Taq error (Janse et al. 2004), or heteroduplex formation (bands resulting from annealing of 2 similar, yet slightly different sequences, e.g. Guldberg & Guttler 1993) could result in >1 band per sequence. In our study, both of these conditions were observed, although the results are reproducible, (i.e. if one band co-migrates with another, or if the same sequence is found at 2 migration points, they will perform reproducibly in separate DGGE experiments). The absolute calculations of phylotype richness could then be compromised, as can the similarity calculations, although less so, particularly in the case of >1 band per sequence. Recovery of high quality sequences was not achieved in all bands excised. This issue may be circumvented by cloning the DNA from excised bands (Fandino et al. 2001). Several phylotypes (AntEuk13-24, AntEuk13-5, AntEuk13-107 and [possibly] AntEuk16-8) were detected in >1 band. Ultimately, awareness of the issues influencing sequence detection is important, as is gaining a better understanding of the potential for the balance between copy number and cellular abundance. At present, PCR-DGGE is not quantitative, but it does address many of the previous limitations concerning detection of the full complement of the diet population.

One clear advantage of this approach in comparison to other methods of diet analysis in a grazing zooplankter is that we detected several different heterotrophic taxa in addition to the predominant autotrophic taxa. Sequences detected in the krill diet fell into many eukaryotic taxa including diatoms (Bacillariophyta, the most prevalent group detected), dinoflagellates, cryptomonads, prasinophytes, ciliates, cercozoans, choanoflagellates, turbellarians, and possibly sponge larvae. Sponges are among the more common benthic invertebrates at our study site (Martin pers. obs.), and turbellarians can comprise 45% of the Antarctic sympagic meiofauna biomass (Schnack-Schiel et al. 2001). In the laboratory feeding trials, unexpected heterotrophic sequences such as the Umbraculum umbraculum-related sequence may have come from imperfect sorting of wild-caught copepods from other zooplankters, from copepod guts, or from internal parasites of the krill in the case of the putative apicomplexan sequence. However, the gut contents of heterotrophic prey are likely to be below detection limits, as prey DNA swamps the signal from their own gut contents. Note that because of the limits of detection, care must also be taken to avoid as much host DNA as possible, as evidenced by the krill bands in the first laboratory feeding trial, where the gut tissue itself probably masked the algal signal, given that they migrated to the same gel position. The laboratory trials also indicate that eukaryotic gut flora, if present, are largely below these detection limits.

Given the 3-fold range in phylotype richness of environment samples across dates, we also addressed whether such changes might be reflected in the krill diet. When compared, phylotype richness of the diet generally tracked changes in overall environment richness (Fig. 3). Comparisons of DGGE phylotype profiles also revealed that the sea ice microbial community and the surrounding seawater became more alike (Cs = 0.60, ISF-2) as richness declined concomitant with sea ice melting and the associated phytoplankton bloom. Although we did not compare phylotype profiles among dates, the different environmental samples (sea ice and seawater) compared within each date were more similar than samples in a comparable study (Gast et al. 2004).
Many questions remain unanswered regarding the behavior and feeding ecology of *Euphausia superba*, particularly in reference to the winter feeding of larvae, and when and where omnivory plays an important role. Over a decade ago, several investigators (e.g. Smetacek et al. 1990) suggested that sea ice microbial communities might be an essential food resource for winter survival of larval and juvenile krill. Both behavioral and physiological evidence continues to support this concept (e.g. Frazer et al. 2002, Quetin et al. 2003). In our study, we hand-collected small swarms of larval krill actively feeding on sea ice surfaces with a well-developed sea ice microbial community (ISF-1, -2), and thus it is interesting to note that the young krill analyzed appeared to be feeding on the sea ice microbial community given that food (inferred from chlorophyll a levels) was plentiful in the water column in the austral spring period sampled. A number of phylotypes in the sea ice were present in the diet samples, including the sequenced tubellarian band. It is unknown whether the heterotrophs consumed in this study were taken as by-catch or were selected for, but their detection suggests that this approach may assist in future investigations of omnivory in this species. Our results also reflect grazing studies in which krill were shown to select diatoms over prymnesiophytes (Haberman et al. 2003): *Phaeocystis pouchetii* was present in the sea ice microbial community but not in the diet (Fig. 2b, Band 16), while diatoms in general dominated the diet (Table 1, Fig. 2). Furthermore, differences between juvenile and adult gut samples in this study (*C = 0.29, ISF-3*) may be evidence of an ontogenetic or seasonal shift in feeding and/or habitat choice, and point to the utility of molecular dietary analysis in revealing behavioral attributes of *E. superba*. Although the numbers of samples studied here are limited, the data demonstrate that this technique can be used on individuals as well as pools of individuals to identify dietary constituents with fewer taxa-specific biases. In future studies it will be interesting to consider both variation in the diet between individuals and swarms feeding in different water masses or ice habitats.

In conclusion, there is a need to develop better methods to understand the diversity of organisms contributing to the diet of *Euphausia superba*, a key member of the Antarctic ecosystem, with the implications of such work extending well beyond this application. The PCR-DGGE approach used in this study is a step in that direction, and proved useful not only in describing the diversity of eukaryotic micro- and nanoplanクトn under very different environmental conditions, but in linking changes in community composition to *in situ* changes in krill diet. Quantification of mixed-diet constituents is desirable in diet analyses, but quantitative approaches such as real-time PCR require *a priori* knowledge of the sequences to target. Thus, identification of putative diet components is a necessary first step, in which the approach described should be useful.

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**LITERATURE CITED**


