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IN-SITU FEEDING IN THE NORTHERN KRILL, MEGANYCTIPHANES
NORVEGICA:

A DNA ANALYSIS OF GUT CONTENTS

BY

ALISON CLEARY

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

OCEANOGRAPHY

UNIVERSITY OF RHODE ISLAND

2010

MASTER OF SCIENCE THESIS

OF

ALISON CLEARY

APPROVED:

Thesis Committee:

Major Professor

The image shows four handwritten signatures in blue ink, each written over a horizontal line. The first signature is at the top, followed by a second, third, and fourth signature below it. The signatures are stylized and cursive.

DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND

2010

Abstract

The northern krill, *Meganyctiphanes norvegica*, is an important member of North Atlantic shelf ecosystems, serving both as prey for whales, fish and seabirds, and as a predator on phytoplankton and other zooplankton. However, understanding *in-situ* krill feeding is technically challenging; incubations may not be representative of krill feeding *in-situ*, while biomarkers and microscopic examination of gut contents suffer from limited prey type resolution and have a limited range of detectible prey. Analyzing DNA in gut contents may offer insight into *M. norvegica* feeding *in-situ* unobtainable using other methodologies. The major technical difficulty in using DNA as a marker of gut contents is the overwhelming quantity of predator DNA; two approaches are taken here to exclude predator DNA from analysis, firstly the use of species specific primers, and secondly the use of a krill-specific peptide nucleic acid probe. Species specific primers were used to sequence and quantify known phytoplankton prey (*Thalassiosira weissflogii* and *Rhodomonas* sp.) from the guts of captive krill, showing that even low abundance prey items can be successfully detected using DNA in gut contents. A krill-specific peptide nucleic acid (PNA) probe was used to sequence all non-krill 18S DNA present in the guts of krill collected *in-situ* in the Gulf of Maine. PNA is a synthetic, uncharged, DNA analog which binds strongly and specifically to complementary DNA, and thus inhibits PCR amplification of the target sequence. Including a krill-specific PNA probe in a PCR using universal 18S primers allowed for amplification of all eukaryotes present in the krill fore-gut. Gut contents amplicons were sequenced from a clone library and compared with known sequences to determine their identity. The most common prey item, found in

M. norvegica guts at every station, was an uncultured and poorly known protist, which previous studies suggest represents a novel kingdom of eukaryotes, and may suggest krill feeding on the sediment interface. *M. norvegica* in the Gulf of Maine also consumed the copepod *Calanus finmarchicus* at 7 of the 8 stations samples, in agreement with results found using other methods. Additionally, *Centropages* sp., 4 other protists, another copepod, 5 phytoplankton and the salp *Thalia democratica* were found as gut contents. In addition to providing interesting information about *M. norvegica* feeding *in-situ* in the Gulf of Maine, these results demonstrate the utility of the PNA-PCR clone library approach to gut contents analysis, elucidating prey, such as protists and *T. democratica*, which would have been difficult or impossible to detect with other methodologies.

Acknowledgments

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This research is based in part upon work conducted using the Rhode Island Genomics and Sequencing Center which is supported in part by the National Science Foundation under EPSCoR Grant No. 0554548.

Preface

This thesis is composed of two manuscripts, “DNA as a marker of gut contents in *Meganyctiphanes norvegica*: sequencing and quantification of known prey items in captive krill”, and “*In-situ* feeding by *Meganyctiphanes norvegica* in the Gulf of Maine: a PNA-PCR analysis of gut contents”, both of which are formatted to be submitted to the journal Marine Ecology Progress Series. Both manuscripts address the question of investigating feeding by *M. norvegica* using DNA in gut contents, although each takes a different approach to the DNA analysis.

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DNA as a marker of gut contents in *Meganyctiphanes norvegica*:

sequencing and quantification of known prey items in captive krill

(captive krill gut contents DNA)

Alison Cleary*, Edward Durbin

University of Rhode Island Graduate School of Oceanography, South Ferry Road,
Narragansett, Rhode Island, 02882, USA

DNA as a gut contents marker in northern krill, *Meganyctiphanes norvegica*, was investigated using laboratory feeding incubations. DNA from two different phytoplankton species was successfully sequenced from the guts of captive *M. norvegica* using species specific primers. Phytoplankton DNA was sequenced both from krill individuals fed a single species of phytoplankton, and those fed a mixture of known phytoplankton. Quantitative PCR indicated that even very low copy number samples were successfully sequenced using the species specific primer approach, and suggest krill were feeding at low levels in the experimental incubations. DNA may potentially be used as a marker of *in-situ* gut contents, and the methods described here successfully recovered sequenceable DNA from *M. norvegica* guts, and thus may be applicable to *in-situ* investigations.

Key Words: *Meganyctiphanes norvegica*, feeding, DNA

* corresponding author: acleary@gso.uri.edu

Introduction:

Meganyctiphanes norvegica, the northern krill, is an important component of food webs in the North Atlantic, serving both as prey for whales, fish and seabirds, and as a predator on phytoplankton and smaller zooplankton (Kaartvedt et al 2002, MacDonald 1927, Thomasson et al 2003, Cotté and Simard 2005, Lass et al 2001). Understanding the feeding behavior of *M. norvegica* is important to understanding the functioning of these ecosystems, and their potential to adapt to changing environmental conditions. However, measuring feeding of small organisms consuming microscopic prey in a marine environment is technically challenging. While many approaches have been used to measure krill feeding, all have advantages and drawbacks. Incubations may not be representative of *in-situ* feeding, biomarkers have low temporal and prey type resolution, and gut contents microscopy is limited to organisms with hard parts (McClatchie 1985, Torgersson 2001, Schmidt et al 2003, Rossi et al 2008, Dalpadado et al 2008, Kaartvedt et al 1998, Båmstedt and Karlson 1998). Recent studies with Antarctic krill, *Euphausia superba*, have used DNA as a marker of gut contents (Martin et al 2006, Passmore et al 2006). In this work, the use of DNA as a marker of gut contents in *M. norvegica* is investigated. Species specific primers were employed in sequencing and quantitative PCR reactions on krill fed a known phytoplankton diet.

For this study 18S ribosomal DNA is used. 18S rDNA is the DNA region encoding for the 18S rRNA which makes up a structural component of the small sub-unit of eukaryotic ribosomes (Nelson and Cox 2005). 18S rDNA is around 1,900

nucleotides in length, although this varies somewhat between species (Nelson and Cox 2005). 18S is also a multi-copy gene, that is, it occurs many times in the genome of a single cell unlike most genes which occur once per cell. This increases the probability of detecting an organism, which is particularly important when working with partially digested gut contents.

18S rDNA is often used as a “bar-coding” region of DNA, a region used to identify different species. Both previous studies using DNA to investigate krill feeding have used 18S (Martin et al 2006, Passmore et al 2006). Due to its role in the structure of the ribosome, 18S contains regions of sequence which are highly conserved, as well as quite variable regions. This is ideal for use as a “bar-coding” gene because it allows for the design of universal primers amplifying all eukaryotes in the highly conserved regions, while providing good information content and species resolution in the highly variable regions, as well as allowing for the design of species specific primers, as done in this study. The popularity of 18S as a bar-coding marker also means that the greatest amount of sequence data is available for it. GenBank (National Center for Biotechnology Information, National Institute of Health), contains 273,609 sequences for “18S” (<http://www.ncbi.nlm.nih.gov>, January 3, 2010). By contrast none of the other bar-coding type genes contained even 70% of that number, with “COI” having 187,353, “Cox” having 123,027, and “Cob” with 6,497 (<http://www.ncbi.nlm.nih.gov>, January 3, 2010). For gut contents studies an ideal marker will have a large database of sequences of the region from a variety of known organism, thus providing a way of identifying unknown sequences through homology with sequences of known origin.

Methods:

Collection and maintenance:

Krill for this study were collected on NOAA ship Delaware II during the Ecosystem Monitoring (ECOMON) cruise in January/February 2009. Live euphausiids were collected using a bongo net with 333 μm mesh and solid plastic 1 liter cod-ends in the Gulf of Maine (Table 1). Tows were oblique from the surface to 100 meters, with a forward speed of 1.5 to 2 knots, and a wire speed of 50 m min^{-1} out, and 10 to 15 m min^{-1} in, and were conducted mainly at night. When the net reached the surface cod ends were immediately submerged in buckets of seawater, and their contents gently inverted to minimize sheer forces on the krill. Individual krill were transferred from the bucket into holding tanks on deck using large white plastic ladles. Methods were designed to be as gentle as possible and minimize handling time of the krill, to improve krill survival (King et al 2003).

While at sea krill were maintained in two flowing seawater tanks on deck, with each tank containing approximately half the population, at maximum approximately 0.5 krill L^{-1} . These tanks were 120 liter insulated coolers, modified to include external adjustable height stand pipes, internal large surface area water outflows, and adjustable rate seawater inflow. While closed, these tanks were tightly shut using external ratchet straps, and water level was maintained completely full, with minimal air space to reduce sloshing due to ship movement. Tanks were secured on deck to maintain *in-situ* temperatures. Krill were fed *Artemia salina* larvae (2 to 7 days old) which were raised in a heated, aerated, 4 liter aquarium with a salinity around 50 psu. Krill were fed approximately 500 ml of dense *Artemia* culture daily, additionally the seawater flowing through the tanks was coarsely filtered, so small prey items similar

to those found *in-situ* were available to the krill. Krill were fed and checked daily; any dead individuals or molts were removed and recorded. Krill were maintained onboard up to one week.

In the lab, the krill were maintained in a round tank, 70 cm high and 150 cm diameter, with a water volume of 1,240 liters. Krill were transferred from the ship's return to port in Woods Hole, MA to the lab in Narragansett, RI in 5 gallon buckets of seawater, which were then allowed to temperature acclimatize by floating in the laboratory tank, before being gently transferred to the tank. At maximum population size immediately upon return to shore, 123 krill were maintained in this tank, 0.1 krill L^{-1} . The tank had 5 μm cartridge filtered flowing filtered seawater from Narragansett Bay, initially 6-7° C warming to 12° C by April 22 when the experiment was concluded with a flow rate of approximately 3 $L\ min^{-1}$. The central stand pipe outflow was modified with a large cone of plastic mesh, effectively increasing the inflow surface to approximately 50 linear cm. The tank was of dark blue slightly translucent polyethylene plastic and was covered by a teepee of thin black plastic in order to create a darkened environment for the krill which is optimal for maintaining euphausiids in captivity (King et al 2003). Krill were observed daily and any molts or dead individuals were removed and noted, as was excess dirt or fecal matter. Krill were fed two to ten day old larval *Artemia salina* daily at a final krill tank concentration of approximately 60 *Artemia* L^{-1} .

Feeding experiments:

Feeding experiments were carried out 10 days after the krill were transferred to the laboratory aquarium, ensuring that remaining krill were healthy and acclimated (King et al 2003). Three different feeding treatments were conducted: *Rhodomonas* sp., *Thalassiosira weissflogii*, and combined *Rhodomonas* sp. and *T. weissflogii*. *Rhodomonas* sp. (CCMP 768) and *T. weissflogii* (CCMP 1048) were cultured in sterile f/2 media at 20° C and constant light (15 $\mu\text{mole photons m}^{-2} \text{s}^{-1}$) to high concentrations in 2 L polycarbonate containers. Feeding experiments were conducted by filling 5 L wide mouth plastic jars approximately two thirds full of filtered seawater and a high concentration of prey (visibly colored the water), to promote high feeding rates (McClatchie 1985). These jars were floated within the main tank to temperature acclimate. Krill were captured as gently as possible from the main tank using small aquarium nets and beakers without bringing them into the air. All feeding experiments were 5 hours, from approximately 10 am to 3 pm in the dark. While this is a time when krill would normally be at depth and thus unlikely to be actively feeding on phytoplankton, work by Båmstedt and Karlson (1998) suggests that krill do not have endogenous rhythms in feeding activity. After 5 hours krill were removed from the feeding containers as rapidly as possible and with minimal stress to the animals and placed in 80% ethanol. Ethanol was changed once after 24 hours to maintain preservation.

Laboratory analysis:

Prey DNA was sequenced from the same cultures used in the feeding experiments. 10 ml of culture was vacuum filtered onto 13 mm diameter 0.5 μm membrane filters (nucleopore). DNA was extracted using the DNeasy plant kit

(Qiagen) as per manufacturer's instructions. DNA extracts were diluted 10x to 100x and amplified using universal eukaryote 18S primers Euk A and Euk B (Table 2) (Medlin 1988). Each 50 μ l reaction contained 1x GoTaq Green Master Mix (Promega), 0.5 μ M forward primer EukA, 0.5 μ M reverse primer EukB, and 0.5 – 1 ng μ l⁻¹ template DNA. Thermal cycling was run on a Mastercycler (Eppendorf) as follows: 95°C for 30s, 35 cycles of 94°C 30s, 60°C 1 min, 72°C 2 min, final extension 72°C 10 min, 4°C hold up to 12 hours.

PCR products were visualized on agarose gels (200 ml of 1% (weight/volume) agarose in 1x TAE buffer, 125 volts for 90 minutes). PCR products were purified using the Qiaquick clean-up kit as per manufacturer's instructions (Qiagen) and DNA concentration was measured spectrophotometrically (Nanodrop). DNA samples were prepared for sequencing by combining 180 ng purified 18S PCR product, 10 picomoles primer, and ultra pure H₂O to a total volume of 24 μ l. Each sample was sequenced with both forward (Euk A) and reverse (Euk B) primers to cover the entire 1,800 bp 18S amplicons. Sequencing was conducted by the Rhode Island Genomics and Sequencing Center using the Applied Biosystems ABI 3130xl genetic analyzer and POP7 polymer, a 50 cm 16-capillary array and the KB Basecaller software (URI RIGSC).

Four krill were analyzed from each of the single prey feeding experiments, and six from the mixed prey experiment. Krill were patted dry, wet weighed using an electronic balance to the nearest 0.01 gram, and measured under the microscope for total length, using Mauchline's standard one length measure as the lateral or dorsal distance from the tip of the rostrum to the end of the uropods, excluding terminal setae

(Mauchline 1980 in Everson 2000). Krill were dissected in disposable translucent weigh boats using flame sterilized sharp forceps. The front of the carapace was detached to reveal the foregut which was carefully removed with forceps. Foreguts were placed in individual microcentrifuge tubes and kept on ice for the duration of the dissections, up to 2 hours. Following dissections, DNA was immediately extracted from whole krill fore-guts using the DNeasy Blood and Tissue kit as per manufacturer's instructions (Qiagen), with overnight lysis, and 200 μ l elution volume.

Species specific primers were designed for *Rhodomonas* sp. and *Thalassiosira weissflogii* based on full length sequences from culture. These sequences were aligned with *Meganyctiphanes norvegica* and 16 other potential prey items, and primers were designed to maximize base pair mis-matches between the target organism and all other organisms, with special attention to *M. norvegica*. Base pair mismatches were located as close to the 3' end of the primer as possible to maximize destabilizing effects and primers were designed following the recommendations of Innis and Gelfand regarding length, GC content, T_m , and possible secondary structures (Innis and Gelfand 1990) (see table 2).

A thermal gradient PCR was run to optimize primer specificity with 10 μ l reactions of 1x GoTaq Green Master Mix (Promega), 0.5 μ M forward primer (T.w. 1f or Rh 1f), 0.5 μ M reverse primer (T.w. 1r or Rh 1r), and 0.5 – 1 ng μ l⁻¹ template DNA. Thermal cycling was run on a Mastercycler (Eppendorf) as follows: 95° for 30s, 35 cycles of 94°C 30s, 51°C – 71°C 1 min, 72°C 2 min, final extension 72°C 10 min, 4°C hold up to 12 hours. Primer specificity was tested against: *Ditylum brightwelli*, *Thalassiosira nordenskioldi*, *T. weissflogii*, *Rhodomonas* sp., *Artemia salina*, *Acartia*

tonsa, *Meganycitophanes norvegica*, *Isochrysis galbana*, and *Tetraselmis sp.* Reaction chemistry and thermocycling were run as above, with an annealing temperature of 67°C for T.w. 1f1r, and 65°C for Rh 1f1r. No amplification was observed for non-target organisms. Both sets of primers amplify an approximately 450 base pair region of the 18S.

Gut DNA extracts of all krill were amplified using both sets of species specific primers, T.w. 1f1r and Rh 1f1r, in 10 µl PCR reactions. Thermal cycling was conducted on a Mastercycler (Eppendorf) as follows: 95°C for 30s, 35 cycles of 94°C 30s, 65°C (Rh 1f1r)/ 67°C (T.w. 1f1r) 1 min, 72°C 2 min, final extension 72°C 10 min, 4°C hold up to 12 hours. Krill guts were again amplified in 50µl PCR reactions under the same conditions as above for only the prey items they were fed, and the resulting amplicons were purified with Qiaquick (Qiagen) for sequencing, which was conducted as described for phytoplankton cultures, but with 45 ng of DNA due to shorter length of the PCR amplicons.

A quantitative PCR (qPCR) experiment was run to investigate the sensitivity of the species specific primer gut contents sequencing approach using the *Thalassiosira weissflogii* specific primers. Cycling was run on a Stratagene Mx3005P. Each 25 µl reaction contained: 1x SYBR green qPCR master mix, 0.1µM T.w. 1f forward primer, 0.1 µM T.w. 1r reverse primer, 0.03 µM Rox reference dye, and template DNA. Thermocycling was run as above, and fluorescence was detected at the end of each annealing step. A standard curve of 18S *T. weissflogii* amplicons (as used in sequencing) was run at 10⁷, 10⁵, 10³, 10¹, and 10⁰ copies µl⁻¹; 18S copy number µl⁻¹ was determined as follows: copies µl⁻¹ = (6.02 * 10²³) * (C) *

$(1800 \times 650)^{-1}$, where C is the concentration of PCR product in $\text{ng } \mu\text{l}^{-1}$, 1800 is the bp length of the PCR amplicon, and 650 is the average base pair weight. Gut extracts from every *M. norvegica* individual were amplified, all samples and standards were run in duplicate.

Results:

The system developed for collecting and maintaining live euphausiids was fairly effective. *M. norvegica* were collected between February 6 and 12 2009, and the last krill in captivity died April 22 2009, for a total life in captivity of 61 ± 3 days, or about 9 weeks (Fig.1). Krill in the large tank were observed to spend significant amounts of time on the bottom of the tank and also near the walls of the tank, and relatively little time swimming around the central part of the tank. When disturbed, krill bioluminesced brightly; bioluminescence was not observed in the absence of mechanical disturbance to the krill.

Sequences derived from DNA extracts of krill guts using species specific primers were of good quality and were identical to the sequences derived from phytoplankton culture. All 4 of the krill who were fed only *Thalassiosira weissflogii* resulted in sequences identical to that of pure culture. Additionally 3 of the 6 krill fed a mixed diet of *T. weissflogii* and *Rhodomonas* sp. resulted in sequences identical to pure *T. weissflogii* culture when amplified with the species specific primers. Using the *Rhodomonas* sp. specific primers gut DNA extracts of 3 of the 4 krill fed only *Rhodomonas* sp. resulted in sequences identical to that of pure culture and 3 of the 6 krill fed a mixed diet resulted in sequences identical to that derived from pure culture,

including one individual which also yielded a sequence for *T. weissflogii*. The remaining 7 reactions had low signal level and did not produce any meaningful sequence data.

qPCR results indicated that prey DNA 18S copy numbers in the krill gut extracts were very low (Fig. 2). The krill gut samples which resulted in successful sequences identical to that of pure phytoplankton culture contained approximately 0.1 to 2.5 copies μl^{-1} , about 1.5 to 30 copies per reaction, equivalent to 60 – 1,200 copies gut^{-1} . Samples which had not resulted in sequences showed little or no amplification. (Fig. 2)

Discussion:

The krill maintenance in captivity was fairly successful, with krill consuming at least some food, and swimming actively around the tank. The maximum life span of the *Meganyctiphanes norvegica* maintained in this study, about 9 weeks, was equivalent to the longest time they have been maintained in captivity in published literature, (McDonald 1927) Other species of krill, notably *Euphausia superba*, have been maintained for significantly longer than this in captivity, however doing so tends to involve significant infrastructure and be fairly labor intensive (King et al 2003). Earlier efforts for this study to maintain *M. norvegica* in captivity suffered from high mortality rates, and were labor intensive. Factors that appear to be important to maintaining captive krill include stable, *in-situ* temperatures, relatively large volumes of water per krill, flowing water to maintain good water quality, and gentle capture of the krill. However, this system is probably less than ideal in some respects. Krill did

suffer some mortality; microscopic necropsies did not provide a consistent answer on the cause of death. Additionally, krill exhibited behaviors in captivity different from those one would expect to find *in-situ* in a largely open ocean organism; krill were frequently observed near the bottom and sides of the tank, and particularly where the bottom and sides met, while they were relatively rarely seen in the central open water area of the tank. Krill feeding levels may also have been quite low, as discussed further in the qPCR.

This species specific primer technique was successful in sequencing two different prey items in the guts of *Meganyctiphanes norvegica*, including sequencing both items from the same krill individual. This suggests that species specific primers could be used in studies of *in-situ* krill feeding, focusing on specific prey items. However, this technique does require significant *a-priori* assumptions as to what the krill are consuming, and so is probably most applicable to very directed questions, such as the role of krill grazing on HAB formation, krill predation on common prey items such as *Calanus finmarchicus*, or predation of krill on eggs and larvae of fish species.

qPCR results indicated that prey 18S copy number in *Meganyctiphanes norvegica* guts was very low, for which there are two main explanations. Either krill rapidly digest prey DNA leaving little to be detected by qPCR, or krill in captivity were eating at very low levels due to stress of being in a confined, laboratory environment. While it is certainly true that krill have particularly strong digestive enzymes, as seen by the rapid degradation of photosynthetic pigments in *Euphausia superba* guts, low feeding rates are also likely in this case (Perissinotto and Pakhomov

1996). Previous studies of *M. norvegica* feeding in captivity have shown low and variable feeding rates under laboratory conditions. For example at a copepod concentration of 1.6 copepods L⁻¹. McClatchie (1985) found a feeding rate of < 1 copepod krill⁻¹ hour⁻¹ in 4 L aquariums, whereas Torgerson (2001) found a feeding rate of 7 copepods krill⁻¹ hour⁻¹ in 50L aquariums. Phytoplankton may also not be a preferred prey of krill. *M. norvegica* has been observed to consume significant amounts of photosynthetic prey, within an order of magnitude as much carbon as carnivorous feeding, (Kaartvedt et al 2002, McDonald 1927, Fisher and Goldie 1959, Dalpadado et al. 2008). However, in incubation experiments *M. norvegica* were not able to support metabolism feeding on phytoplankton alone, and mandible morphology suggests *M. norvegica* is mainly carnivorous (McClatchie 1985, Båmstedt and Karlson 1998). Krill were accustomed to being provided *A. salina* as prey, and thus feeding on phytoplankton may not have been necessary or energy efficient for these krill.

Low prey 18S copy numbers found in qPCR indicate the sensitivity of the technique. Samples containing as few as 2 copies of the target prey DNA resulted in successful sequences using species specific primers. This further suggests the utility of this technique in detecting relatively rare or rapidly digested prey items. Species specific primers may be applicable to detecting krill feeding on particular prey species *in-situ*, such as dominant copepods, or harmful algal bloom organisms.

Figure 1: Krill longevity in captivity, with individuals alive as time progresses from capture. Black arrow indicates when 24 krill were removed for use in feeding experiments.

Krill Longevity in Captivity

Figure 2: Standard curve of *T.weissflogii* specific primers with krill guts, krill guts resulting in sequences light grey squares, krill guts not resulting in sequences dark grey triangles

T. weissflogii specific qPCR

Table 1: Stations where live krill were collected: Latitude, Longitude, depth, date, and time

Station	Latitude	Longitude	Depth (m)	Date	Time
1					
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99					
100					

Station #	Date	time EST	Latitude	Longitude	Depth	# of krill
82	6-Feb-09	20:15	40°40.1'	66° 53.1'	210	15
94	8-Feb-09	2:13	42° 13.0'	65° 45.0'	228	3
104	9-Feb-09	19:30	42° 06.4'	68° 23.4'	183	15
107	10-Feb-09	4:30	42° 41.3'	68° 39.3'	180	16
113	10-Feb-09	19:30	43° 36.2'	67 °21.5'	219	17
116	11-Feb-09	5:00	43° 40.8'	67° 56.3'	211	22
123	12-Feb-09	3:00	42° 31.4'	69° 35.5'	263	16
127	12-Feb-09	16:30	41° 52.7'	69° 36.9'	184	34

Table 2: Primers used, sequences, names, references, annealing temperatures. Position is relative to *Meganyctiphanes norvegica* sequence (GenBank accession number GU595169)

Primer	Sequence	Anneal Temp	Position	Reference
Euk A	AACCTGGTTGATCCTGCCAGT	60	<0	Medlin 1988
Euk B	GATCCTTCTGCAGGTTACCT AC	60	>1747	Medlin 1988
T.w. 1f	CACACCCTGTGTGAGAACTTG TG	67	531 - 553	This study
T.w. 1r	CGGAGTCAAAAAACAACCGC CAATCCT	67	1026 - 1056	This study
Rh 1f	ATGTCCGGGCCTTTCTGC	65	666 - 683	This study
Rh 1r	GGAGTCGCAAATTGACATCCA CTG	65	1031 - 1055	This study

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In-situ feeding by *Meganyctiphanes norvegica*: a PNA-PCR analysis of gut contents

(Krill gut contents DNA analysis)

Alison Cleary*, Edward Durbin, Tatiana Rynearson

University of Rhode Island Graduate School of Oceanography, South Ferry Road,
Narragansett, Rhode Island, 02882, USA

Meganyctiphanes norvegica feeding in the Gulf of Maine was investigated using DNA in gut contents. Krill were found to have consumed *Calanus finmarchicus* at all but one station, and an uncultured and poorly known protist at every station sampled. Additionally, *Centropages* sp., another unidentified copepod, a *Prorocentrum* dinoflagellate, a green alga, another phytoplankton, two heterotrophic alveolates, three other protists, and the salp *Thalia democratica* were found as prey items at one or two stations. The common protist gut contents item potentially suggests *M. norvegica* may be feeding at the sediment interface. A krill specific peptide nucleic acid (PNA) probe was incorporated into a PCR reaction with universal primers to amplify 18S rDNA of all gut contents eukaryotes while selectively blocking amplification of krill 18S rDNA. Sequencing of clone libraries of these amplicons resulted in 255 prey sequences, 13 OTUs, from a total of 80 *M. norvegica* at 8 stations. These sequences were classified by homology with known sequences. This technique offers unique insights into *in-situ* feeding, with minimal *a-priori* assumptions, and may be applicable to other small organisms.

Key Words: *Meganyctiphanes norvegica*, feeding, Peptide Nucleic Acid, gut contents, DNA

* corresponding author: acleary@gso.uri.edu

Introduction:

Meganyctiphanes norvegica, the Northern krill, is a pelagic zooplankton important in food webs of the North Atlantic, as prey for whales, fish and seabirds, and as a predator on phytoplankton and smaller zooplankton (Kaartvedt et al 2002, MacDonald 1927, Thomasson et al 2003, Cotté and Simard 2005, Lass et al 2001). While copepods, and particularly *Calanus finmarchicus*, have been commonly found to be important in *M. norvegica* feeding, a variety of other prey items have also been observed, including phytoplankton, tintinnids, terrestrial debris, and marine detritus (Båmstedt and Karlson 1998, Kaartvedt et al 2002, Dalpadado et al 2008, Fisher and Goldie 1959, MacDonald 1927, Lass et al 2001). Understanding the feeding behavior of these krill is important to understanding the functioning of this ecosystem, and yet is also challenging. Incubations may not be representative of *in-situ* feeding, and biomarkers, such as stable isotopes and fatty acids, have low temporal and prey type resolution (McClatchie 1985, Torgersson 2001, Schmidt et al 2003, Rossi et al 2008). Current methods of gut contents analysis have a limited range of detectable prey, with gut contents microscopy limited to organisms with hard parts and gut pigments limited to photosynthetic prey, and suffer from challenges in identifying macerated prey items (Dalpadado et al 2008, Kaartvedt et al 1998, Båmstedt and Karlson 1998).

DNA as a marker of gut contents has the potential to provide information about *in-situ* krill feeding on a variety of prey. The major technological issue facing such studies is the overwhelming quantity of non-information containing predator DNA.

Previous studies of DNA in Antarctic krill gut contents have used either group specific diatom primers (Passmore et al 2006) or dissection of all krill tissue away from gut contents (Martin et al 2006). An ideal method of gut contents DNA analysis would allow for the detection of all potential prey within the gut, while excluding or ignoring krill DNA. One such potential method is peptide nucleic acid mediated PCR clone library sequencing. This approach consists of sequencing clone libraries of “bar-coding” genes from krill guts, incorporating a peptide nucleic acid probe to block amplification of krill DNA in the initial polymerase chain reaction (PCR). This approach has not previously been applied to gut contents studies, and this study will develop methods which may be applied to other organisms and ecosystems.

Peptide nucleic acid, PNA, is a synthetic DNA analog. Like DNA it consists of a relatively rigid helical backbone bearing a string of the nucleotide bases whose order determines binding kinetics (Nielson and Egholm 1999). Unlike DNA, whose backbone is composed of sugar and phosphate groups bearing a negative charge, in PNA this backbone is composed of uncharged peptides (Nielson and Egholm 1999). This uncharged backbone has important effects on the binding kinetics of PNA-DNA interactions. Binding of complimentary PNA-DNA strands is stronger than binding of DNA-DNA strands (Nielson and Egholm 1999). PNA-DNA has a higher binding specificity than DNA-DNA interactions (Nielson and Egholm 1999). For DNA-DNA interactions the minimum ΔT_m for a single base-pair mismatch is 4° , whereas for PNA-DNA interactions the minimum ΔT_m for a single base pair mismatch is 8° , twice that observed for DNA-DNA interactions (Nielson and Egholm 1999). This means that a single base pair mismatch is much more destabilizing to PNA-DNA binding

kinetics, and thus PNA-DNA interactions are more sequence specific than DNA-DNA interactions.

One application of the unique features of PNA is in PCR –clamping, the inclusion of a PNA probe in a PCR reaction (Ørum 2000). PCR-clamping works in one of two ways, either through competition for the primer site, or through arresting polymerase elongation (Ørum 2000). Both of these techniques depend on differences in binding kinetics between the PNA probe and the DNA primer. The PNA probe must have a higher T_m than the DNA primer, allowing the PNA to bind under conditions where the DNA primer will not (Ørum 2000).

This study uses PNA to arrest polymerase elongation. In this approach a PNA probe is designed which binds the target DNA sequence between the two PCR primers, and blocks PCR amplification by stopping the progress of the polymerase and thus preventing the creation of full length complimentary strands (Ørum 2000). PCR clamping employs a 4 step PCR. Initial high temperature denaturing is followed by a step at the PNA binding temperature, which is a temperature between the PNA T_m and the DNA primer T_m . Primer binding is then conducted at a temperature below the DNA primer T_m , and elongation is run at a temperature at least 10° below the T_m of the PNA to prevent PNA disassociation (Ørum 2000, Nielson pers. com.)

In previous studies, a PNA probe increased the sensitivity of a PCR-DHPLC detection of parasites in the blue crab (Troedsson et al 2008). Incorporating a PNA probe dramatically increased the detection of parasites, and allowed for the detection and sequencing of a previously unknown crab parasite (Troedsson et al 2008).

However, in that study low concentrations of PNA were used and blue crab was still by far the dominant amplicon recovered (Troedsson et al 2008).

In this work a PNA probe specific to *Meganyctiphanes norvegica* 18S ribosomal DNA is designed and incorporated into a PCR amplification using universal primers. The resulting amplicons are sequenced from a clone library to identify krill gut contents. The main advantage of this technique is in minimizing *a-priori* assumptions of the type of prey consumed *in-situ* by *M. norvegica*, assuming only that bacteriophagy and cannibalism are not important contributions to the diet. Because this study uses clone libraries, the results are considered to be purely qualitative, as cloning has been shown to potentially bias the relative proportions of different sequences.

Methods:

Krill field collections:

Krill for this study were collected on NOAA Ship Delaware II on Ecosystem Monitoring cruises in August (August 16 to 30, 2008) and February (January 26 to February 14, 2009) between 18:30 and 04:00, local time. Sampling was concentrated in the Gulf of Maine region where *Meganyctiphanes norvegica* are abundant (figure 1). Krill were collected in oblique bongo tows to 5 meters from the bottom, or 200 meters in water depths greater than 205 meters, using was a 61 cm bongo frame fitted with 333 μ m mesh nets without cod-ends, net ends folded and tied. The use of tied net ends as opposed to solid cod-ends should minimize the potential for net feeding, as the flow of water through the net keeps both krill and potential prey pushed against the

back of the net and thus unable to filter feed. The net was towed with a forward velocity of between 1.5 and 2 knots, and wire speeds of 50 m min⁻¹ out, 20 m min⁻¹ in. A bongo net is good for capturing euphausiids as it has no bridle in front of the net opening, thus minimizing the potential for net avoidance (Everson 2000). Minimizing net avoidance is important in investigations of feeding as gear which allows for significant net avoidance may select for the weaker and less able to escape krill. Work with *Euphausia superba* has observed white-ish, poor condition krill individuals weakly swimming at the edges of krill schools, and these individuals may consume a very different diet from the healthy individuals making up the bulk of the krill population (Hamner and Hamner 2000). In this work, all krill appeared to be still alive or recently dead when collected and no white-ish individuals were collected.

The net was immediately rinsed down, and contents washed from the net into a plankton sieve, from which krill were rapidly picked with forceps and placed directly into jars of 80% ethanol to preserve gut contents. The time from the net reaching the surface to the krill being placed in ethanol averaged less than 5 minutes. Most krill still appeared to be alive at the time they were placed in ethanol, actively attempting to escape the forceps and/or ethanol. Ethanol has been shown to more effectively preserve krill gut contents DNA than freezing, through immediately and permanently deactivating the digestive enzymes (Passmore et al 2006). 80% ethanol is used as opposed to 95% as 95% has been seen to result in krill to brittle for dissection (Passmore et al 2006). Ethanol was changed once after 12 to 24 hours to maintain effective preservation. Krill were preserved in 125 ml jars, with no more than one third of the jar composed of krill biomass; a maximum of approximately 15 to 20 krill

per jar. The only selective criteria were an appearance of health, as indicated by clear and red coloration, and relatively large size. Different stages and sizes of krill may consume different diets, and this study focuses on adult krill, thus juvenile krill (as determined practically as small individuals, less than about 20 mm) were not collected.

Laboratory analysis of krill guts:

Krill were dissected to remove their foreguts under a stereo microscope. Eight krill were analyzed from each station, with the exception of station 5, Bay of Fundy summer, where three replicate groups of eight krill were analyzed. Krill were selected at random from the adult krill collected, patted dry, and wet weighed using an electronic balance to the nearest 0.01 gram. Krill were measured under the microscope for total length, using Mauchline's standard one length measure as the lateral or dorsal distance from the tip of the rostrum to the end of the uropods, excluding terminal setae (Mauchline 1980), and krill sex was determined morphologically (Everson 2000). Krill were dissected in disposable translucent weigh boats using flame sterilized sharp forceps. The front of the carapace was detached and the foregut was removed with forceps, taking care not to break the outer membrane. Foregut fullness was classified as "empty", "< ½ full", "> ½ full" or "full", similar to the classification schemes used by Båmstedt and Karlson (1998), and Dalpadado et al (2008). Foreguts were placed in numbered individual microcentrifuge tubes and kept on ice for the duration of the dissections, up to 2 hours. Following dissections, krill gut DNA was immediately extracted.

DNA was extracted from whole krill fore-guts using the DNeasy Blood and Tissue kit as per manufacturer's instructions (Qiagen). This kit has been used successfully in previous work for DNA extraction from both zooplankton and phytoplankton, including dinoflagellates and diatoms. Krill guts were first mechanically disrupted using sterilized toothpicks, to ensure complete lysis of gut contents. Lysis incubation was conducted overnight, and DNA was eluted in 200 μ l. DNA extracts consistently had high DNA concentrations of 25 to 35 ng μ l⁻¹ (Nanodrop). 2 μ l aliquots from each of the eight krill at a station were combined for use in the analysis.

A PNA probe was designed from an alignment of *Meganyctiphanes norvegica* and 16 diverse potential prey items. The probe was designed to sit between well established universal eukaryote primers on the 18S gene (Gast et al. 2004), to be complimentary to *M. norvegica*, and to contain as many differences as possible with all potential prey items (Table 1). Design followed the recommendations of Applied Biosystems and associated sequence analyzer function for T_m, GC content and to minimize self complementarity (ABI 2009). The primers developed by Gast et al. (2004) were used in this study because they have been well established to amplify a wide range of Eukaryotic organisms, and create relatively short amplicons, 250 bp, which helps to minimize the effects of digestion on sequence recovery.

The effectiveness of the PNA was tested using a quantitative PCR (qPCR) experiment. qPCR was run with samples of full length 18S gene PCR products from *Meganyctiphanes norvegica* and *Thalassiosira weissflogii* with and without PNA. These PCR products were produced using universal eukaryote primers (Medlin 1988)

in 50 μl reactions containing (final concentrations) 1x GoTaq Green Master Mix (Promega), .5 μM forward primer EukA, .5 μM reverse primer Euk B, and 0.5 – 1 ng μl^{-1} DNA template of DNA extracted using the DNeasy Blood and Tissue kit from *M. norvegica* eyes, and DNeasy Plant kit from *T. weissflogii* culture filters (Qiagen). Thermal cycling was conducted on an Eppendorf Mastercycler as follows, initial denaturation at 95 °C for 30s, followed by 35 cycles of 94°C for 30s, 60°C for 1 min, 72°C for 2 min, then a final extension of 72°C for 10 min. 18S copy number μl^{-1} of these 18S PCR products was determined as follows: copies $\mu\text{l}^{-1} = (6.02 * 10^{23}) * (C) * (1800*650*10^6)^{-1}$, where C is the concentration of PCR product in ng μl^{-1} , 1800 is the bp length of the PCR amplicon, and 650 is the average base pair weight.

In a qPCR, a standard curve of *M. norvegica* amplicons was run at 10^1 , 10^4 , 10^6 , 10^8 , and 10^{10} gene copies μl^{-1} , and these same *M. norvegica* amplicons were run in the presence of PNA at 10^4 , 10^6 , and 10^8 copies μl^{-1} . *T. weissflogii* 18S amplicons were run with and without PNA at the same DNA concentration to measure the non-specific effect of the krill PNA probe. All samples were run in duplicate. Cycling was run on a Stratagene Mx3005P. Each 25 μl reaction contained: 1x SYBR green qPCR master mix, 0.1 μM Gast forward primer, 0.1 μM Gast reverse primer, 0.03 μM Rox reference dye, 20 μM PNA probe when required and template DNA. Thermal cycling was run with an initial denaturing step of 95°C for 10 minutes, followed by 40 cycles of 94°C for 30 s, 67°C for 30 s, 58°C for 30 s, 60°C for 45 s (detection at the end of this step in every cycle).

A PCR reaction using the universal Gast F and R primers and incorporating the krill-specific PNA probe was run on the combined krill gut contents DNA extracts

for each station. This 20 μ l reaction contained 1x GoTaq green master mix (Promega), 0.5 μ M Gast forward primer, 0.5 μ M Gast reverse primer, 20 μ M PNA probe, and 0.5 - 1 ng μ l⁻¹ of krill gut DNA. Thermocycling was as follows: 95° 30 s, followed by 25 cycles of 94°C 30 s, 67°C 30 s, 58°C 30 s, 60°C 45 s, then 60°C 5 min, and 4°C hold up to 12 hours.

PCR products were run on an agarose gel (35 ml of 1% agarose in 1x TAE buffer) and electrophoresed at 100 volts for 30 minutes. Gels were stained by submersion in a dilute Ethidium bromide solution for 10 minutes and viewed under UV light with minimal UV light exposure time to minimize damage to the DNA. PCR products were extracted from gel slices (SafeXtractor) using the wizard PCR and gel clean-up kit (Promega) as per manufacturer's instructions. DNA concentration was estimated by comparison with a DNA ladder size standard (exACTGene 100 bp DNA ladder, Fisher Scientific).

Cloning reactions were completed using the pGEM-T Easy Vector system (Promega), as per manufacturer's instructions with modifications as follows. Insert: vector molar ratios were 1:1. Ligation reactions were run in 11 μ l, with 4 μ l PCR product, 5 μ l 2x rapid ligation buffer, 1 μ l vector, and 1 μ l T4 DNA ligase. A very high white colony : blue colony ratio of approximately 100:1 was observed. Light blue or blue centered white colonies were relatively frequently observed and usually contained the insert; dark blue colonies consistently did not contain the insert.

Gut contents amplicons were PCR amplified directly from clone colonies for sequencing. Clone colonies were touched with an autoclaved toothpick or flame

sterilized loop, transferred to a numbered reference plate, and the toothpick/loop mechanically disrupted in a 200 µl tube containing PCR reaction mixture. Between 30 and 70 colonies were PCR amplified from each cloning reaction. PCR reactions were conducted in 30 µl and contained 1x GoTaq Green Master Mix (Promega), 0.5 µM M13 forward primer, and 0.5 µM M13 reverse primer. Thermocycling was run on an Eppendorf Master cycler, as follows: 95°C 30 s, followed by 30 cycles of 94°C 30 s, 58°C 30 s, 72°C 45 s, with a final extension of 72°C 5 min, and 4°C hold up to 12 hours.

PCR products were visualized on a 1% agarose gel, stained with ethidium bromide, and viewed under UV illumination. PCR products of the correct size, ~450 bp, were purified with ethanol precipitation, using a protocol modified from Zeugin and Hartley (1985). Samples were prepared for sequencing by combining 200 ng of PCR product, or 2 µl, whichever was greater, as determined spectrophotometrically (Nanodrop), 10 pmoles of M13 forward primer, and H₂O to a final volume of 24 µl. Samples were sequenced at the Rhode Island Genomics and Sequencing Center (RIGSC) using the Applied Biosystems ABI 3130xl genetic analyzer and POP7 polymer, a 50 cm. 16-capillary array and the KB Basecaller software.

Prey Field Sequencing:

To identify the sequences obtained from gut contents, a variety of potential prey items for krill were sequenced to complement the data available on Genbank. Mesozooplankton sequenced for this were collected on R/V Endeavor cruise EN446 in the Great South Channel, and Southern Flank of Georges Bank area, during June

2008. Mesozooplankton samples were collected using a plankton pump. Zooplankton were picked individually from ethanol preserved samples under stereomicroscopy. Mesozooplankton sequenced include *Microcalanus pusillus*, *Pseudocalanus* sp., *Calanus finmarchicus*, a hyperid amphipod sp., and *Metridia lucens*. Additionally, *Acartia tonsa* (Narragansett Bay, RI), *Centropages typicus* (Narragansett Bay, RI) *Thalassiosira weissflogii* (CCMP 1048), *Rhodomonas* sp. (CCMP 78), *Heterocapsa triquetra* (CCMP 448), and *Artemia salina* (San Francisco Bay brand) were sequenced (Table 3).

Sediment and water samples for sequencing of protists were collected on the NOAA ship Delaware II Ecosystem Monitoring cruise DE0909 in August 2009. Sediment samples were collected using a small ponar grab in the Wilcox Basin (42° 29.8'N, 69° 40.3'W, depth 255 m) and Georges Basin (42° 25.3' N, 69° 00.3' W, depth 365 m). Sediment was soft mud in both areas, and while showing no clear vertical structure, did have indications of epibiotic activity, indicating that the sediment water interface was captured. Sub-samples were transferred to microcentrifuge tubes using sterile popsicle sticks. Water samples were collected by Niskin bottles on a CTD rosette at the same stations. Samples were collected from both near surface (< 2 m depth) and near bottom waters (4 (GB) or 7 (WB) m above sediment). Water samples were filtered onto 25 mm diameter, 0.5 µm membrane filters (nucleopore) using gentle vacuum filtration in triplicate (200 to 500 ml). Water filters and mud samples were frozen at -20° C until analysis.

Zooplankton DNA was extracted from whole individuals using the DNeasy tissue kit as per manufacturer's instructions (Qiagen), with mechanical disruption

using a sterile toothpick, and overnight lysis incubation. Zooplankton extraction and sequencing was done for two to seven replicate samples for each species. Zooplankton DNA was PCR amplified using universal full 18S gene primers, Euk A and Euk B (Medlin et al. 1988). Each 50 μ l reaction contained 1x GoTaq Green Master Mix (Promega), 0.5 μ M forward primer EukA, 0.5 μ M reverse primer EukB, and 0.5 – 1 ng μ l⁻¹ template DNA. Thermal cycling was run on a Mastercycler (Eppendorf) as follows: 95°C for 30s, 35 cycles of 94°C 30s, 60°C 1 min, 72°C 2 min, final extension 72°C 10 min, 4°C hold up to 12 hours.

Sediment DNA was extracted using the DNeasy tissue kit (Qiagen), as per manufacturer's instructions with the following modifications. For each extraction 0.03 to 0.05 g of sediment were transferred to a microcentrifuge tube. Twice the recommended quantity of ATL and proteinase K from the DNeasy Blood and Tissue kit were added to the sample and the sample was vortexed vigorously occasionally during lysis. The sample was then centrifuged to pellet the inorganic particles, and the supernatant was used in the remaining steps of the DNA extraction, with two initial centrifugations to pass the entire sample through the spin column. The resulting DNA contained co-purified humics as seen with spectrophotometry (Nanodrop), but produced successful PCR amplicons, and thus was sufficient for the purposes of this study. Water filters were similarly extracted using the DNeasy tissue kit with initial step double volumes of lysis buffer and proteinase K in order to ensure the filter was submerged and thus all parts of the filter would be completely lysed. Sediment and water filter DNA extracts were PCR amplified using primers designed to be specific to an uncultured eukaryote found in the krill gut clone library sequencing. These primers,

OTU A specific forward and reverse (OTU A sp. f/r) were designed using the clone library sequence and the PrimerBLAST tool from the National Center for Biotechnology Information, (www.ncbi.nlm.nih.gov September 2009) (See Table 1). These OTU A f/r primers were also used in conjunction with universal EukA EukB primers to obtain a longer sequence for this organism from krill guts.

Samples were sequenced at the RIGSC as described above, with sequencing run starting at each primer, in order to cover the entire length of the 18S gene, 1800 bp.

Sequence analysis:

All sequences, gut contents and prey field, were visually checked for read quality using the Applied Biosystems Sequence Scanner (ABI). MegAlign and EditSeq (DNASTar) were used to align, assemble and crop sequences. Clone library sequences were cropped to remove plasmid DNA sequence, and the reverse compliment taken as necessary. Prey field zooplankton sequences were assembled with at least 50 overlapping identical base pairs. All replicates of the same prey species were identical. For sequences which were not sufficiently long to overlap, the region of interest lay entirely on the reverse strand, so this sequence alone was used in further analysis, and both are provided separately in GenBank (Table 4).

Sequences were aligned using ClustalW and default settings (gap penalty 15.0, gap length penalty 6.666, delay divergent seqs 30%, DNA transition weight .5). Gut contents clone library sequences were aligned over all stations, and classified into Operational Taxonomic Units (OTUs) based on a 3% divergence cut-off and assigned

arbitrary letter names (Sogin et al 2006). OTU sequences were aligned with a variety of known sequences, both of species previously found to be taken as prey by krill, and the top named (not “uncultured eukaryote”) BLAST hits for each OTU (Altschul et al 1990). Known prey item sequences were taken from Genbank and the prey field sequencing done as part of this study. OTUs were classified as the most closely related known species within 3%. In cases where there were no identified species within 3%, and it was unclear what the closest species was, OTUs were classified into the lowest taxonomic group where they could confidently be placed based on phylogenetic tree morphology.

Data on the mesozooplankton abundances in the environment was obtained from Ecosystem Monitoring Program of the NOAA Northeast Fisheries Science Center. This data is in the form of counts of each zooplankton species or group per volume filtered, as measured with flow meters mounted on net mouth openings (Kane 2007). Counts come from the other side of the bongo net on the same tows where krill were collected. Mesozooplankton abundance data was obtained for every station where krill gut contents were analyzed for *in-situ* feeding.

Results:

The krill PNA probe reduced amplification of krill DNA to negligible levels, while having a minimal effect on non-target sequences (standard curve $r^2=1.000$) (figure 2). Some amplification is observed after 35 cycles, and may be indicative of single stranded amplification of the unclamped strand.

308 krill gut contents sequences were obtained. These classified into 32 OTUs. Of these OTUs, 19 (59 sequences) clustered most closely with *Meganyctiphanes norvegica* and other krill species. The remaining 13 OTUs represent prey items consumed by the krill *in-situ* in the Gulf of Maine. These include: *Calanus finmarchicus*, a copepod related to *C. finmarchicus*, *Centropages* sp., a *Prorocentrum* dinoflagellate, a green alga, another phytoplankton, the salp *Thalia democratica*, two different heterotrophic alveolates, and three different uncultured protists. Krill guts were more full in summer than in winter, based on microscopic examination (t-test non-parametric, $p < .01$) (figure 4).

Mesozooplankton were common prey items for *Meganyctiphanes norvegica* in this region. *Calanus finmarchicus* was taken as prey by krill at every station except 7. *Centropages* sp. was a gut contents item at station 3, and was the dominant sequence at this location, making up 87% of the prey sequences obtained. The copepod consumed at 2 was very similar to *C. finmarchicus*, more so than to any other available copepod sequence, but was a distinct cluster outside of 3% similarity. The salp *Thalia democratica* was taken as prey by krill in 2 of the 3 replicate cloning experiments at station 5, the Bay of Fundy during the summer (figure 5).

Phytoplankton prey were taken relatively infrequently. Of the five phytoplankton sequences found, only one could be identified to lower taxonomic groupings. This *Prorocentrum* sp. was found as a krill gut contents item at station 7, off the southern flank of Georges Bank in winter. Two of these remaining phytoplankton prey were found as gut content items at station 4. One of these same

phytoplankton, and an additional two types were found as gut contents items at station 5 (figure 6).

The most surprising finding of this study was the abundance of uncultured protists as krill gut contents. A specific uncultured protist was found as a gut contents item at every station investigated in this study. While the relative proportions of different clones within a station is not here considered as quantitative, it may be worth noting that this uncultured protist comprised 93 of the 255 prey sequences obtained in this study, 36%. In addition to this common uncultured protist, three other uncultured protists were found as gut contents, for a total of four distinct protists as krill gut contents items. Two of these additional protists were found as gut contents at station 7, and the third at station 5. Overall gut contents abundance data is presented in figure 9.

Further investigations into the sources of these protists sequenced protist DNA from sediment and surface waters of the Gulf of Maine. Sequences from Georges Basin sediment, surface water and nephroid water, as well as Wilkinson Basin sediment and surface water were identical to that of the abundant krill gut contents uncultured protist. The protist found at station 5 was 99% similar to the sequence obtained from Wilkinson Basin nephroid water. This sequencing was done using targeted primers, designed to amplify only the abundant krill gut contents protist (Table 1). No amplification was observed applying these primers to the guts of krill captured in the Gulf of Maine and maintained in captivity for 10 days feeding on cultured phytoplankton and *Artemia salina*. Combining species specific primers with universal primers produced amplicons 520 bp in length, identical to the gut contents amplicons; this longer sequence is used in all further analysis.

The prey field available to *Meganyctiphanes norvegica* in this study was dominated by *Calanus finmarchicus*. *C. finmarchicus* made up on average 60% of the available mesozooplankton (range 30% at station 7 to 96% at station 8) with an average concentration of 162 indiv. m⁻³ (range 1 indiv. m⁻³ at station 7, to 370 indiv. m⁻³ at station 5). Total concentration of mesozooplankton varied more than an order of magnitude between stations, ranging from 15 indiv. m⁻³ at station 7 to 544 indiv. m⁻³ at station 4, with generally higher abundances in summer (ave. summer 378, ave. winter 85 t-test non-parametric $p < .01$). Other major components of the zooplankton assemblage included *Centropages typicus* (13%), *Thaliaceae* (11%), *Pseudocalanus minutus* (4%) and *Metridia lucens* (3%) (figure 3).

Discussion:

This study identified a variety of prey items taken *in-situ* by *Meganyctiphanes norvegica* in the Gulf of Maine. The two main prey items found were *Calanus finmarchicus* and an uncultured protist. Additional zooplankton prey included *Centropages* sp., another copepod and the salp *Thalia democratica*. Phytoplankton were encountered as prey relatively rarely, but three different phytoplankton were found including a *Prorocentrum* sp. (dinoflagellate) and a green alga. Heterotrophic alveolates (2) and other uncultured protists (3) were also encountered.

Calanus finmarchicus is a large, abundant and oil rich copepod in the Gulf of Maine region. This copepod has been previously found to be taken by *Meganyctiphanes norvegica* as prey in several studies (MacDonald 1927, McClatchie 1985, Båmstedt and Karlson 1998). *C. finmarchicus* is considered to be an important

prey item for *M. norvegica*, and in one study was observed to make up between 63% and 100% of the copepod prey taken (Båmstedt and Karlson 1998). The present study agrees with these previous studies, finding *C. finmarchicus* as a gut contents item for krill at seven of the eight stations sampled.

Salps, *Thalia democratica*, were a surprising prey item found in krill gut contents from the Bay of Fundy. Salps are gelatinous organisms, and consist largely of water. Adult *Thalia democratica* are also fairly large in relation to the size of *Meganyctiphanes norvegica*. DNA identified as belonging to a salp may come from any life stage, thus it is possible that the salps consumed were larval or juvenile and hence closer in size to the more typical prey of *M. norvegica*, the copepods. While there are no previous reports of *M. norvegica* consuming salps, *Euphausia superba* has been observed to consume salps in incubation experiments, and it has been suggested salps may be a preferred food (Kawaguchi and Takahashi 1996). In those experiments, krill “attacked the salps several times within 5-10 minutes, and then grasped the salps with their thoracic endopodites and swam away...and ingested them efficiently” (Kawaguchi and Takahashi 1996). Salps were only found at one of the stations studied, and only in two of three replicate groups of krill from that station, so it is unlikely *T. democratica* plays an important role in *M. norvegica* nutrition, but is interesting none the less. *T. democratica* as a gut contents item also highlights some of the advantages of the PNA-PCR technique. Salps would not have been expected *a-priori* to be a krill gut contents item, and with no hard parts would have been difficult or impossible to detect by gut contents microscopy.

Phytoplankton were relatively rarely encountered as prey items, occurring at only three of the eight stations sampled. Previous work has suggested krill feed on phytoplankton when photosynthetic prey are abundant in the water column, and mainly on copepods at other times of year (Kaartvedt et al 2002). Previous studies of the Gulf of Maine have found very low phytoplankton biomass in February, and moderate biomass in August, with the spring bloom of large diatoms occurring in March and April (Colebrook 1979, Durbin et al 2003). In this study two of the five summer stations analyzed had phytoplankton prey as krill gut contents, as did one of the three winter stations analyzed, with the remaining five stations containing no phytoplankton in krill gut contents. Thus no clear trend in seasonality between late summer and winter of krill consumption of phytoplankton was observed. It does not appear that phytoplankton form an important component of the diet of *Meganyctiphanes norvegica* in the Gulf of Maine region during these times. However, samples were not obtained during the spring bloom period when large diatoms available to the krill may be abundant, thus photosynthetic prey may be important at times of year when large phytoplankters are abundant (Durbin et al 2003, Kaartvedt et al 2002).

The most intriguing result of this study was the finding of a specific uncultured protist in the krill guts at every station sampled. No previous studies have mentioned protists as prey for *Meganyctiphanes norvegica*, and they are generally smaller than the size of organisms typically taken as prey by krill. BLAST searching yields no named organism with a sequence alignment covering more than 73% of the sequence, with 74% maximum identity. The closest sequences to this gut contents

item are derived from clone library studies of marine sediments. Amongst these uncultured, unnamed eukaryotes BLAST hits covering 89% of the sequence with maximum similarities up to 98% are found. This sequence is also 96% similar over 33% of the region to a gut contents item sequenced from guts of *Euphausia superba* near Palmer Station, Antarctica (Martin et al 2006). Previous studies finding highly related sequences have and have concluded these closely related sequences are not chimeric using the program CHECK CHIMERA, (Takishita et al 2007, Dawson and Pace 2002, Berney et al 2004).

Phylogenetic tree analysis suggests this sequence is from a protist of some description, however, it appears to be a poorly known organism. This protist is larger than 0.5 μm or particle associated as seen by its presence on 0.5 μm water filters, and of a related sequence in the >0.5 μm size fraction, but not the 0.2 to 0.5 μm size fraction in López-García et al (2001). Previous studies which have found closely related sequences have not been able to assign it to a known taxonomic grouping. Takishita et al (2007) studying methane seep microeukaryotes found a sequence 98% identical to the protist found here over the overlapping region “that could not be assigned to major eukaryotic groups...[and] possibly represent anoxic tolerant taxa”. That study also helps to put an upper size limit on this unknown protist, as they specifically excluded metazoans from DNA extractions (Takishita et al 2007). Dawson and Pace (2002) found a sequence 89% similar to the krill gut contents protist, which was among the sequences they concluded were “not specifically affiliated with any molecularly described taxonomic group, and therefore indicate novel kingdom-level relatedness groups”. Edgcomb et al (2002) found a sequence 90% identical to the krill

gut contents protist in the Guaymas Basin hydrothermal vent environment, and concluded that this and a few other sequences “are unrelated to those of any other eukaryotes, and they seem to represent early branches in the eukaryotic tree”. A re-analysis of some of these sequences as well as additional sequences from river sediment (93% identical to krill gut contents protist) again concluded these sequences represent a “possibly novel high-level lineage” (Berney et al 2004). A sequence 90% identical to the gut contents protist was found in Weddell Sea deep water and “represents a new lineage emerging in the region of Archeozoa” (López-García et al. 2001)

This leads to the question of what is the role of this poorly known protist as a krill gut contents item. There are several possible explanations for this gut contents item, four of which will be discussed below. These are: 1) this sequence is a parasitic or symbiotic organism resident in the guts of krill, 2) this sequence is a protist parasitic or endosymbiotic with a krill prey item, 3) this sequence is a protist filtered and consumed by krill in the water column, and 4) this sequence is a protist consumed by krill on the sediment or in the near bottom nephroid layer.

Krill endoparasites have been found in a few studies. A gregarine has been found in *Meganyctiphanes norvegica* guts (MacDonald 1927). There is evidence that *M. norvegica* is an important intermediate host of the helminth *Anisakis simplex* (Everson 2000 p. 264). Endosymbiotic bacteria have also been shown to have a digestive function in *M. norvegica* (Everson 2000). The extremely divergent nature of this sequence, as compared to sequences of known organisms, has been suggested to imply it may come from a parasite, whose rRNA evolved rapidly (López-García et al

2001). However, the gut contents protist sequence was not found in krill maintained in captivity. Krill captured in the same regions as those analyzed for *in-situ* feeding were maintained in captivity for 10 days in filtered sea water fed known species of cultured phytoplankton or artemia larvae. PCR amplification of gut DNA extracts of these krill using primers amplifying specifically this uncultured protist gave no detectible amplification. This suggests that the uncultured protist is not a gut parasite or symbiont, as such parasites and symbionts would be expected to remain present in the guts of captive krill. Additionally, this sequence was found in mud and water filters, so for this sequence to come from a parasite it would have to be a parasite with ubiquitous free-living forms.

This gut contents protist could be a parasite on or in prey consumed by *Meganyctiphanes norvegica*. However, this protist was found at every station sampled, and no other prey item was consistently detected, suggesting that the sequence does not belong to a copepod specific parasite. Cannibalism is not detectible using the DNA methods in this study, thus the protist sequence could be a krill parasite. Ectoparasites noted in previous studies of *M. norvegica* include a dinoflagellate, *Staphylocystis racemosus*, on the carapace, and a suctorian found on pleiopods (MacDonald 1927). For this gut contents parasite to be consistent with observations here, it would also need to have a free living form found throughout the water column and in sediments. Cannibalism has been observed in *M. norvegica*, but is not thought to form a major component of the diet (Fisher and Goldie 1959, Lass et al 2001). Thus this explanation also appears unlikely

This uncultured protist may have been filtered by krill from the water column. The sequence was obtained from filters of surface water in Georges Basin and Wilkinson Basin. This indicates that the sequence, and the organism from which it originates, were present in surface waters of the region where krill for this study were collected. It also provides a minimum estimate on the particle size associated with this sequence. The filters used in this study were 0.5 μm membrane filters, and the sequence was retained on these filters. Thus, either the organism containing this sequence is larger than 0.5 μm in some dimension, or it was particle-associated. However, the species specific primer approach used to determine the presence or absence of this sequence in the surface waters is extremely sensitive and non-quantitative, thus the sequence may be in low abundance in these waters. A low abundance of this sequence in surface waters is also suggested by the absence of this sequence from clone library studies of surface waters, including such studies in this region (Savin et al 2004). Additionally, this uncultured protist sequence was found in krill guts at every station, whereas phytoplankton was found in krill guts at only three of the eight stations. If krill were actively filtering sufficient volumes of water to consume this protist, one might expect that they would additionally be consuming phytoplankton.

Krill may have obtained this uncultured protist while feeding on or near the sediment interface. The sequence of this protist has been obtained from sediments of Georges Basin and Wilkinson Basin. To minimize the inhibitory effects of humics, these extractions contained very little starting material and were diluted ten to a thousand fold before PCR. This suggests that the krill gut content uncultured protist

may be relatively common at the sediment interface. All of the BLAST hits showing alignment with more than 70% of the krill gut uncultured protist sequence come from studies of sediment, with the exceptions of: a *Euphausia superba* gut contents item, a benthic bivalve gut contents item, and a study of Weddell Sea deep water (below 2,000 meters), all of which could be expected to contain some amount of sediment, consumed by the organisms, or re-suspended into the near bottom waters. Previous studies have observed *E. superba* feeding on the sediment interface *in-situ*, and *Meganyctiphanes norvegica* has been observed to re-suspend sediment and feed on this mud cloud in captivity (Clarke and Tyler 2008, Hamner and Hamner 2000, MacDonald 1927). Detritus has been observed as a gut contents item *in-situ* (Dalpadado et al 2008, Lass et al 2001, Fisher and Goldie 1959), It has been suggested that debris and detritus may be important components of *M. norvegica*'s diet, "the large amounts usually found indicated that this material probably forms the bulk of the diet" (Fisher and Goldie 1959). Thus it may be that this sequence is indicative of *M. norvegica* feeding on the sediment interface.

The relative abundance of different OTUs found in the clone libraries is not considered to be representative of the proportion of the diet made up by the respective prey items. PCR is known to have some degree of innate bias in the amplification of mixed samples (Dawson and Pace 2000). Slight differences in concentration can be greatly amplified, and stochastic effects can alter the proportions of relatively infrequent sequences. Cloning is also known to bias the proportion of different sequences in a sample. Plasmids preferentially take up the smallest available PCR inserts, and the *Gast* amplicons of the 18S gene is somewhat size variable by species,

with, for example, *Meganycitiphanes norvegica* approximately 15 bp longer than *Thalassiosira weissflogii* for the same PCR amplicon. Previous work with clone libraries of krill gut contents DNA suggest proportions of different OTUs are not necessarily the same as proportions ingested of different organisms (Passmore et al 2006).

The data available for prey field mesozooplankton helps to explain some of the distributions in krill prey items observed. However, since only 4 of the 13 gut contents items found are mesozooplankton, and krill gut contents data obtained here is qualitative, but not quantitative, a thorough analysis of selectivity is not possible. The lower overall mesozooplankton abundance in winter may explain lower krill gut fullness in winter. *Centropages* sp. was consumed only at station 3, where these copepods (*C. typicus* and *C. hamatus*) made up a greater proportion of the available prey (27%) than at other stations, although in absolute abundance, nearly 2x greater concentrations occurred at station 4 where *Centropages* sp. was not found in krill gut contents. *C. hamatus* was only found at station 3 of all stations sampled. Because sequence data is not available for *C. hamatus*, these two congeners cannot be differentiated as krill gut contents items, however, they are morphologically similar, and thus seemingly unlikely to be differentiated by *M. norvegica*. Prey field data also explains the apparently anomalous feature of krill not consuming *Calanus finmarchicus* at station 7. *C. finmarchicus* was in much lower abundance there than at other stations sampled, with only 1 copepod m⁻³ there, as compared to 77 to 370 copepods m⁻³ at other stations sampled where krill did consume *C. finmarchicus*. It is interesting to note that krill consumed *T. democratica* at station 120 where they were

not particularly abundant, but did not consume salps at other stations where they were more abundant, and more abundant relative to other available mesozooplankton.

The method employed in this study of PNA mediated PCR followed by cloning and sequencing has been successful in identifying a variety of prey items consumed by *Meganyctiphanes norvegica in-situ*. The advantages of this technique over other methods of gut contents analysis, including minimizing *a-priori* assumptions, and detecting morphologically indistinct prey are clear from these results. Gut contents items found in this study included soft bodied organisms not previously thought to be important to the diet of *Meganyctiphanes norvegica*, including the salp *Thalia democratica*, and uncultured protists. These items would have been difficult or impossible to detect using traditional methods of gut contents microscopy, as they contain no hard parts which would be identifiable after maceration by the krill. Additionally, as they were not expected to be prey items, they likely would not have been detected using more targeted gut contents studies, such as those employing group or species specific primers.

However, this method does currently have one major draw-back; it does not offer quantitative information on the relative amounts of different prey items consumed. The method could be made more quantitative in a variety of ways. qPCR could be applied to the sequences found to be present at many stations. Some of the newest sequencing technologies based on massively parallel independent sequencing reactions are considered to be quantitative, with abundances of sequences obtained representative of the abundance of the sequence in a starting sample. Incorporating a PNA probe into massively parallel sequencing techniques could potentially be used to

obtain quantitative sequence information directly from krill guts for all prey items consumed. One caveat to increased quantification is the potential for bias from variations in 18S copy number per cell, and differences in digestion rate for different prey types.

This method has provided interesting insights into the *in-situ* feeding of *Meganyctiphanes norvegica* in the Gulf of Maine, both confirming the importance of *Calanus finmarchicus* as a prey item, and suggesting the potential importance of protists to *M. norvegica* feeding. The method could be applied to any small organism whose feeding is poorly known, and offers the potential for new insights into feeding behaviors, particularly feeding on small, soft bodied, or cryptic organisms.

Figure 1: Locations of stations where *Meganyctiphanes norvegica* was sampled for *in-situ* gut contents analysis. Filled circles – August stations, open circles – February stations

44°30'N

42° 30

Stations:

● August 2008

● February 2009

Figure 2: qPCR amplification of *Meganyctiphanes norvegica* and *Thalassiosira weissflogii* with and without krill PNA. black diamonds-without PNA, grey squares – with PNA, A) *M. norvegica* 18S at 10^8 copies μl^{-1} B) *T. weissflogii* 18S

A.

B.

Figure 3: Abundances of mesozooplankton potential prey for stations where krill gut contents were analyzed (data from NMFS)

Prey Field Abundances

Data from the Ecosystem Monitoring Program of the Northeast Fisheries Science Center (NOAA)

Figure 4: Krill had greater gut fullness in summer than in winter ($p < .01$)

Seasonal variation in Gut Fullness

Figure 5: Mesozooplankton prey consumed by krill at each of the stations sampled

- ★ *C. finmarchicus*
- *Centropages* sp.
- ▲ *Copepod* sp.
- *T. democratica*

Stations:

- August 2008
- February 2009

Figure 6: Phytoplankton prey consumed by krill at each station

★ *Phytoplankton* sp.

■ *Green alga* sp.

◆ *Prorocentrum* sp.

Stations:

● August 2008

● February 2009

Figure 8: Protist prey consumed by krill at each station sampled

- Protist sp. A
- Protist sp. E
- Protist sp. F
- Protist sp. G
- Heterotrophic Alveolate sp. I
- Heterotrophic Alveolate sp. J

Stations:

- August 2008
- February 2009

Figure 9: Krill gut contents in the Gulf of Maine A) total sequences B) presence at different stations

A)

B)

Table 1: Sequences, names, references and annealing temperatures of primers and PNA. Position on 18S is referenced to *Meganyctiphanes norvegica* sequence (GenBank accession number GU595169).

Primer	Sequence	Annealing Temp	Position on 18S	Reference
Euk A	AACCTGGTTGATCCTGCCAGT	60	<0	Medlin 1988
Euk B	GATCCTTCTGCAGGTTACCTAC	60	>1747	Medlin 1988
Gast F	GGCTTAATTTGACTCAACRCG	58	1165 - 1185	Gast et al. 2004
Gast R	GGGCATCACAGACCTG	58	1433 - 1448	Gast et al. 2004
M13 F	GTTTTCCCAGTCACGAC	58	N/A	Messing 1983
M13 R	CAGGAAACAGCTATGAC	58	N/A	Messing 1983
OTU A sp. F	CGGGAAACCTTACTAGGGTAAG	56	1184 - 1205	This study
OTU A sp. R	TCACAGACCTGATTTAGCCCG	56	1423 - 1443	This study
Krill PNA	CGTCGGGTTGTCTTG	67	1338 - 1352	This study

Table 2: Sequence distance matrix comparing PNA probe with potential prey items

Table 3: Prey sequences obtained, sources, and GenBank accession numbers

Organism	Source	GenBank ID
<i>Calanus finmarchicus</i>	Damariscotta Bay	
<i>Centropages typicus</i>	Narragansett Bay	GU594639
<i>Metridia lucens</i>	Georges Bank	GU594642
<i>Microcalanus pusillus</i>	Georges Bank	GU594645 GU594646
<i>Pseudocalanus</i> sp.	Georges Bank	GU594644
<i>Oithona</i> sp.	Georges Bank	GU594643
Hyperiid amphipod sp.	Georges Bank	GU594647 GU594648
<i>Acartia tonsa</i>	Narragansett Bay	FJ422281
<i>Artemia salina</i>	San Francisco Bay Brand	GU594637
<i>Thalassiosira weissflogii</i>	CCMP 1048	GU594641
<i>Rhodomonas</i> sp.	CCMP 768	GU594640
<i>Heterocapsa triquetra</i>	CCMP 448	GU594638
<i>Meganyctiphanes norvegica</i>	Gulf of Maine	GU595169

Table 4: OTU sequences obtained, GenBank accession numbers, putative identities, and stations where found

OTU	GenBank #	Putative Identity	Stations where found
A	GU569090	Protist	7, 8, 7, 3, 4, 5, 2, 1
B	GU569089	<i>Calanus finmarchicus</i>	8, 7, 3, 4, 5, 2, 1
C	GU569088	copepod	5
D	GU569087	Copepod	2
E	GU569086	Protist	5
F	GU569085	Protist	7
G	GU569084	Protist	7
H	GU569083	Phytoplankton	4, 5
I	GU569082	Phytoplankton	5
J	GU569081	Phytoplankton	4
K	GU569080	<i>Prorocentrum</i> dinoflagellate	7
L	GU569079	Phytoplankton	5
M	GU569078	<i>Thalia democratica</i> (salp)	5

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Appendix A: Background Information

The northern krill, *Meganyctiphanes norvegica*, is a pelagic zooplankton of great importance in North Atlantic ecosystems (Båmstedt and Karlson 1998, MacDonald 1927, Fisher and Goldie 1959). *M. norvegica* plays a key role as prey for many diverse organisms in the region, including fish, seabirds and whales (Båmstedt and Karlson 1998, Cotté and Simard 2005). *M. norvegica* may also be an important predator on smaller plankters in the region (Båmstedt and Karlson 1998, Kaartvedt et al 2002). In this study *M. norvegica* feeding *in-situ* in the Gulf of Maine is investigated using DNA analysis of gut contents.

Meganyctiphanes norvegica is a euphausiid crustacean, and is fairly large for a zooplankton, 40 to 44 mm in length, with no sexual dimorphism (MacDonald 1927, Thomasson et al 2003). *M. norvegica* is thought to live between 2 and 3 years, reaching sexual maturity after year one, with spawning occurring in the summer, April through October, although this varies over their geographic range (Everson 2000).

Meganyctiphanes norvegica are bioluminescent, with 10 photophores along the dorsal side, one between each pair of pleiopods, 4 on the thorax, and two below the eyes (MacDonald 1927, Pers. obs.). *M. norvegica* bioluminesce bright blue, either in a single flash or repeated flashes when disturbed, however the extent of this behavior is variable between individuals (MacDonald 1927, Pers. obs.).

Meganyctiphanes norvegica is a strong swimmer, to the extent that it may be more appropriately termed a micronekton (Cotté and Simard 2005, Thomasson et al 2003). Maximum swimming speed has been measured at 15 cm s^{-1} , or about 4 body

lengths per second (Cotté and Simard 2005). Krill are negatively buoyant, and must actively swim to maintain their position in the water column (Thomasson et al 2003). Males and females have similar swimming capacity, however females show a higher pleiopod beat frequency (Thomasson et al 2003). Female pleiopod beat frequency averages 7.4 Hz, and male pleiopod beat frequency averages 6.4 Hz, for 37 mm length individuals (Thomasson et al 2003). *M. norvegica* form discrete, high density swarms estimated between 9,000 and 770,000 krill m⁻³ (Everson 2000).

Meganyctiphanes norvegica exhibit pronounced diel vertical migration (DVM), spending daylight hours at depth and night in the surface waters (Thomasson et al 2003). This migration behavior is believed to be a compromise between exploiting the prey rich surface waters and minimizing predation by avoiding high light environments (Lass et al 2001). These migrations typically transit 500 m, but can be in excess of 1 km vertically (Virtue et al 2000). In some relatively shallow areas, such as the Gulf of Maine, *M. norvegica* encounters the sea bed during its migrations, as evidenced by the occasional finding of *M. norvegica* in sediment samples, and in the guts of benthivorous fishes, such as the velvet belly lantern sharks, *Etmopterus spinax* (Everson 2000, MacDonald 1927)

Meganyctiphanes norvegica is widely distributed in a variety of habitats throughout the North Atlantic. Along the coast of North America, it is found from around Cape Cod at 40° N up along the continental margin through the Gulf of Maine, up into the Gulf of St. Lawrence, and along the continental slope around Labrador and West Greenland to 90° N (Everson 2000, MacDonald 1927). In the Eastern side of the Atlantic, *M. norvegica* ranges from the marginal ice zone in the Western Barents Sea

through the Norwegian Sea, North Sea, and Skagerrak Sea, down along the coast of France throughout the Mediterranean and into the Aegean and Marmora (Everson 2000, MacDonald 1927, Dalapadado et al 2008). *M. norvegica* is a shelf species, and while distributed around the edges of the North Atlantic, has not been found in the central part of the basin (MacDonald 1927).

Assessing the abundance and biomass of *Meganyctiphanes norvegica* is challenging due to net avoidance, and patchiness due to schooling and swarming behaviors, and diel vertical migration, however some limited estimates are available (Båmstedt and Karlson 1998). In Kosterfjorden, Sweden, krill biomass ranged from zero to 1,600 mg dry weight m^{-2} and was seasonally variable, with highest biomass observed in August/September (Båmstedt and Karlson 1998). In the Laurentian Channel krill density in patches ranged from approximately 25 grams m^{-3} to 500 grams m^{-3} , about 1,500 *M. norvegica* m^{-3} and was correlated with tidal forcing (Cotté and Simard 2005). The density of krill swarms in the Bay of Fundy was estimated as ranging from 78 to 780 grams m^{-2} , with a total *M. norvegica* biomass in the Bay of Fundy and Jordan Basin region of 15,000 tons (Everson 2000).

***Meganyctiphanes norvegica* as prey**

Meganyctiphanes norvegica is an important prey item for many fish, whales and seabirds of the Gulf of Maine region. Hake, including Silver and Red hake on Georges Bank, feed on *M. norvegica*, and it is considered to be a preferred food for them (Everson 2000, MacDonald 1927). Hake have even been suggested to follow *M. norvegica* prey in their daily vertical migrations (MacDonald 1927). *M. norvegica* is

considered to be one of the most important food sources for Atlantic herring, on both sides of the basin, and areas of high krill concentration are thought to attract herring (MacDonald 1927, Everson 2000). Capelin and Atlantic mackerel also consume *M. norvegica* (Cotté and Simard 2005, Everson 2000 p. 191). The physonect siphonophore, *Nanomia cara*, was shown to prey on *M. norvegica* in the North Atlantic (Rossi et al 2008).

Whales feed extensively on krill. Fin whales, *Balaenoptera physalus*, have been seen to consume almost exclusively *M. norvegica*; 63 of 67 stomachs examined were full of krill (Brodie et al 1978). With a stomach volume of 550 liters this represents roughly half a metric ton of krill consumed to fill a whale stomach once (Brodie et al 1978). In the north Atlantic rorqual whales feed on *M. norvegica* and make extensive use of areas in which the krill are concentrated, such as the Bay of Fundy (Cotté and Simard 2005). Whales depend on the schooling behavior of krill to concentrate them to levels at which whales can successfully meet their metabolic requirements feeding on such small organisms. It was calculated that *B. physalus* feeding on *M. norvegica* off Nova Scotia would need to swim at 900 km per hour constantly to fill their stomachs on “average” densities of euphausiids! (Brodie et al 1978).

Seabirds also make use of *Meganyctiphanes norvegica* as prey. In the Western Atlantic the sooty shearwater, *Puffinus griseus*, takes as prey mainly krill (Everson 2000 p. 192). *M. norvegica* is an important prey item for Leach’s storm petrel, *Oceanodroma leucorhoa* and the razorbill, *Alca torda*, feeds on *M. norvegica* and sculpins (Everson 2000).

Humans can also be considered predators on *Meganyctiphanes norvegica*. A fishery for *M. norvegica* was conducted in the Mediterranean in the 19th century, with the catch used for fishing bait (Everson 2000, p. 228). In the North Atlantic an exploratory fishery in the Laurentian Channel in 1995 caught 6.3 tons of *M. norvegica*, and the fishery in this region is estimated at a potential value of 3.75 million Canadian dollars (Nicol and Endo 1997). The proposed fishery in this area would catch krill for freezing and freeze-drying for home and public aquarium food, aquaculture feed, and as a flavourant for use in food for human consumption (Everson 2000).

***Meganyctiphanes norvegica* as predators**

Meganyctiphanes norvegica is omnivorous, consuming a variety of phytoplankton, zooplankton and other prey, but carnivory is thought to make up the majority of the diet, more so than for other species of Euphausiid (Torgerson 2001). *M. norvegica*'s mandibles with sharp *pars incivia* may be an adaptation to carnivorous feeding, (Båmstedt and Karlson 1998). The degree to which krill are carnivorous may be seasonally and spatially variable, depending on the available prey and the dietary needs of the krill. In spring and summer, when phytoplankton is more abundant, carnivorous feeding is relatively less important, with carnivory accounting for 23% of the diet in the coastal waters around Norway in the summer time (Kaartvedt et al 2002, Båmstedt and Karlson 1998).

Copepods are by far the most common zooplankton prey of *Meganyctiphanes norvegica*. A variety of copepods have been found as gut contents of *M. norvegica*

throughout its geographic range in Loch Fyne, the Barents Sea, Oslofjorden, the Clyde Sea, and the Kattegat Sea (MacDonald 1927, Dalpadado et al 2008, Kaartvedt et al 2002, Lass et al 2001). *Calanus finmarchicus*, is the most common copepod found as a krill gut contents item (MacDonald 1927, Dalpadado et al 2008, Kaartvedt et al 2002, Lass et al 2001). *Eucheata norvegica*, *Paracalanus*, *Pseudocalanus elegans*, *Pseudocalanus* spp., *Temora longicornis*, and *Calanus* spp., were also relatively common, with *Oithona* spp, *Oithona helgolandicus*, *Acartia* spp., and *Acartia clausii* taken as prey less frequently, mainly when particularly abundant in the water column (MacDonald 1927, Dalpadado et al 2008, Kaartvedt et al 2002, Lass et al 2001. In addition to adult copepods, copepod eggs were a common gut contents item in the Kattegat Sea in summer (Lass et al 2001). In incubation experiments, McClatchie showed that *M. norvegica* could not meet its metabolic needs feeding on phytoplankton alone, nor when feeding on small copepods such as *Acartia* spp. or *Pseudocalanus* spp., suggesting *M. norvegica* relied on high concentration $2.265 \text{ mg DW L}^{-1}$, about $190,000 \text{ } C. \text{finmarchicus}$ -sized copepods m^{-3} , patches of large copepods, such as *Calanus* spp. or *Centropages* spp., or was unable to feed efficiently in his small incubation chambers (McClatchie 1985). All of these studies point to the importance of copepods to the diet of *M. norvegica* throughout its geographic range.

Calanus finmarchicus is probably the most important single prey species for *Meganyctiphanes norvegica*. *C. finmarchicus* is a relatively large and oil rich copepod which is abundant and biomass dominant in many of the same areas of the North Atlantic where *M. norvegica* is found, and has commonly been observed as a gut contents item (Durbin et al 2003, MacDonald 1927, Båmstedt and Karlson 1998, Lass

et al 2001). *C. finmarchicus*, with a prosome length distribution centered around 1.00 mm, stage three copepodites (C3), made up 64% to 100% of the copepods consumed by *M. norvegica* in the coastal waters of Norway (Båmstedt and Karlson 1998). *M. norvegica* was calculated based on measured *in-situ* feeding rates to consume .3 to 6.4% in Norway and 1.3% to 2.7% in the Gulf of Maine of the total *C. finmarchicus* biomass daily (Båmstedt and Karlson 1998, Thal 2004). Tintinnid lorica have also been seen as *M. norvegica* gut contents (Dalpadado et al 2008).

Cannibalism is another form of carnivory which has been observed in *M. norvegica*, though to a fairly limited extent. Compound eyes identified as belonging to euphausiids were found relatively infrequently, and more commonly in winter than summer in the guts of *M. norvegica* in Loch Fyne (MacDonald 1927). Euphausiid ommatidia were common in krill guts in both summer and winter in the Clyde and Kattegat Seas (Lass et al 2001). *Euphausia superba* have been observed to hold and eat krill carcasses (Hamner and Hamner 2000). It is not clear why or under what conditions krill feed cannibalistically, though cannibalism has been suggested to occur when other food is relatively unavailable, and krill are at high densities (Lass et al 2001).

Photosynthetic prey can be seasonally important for *M. norvegica*, and grazing on phytoplankton may provide significant carbon and nutrients to the krill, particularly during the spring bloom period. Phytoplankton were a major component of *M. norvegica* diet in spring (March and May) in Oslofjorden, contributing within an order of magnitude as much carbon as carnivory (Kaartvedt et al 2002). It was concluded

that “*M. norvegica* is very versatile in its ability and motivation to exploit algal food” (Kaartvedt et al 2002).

Diatoms have been observed as prey in the Barents Sea, Clyde and Kattegat Seas and Loch Fyne (Dalpadado et al 2008, Virtue et al 2000, MacDonald 1927, Fisher and Goldie 1959). Diatoms, especially *Thalassiosira* spp., and less commonly pennates, including *Fragilariopsis* spp., *Pseudonitschia* spp., and *Navicula* spp., were the most numerous photosynthetic prey in *Meganyctiphanes norvegica* guts in the Barents Sea (Dalpadado et al 2008). In Loch Fyne a variety of diatoms were found as prey items in gut contents (MacDonald 1927, Fisher and Goldie 1959). *Thalassiosira nordenskioldi*, *T. gravid*, *Coscinodiscus* spp., and *Paralia* spp. Were the most commonly consumed, while *Fragillaria*, *Navicula* spp., *Pleutosigma*, and *Nitschia* spp. were eaten occasionally (MacDonald 1927, Fisher and Goldie 1959).

Dinoflagellates may also form part of the diet of *Meganyctiphanes norvegica* in certain areas and seasons. Flagellates were found to be important in the diet of *M. norvegica* in the Mediterranean, but not in the Clyde or Kattegat Seas (Virtue et al 2000). In Loch Fyne dinoflagellates were found as gut contents of *M. norvegica* mainly when they were abundant in the environment, particularly in the late summer and autumn, with a seasonal cycle evident in the species of dinoflagellates consumed with *Ceratium*, *Dinophysis*, *Phalacroma*, *Prorocentrum* and *Peridinium* consumed in summer, and *Peridinium pellucidans*, *P. depressum*, *Phalocronus* spp., and *Heterocapsa* spp., were consumed in winter (Fisher and Goldie 1959, MacDonald 1927). Thus, while not as common a krill prey as diatoms, dinoflagellates may also be seasonally important.

In addition to zooplankton and phytoplankton, *Meganyctiphanes norvegica* have been observed to consume a variety of other, less common, prey, including marine detritus, benthic prey, and terrigenous prey. Detritus can be consumed in the water column, either as small particulate detritus, or from aggregates or marine snow. In the Barents Sea microscopic examination of *M. norvegica* gut contents indicated krill had consumed detritus (Dalpadado et al 2008). Krill in the Clyde and Kattegat Seas also consumed detritus, as indicated by analysis of gut contents lipids, with high levels of branched fatty acids and stanols, characteristic of bacteria and their breakdown of sterols in detritus, found in all krill stomach samples (Lass et al 2001). Fisher and Goldie found that amongst the *M. norvegica* of Loch Fyne organic debris and inorganic detritus were found in 60% to 100% of the krill samples each week, and it was concluded that “the large amounts usually found indicated that this material probably forms the bulk of the diet” (Fisher and Goldie 1959).

Meganyctiphanes norvegica has been observed to consume terrigenous prey, such as pieces of terrestrial plants, insects, or insect eggs, surprising gut contents in a largely oceanic species like *M. norvegica*. Terrigenous prey has been particularly noted in the deep fjords where *M. norvegica* exists relatively close to land, but has also been observed in *M. norvegica* in open seas, such as the Kattegat. In the Cumbrae deep in Loch Fyne terrigenous plant debris was found to be an important prey item in krill diets; krill abundance was correlated with detrital abundance, and most or all of the krill examined had consumed chiefly detrital decaying vegetable material, most commonly fern sporangia (MacDonald 1927). Later work in the same region

confirmed these observations with fern sporangia found in up to 25% of the krill guts sampled (Fisher and Goldie 1959). Fern sporangia were most common as prey in the fall, and more commonly at night than during the day, suggesting they were consumed in the water column (Fisher and Goldie 1959). “The fern sporangia found in small numbers in the stomachs of *M. norvegica*, indicate that there may be an inkling of truth in the long held belief that the bracken-clad hills bordering the loch nourish Loch Fyne herring, one of the predators of *M. norvegica*, and impart to them and the kippers made from them their renowned high quality” (Fisher and Goldie 1959). In addition to fern pieces, dipteran egg membranes were found as *M. norvegica* gut contents, most commonly in spring, when they were found in up to 35% of the guts sampled, coinciding with the breeding season of these insects (Fisher and Goldie 1959). In the Kattegat, a much more open ocean like environment, pine pollen was found to be a common gut contents item in the summer (Lass et al 2001). Pine pollen was present in all sampled krill guts in summer, and was fairly abundant in the guts (Lass et al 2001). This observation was further substantiated by the lipid analysis of the gut contents which showed sterol profiles typical of higher plants (Lass et al 2001).

While detritus can occasionally be abundant in the water column or as neuston, it is also present at the sediment surface. The above studies did not differentiate between detritus captured in the water column, and that potentially grazed from the sediment surface. Krill are considered to be mainly filter feeders, consuming prey in the water column, and they are well adapted to such a lifestyle with their filtering basket of thoracic limbs. However, some studies have observed more benthic feeding behaviors in *Meganyctiphanes norvegica* and other krill species. *M. norvegica*

maintained in clear tanks containing natural sediment were found to feed on this sediment (MacDonald 1927). They were observed to lie flat on the sediment and beat their pleiopods, thus raising a cloud of re-suspended sediment and then filtered and consumed particles from this sediment cloud by creating a feeding current, (MacDonald 1927). *Euphausia superba* have been observed to consume benthic materials *in-situ*. They have been seen to use their extended thoracic appendages to graze on benthic materials (Hamner and Hamner 2000). ROV observations of *E. superba* have seen them actively feeding on benthic material down to abyssal depths of 3,000 meters near Marguerite Bay, and the researchers “frequently observed a characteristic behavior whereby the krill would nosedive into the sediment and then rise up and feed actively on the re-suspended sediment. Typically krill would dive head first from a height of less than 1 meter above the seabed and at a fairly steep angle of 30° – 50°” (Clarke and Tyler 2008).

It is an open question to what extent *Meganyctiphanes norvegica* is a selective feeder. Some authors have suggested that *M. norvegica* consumes whatever prey is available, whereas other studies have suggested *M. norvegica* feeds selectively on preferred prey. Selectivity may be based on detection, with larger organisms and more motile organisms easier to detect, or it may represent selection for higher food quality. Suggestions that *M. norvegica* is not a selective feeder tend to be based on similarities between krill diet and water column plankton abundances, “*M. norvegica* are opportunists, feeding on whatever they may find” (Dalpadado et al 2008). In incubation studies of *M. norvegica* using a mixture of copepod species, 75% *Centropages typicus*, 15% *Calanus finmarchicus*, and 10% *Pseudocalanus* sp.,

varying in size and swimming behaviors no selection was found (McClatchie 1985). Some studies have seen krill gut contents mirror changes in water column abundances over time or space leading to suggestions that krill will readily consume whatever is available; “the northern krill can switch between herbivory and carnivory quite opportunistically, depending on food availability” (Lass et al 2001). One study which illustrated the non-selective feeding behavior of krill was aimed at assessing gut evacuation rate in *Euphausia superba* and offered krill phytoplankton-sized charcoal particles in an effort to create a continuous feeding environment while observing the evacuation of photosynthetic prey, and surprisingly “Charcoal particles are readily ingested by krill and gradually displace previously digested food” (Perissinotto and Pakhamov 1996). As charcoal particles are clearly a poor food for krill and contain little if any useable nutrients, this consumption of inert particles suggests that krill are not picky eaters, at least in the absence of preferred prey.

Other studies have suggested *Meganyctiphanes norvegica* is indeed a selective feeder, and consumes prey in different proportions to those found in the environment. Selectivity was seen for *Temora longicornis* and to a lesser extent for *Calanus* spp., and against *cyclopoid* copepods (Kaartvedt et al 2002). Several possible explanations were offered for this selection for *Temora*, swimming behavior, patchiness, and pigmentation (Kaartvedt et al 2002). *Temora* swims continuously, which would make it relatively easy for a krill to detect, is darkly pigmented, which makes it more visually conspicuous and is distributed particularly patchily, which while making the potential searching distance for a krill greater, might optimize foraging by allowing for rapid feeding once a patch was located (Kaartvedt et al

2002). Selection against *cyclopoids* was suggested to be due to their relatively slow and torpid behavior in comparison with calanoids in addition to their fairly small size (Kaartvedt et al 2002). *Para/Pseudocalanus* were the preferred and selected prey of krill studied in the Kattegat and Clyde Sea areas, with *Temora* as the second choice, and consumed when *Para/Pseudocalanus* was below the levels required for optimal foraging efficiency (Lass et al 2001). *M. norvegica* is a visual predator, in addition to using mechanosensory reception (Torgerson 2001). Krill showed much higher predation rates under dim light than in total darkness, and the selective pressure on different copepod species was different in the light as opposed to dark regimes with *Metridia lucens* consumed significantly more than *Calanus* in the dark, because it tends to swim faster and more constantly (Torgerson 2001). This selectivity was still evident in dim light conditions, with *Metridia* consumed more than *Calanus*, but the difference was much less, suggesting that visual predation is both more efficient than mechanosensory predation, and also allows for the detection and capture of a wider range of prey (Torgerson 2001).

Results are mixed with respect to when during the diel cycle *Meganyctiphanes norvegica* does most of its feeding. The traditional hypothesis has been that krill feed actively during the night while in the zooplankton rich surface waters, and little if at all in the food poor deep waters where they spend their days. Some studies confirm this hypothesis, finding that *M. norvegica* consumes more during the night than the day (Båmstedt and Karlson 1998, Lass et al 2001). These studies found that the copepod mandibles found in krill guts belonged to copepods living in the surface waters, and therefore must have been consumed at night (Lass et al 2001). However,

they also note with caution that gut fullness may not be a measure of recent feeding activity, as when feeding activity decreases or ceases digestion rate may also decrease, such that krill captured at deep day-time depths contained many copepod mandibles, but these mandibles were of surface dwelling copepods, and thus were the remains of the previous nights feeding (Lass et al. 2001). Other studies, such as those finding benthic detritus as a major food item, suggest daytime feeding may be significant, at least in relatively shallow areas where *M. norvegica*'s diel vertical migration takes it to the sediment interface, and have concluded that *M. norvegica* do not show consistent diel rhythms in feeding activity (Fisher and Goldie 1959).

Measuring krill feeding

Measuring feeding in very small pelagic organisms such as zooplankton is challenging, and four basic approaches and varying methodologies within them have been taken to gain an understanding of krill feeding *in-situ*, direct observation, incubations, biomarkers, and gut contents analysis. All of these approaches have their advantages and limitations. Direct observation is challenging in oceanic environments, Incubations may induce behaviors different from those found *in-situ* and are difficult to scale appropriately, biomarkers suffer from poor temporal and prey type resolution, and gut contents analysis are often limited in prey type and prey resolution and must contend with partially digested samples.

Direct observation of krill feeding has been done using ROVs, scuba divers and surface based observers. ROV observations discovered Antarctic krill, *Euphausia*

superba, feeding on benthic phytodetritus at abyssal depths, as deep as 3,000 m (Clarke and Tyler 2008). Scuba divers have observed *E. superba* feeding on ice algae (Hamner and Hamner 2000). Surface based observers noted a previously undiscovered feeding behavior in *E. superba*, in which the krill swim just below the surface and “hold one branch of each antennules out of the water. Floating particles are flicked out of the surface film for inspection and sometimes eaten” (Hamner et al 1983). While direct observation has the potential to offer much information on krill feeding, it can be difficult to see exactly what the krill are eating, it is uncertain to what extent the presence of observers perturbs the natural behaviors of the krill, results tend to be non-quantitative, and the technique is labor intensive, with limited spatial and temporal scope.

Incubations have been used to look at several aspects of krill feeding. Incubations have several advantages. They are highly controlled, with prey available, light, temperature, feeding time, and pre-feeding treatment all controlled by the investigator. Incubations can also be very quantitative, individual prey can be counted before and after a certain time of krill feeding to determine exactly how many of each prey item the krill consumed during that time. McClatchie conducted a series of feeding experiment incubations in the early 1980s looking at feeding rate and selectivity of *Meganyctiphanes norvegica* on a variety of copepod and phytoplankton prey in 4 liter containers (McClatchie 1985). When fed different concentrations of the diatom *Thalassiosira weissflogii*, *M. norvegica* fed at low rates and could not meet its metabolic needs feeding on this phytoplankton alone (McClatchie 1985). In incubations with a mixture of copepods, *M. norvegica* fed at rates correlated with prey

concentration, and showed no selectivity amongst different copepods, despite order of magnitude size differences (McClatchie 1985). Torgerson investigated the extent to which krill are visual predators using illuminated and dark incubations in 50l slightly conical tanks (Torgerson 2001). He found that krill were indeed visual predators and fed at significantly higher rates under low light than under total darkness. Torgerson also found differences in the rates of different copepod prey under different light conditions, with *Metridia* relatively more susceptible than *Calanus* in the dark as compared to illuminated conditions, which may be due to differences in swimming behavior, and hence differences in the ability of krill to detect the copepod mechanosensorially (Torgerson 2001). All of Torgerson's experiments contained 40 *Calanus* spp. and 40 *Metridia longa*, for a total copepod concentration of 1.6 copepods l^{-1} , less than the lowest concentration used by McClatchie where he found fewer than 1 copepod $krill^{-1} hour^{-1}$, yet Torgerson measured feeding rates of 3 (dark) to 7 (dim light) copepods $krill^{-1} hour^{-1}$, a rate which McClatchie measures at a copepod concentration around 50 copepods $liter^{-1}$ (Torgerson 2001, McClatchie 1985). This discrepancy highlights one of the potential problems with incubation experiments: captive krill may not exhibit the same feeding behaviors as krill *in-situ*. Krill may be damaged in capture and handling, resulting in lower feeding rates than *in-situ*, and potentially shift to easier to capture prey. Light levels in incubations may not mirror those found *in-situ*, which would also affect the feeding behavior of the krill, potentially changing the detectability of different prey items. Additionally, krill are relatively large and mobile organisms, potentially able to cover large distances and

seek out patchy prey. Thus the prey field presented to the krill in a relatively small incubation may not be representative of the prey field available to them *in-situ*.

Biomarkers, including stable isotopes and fatty acids, are molecules in prey which are incorporated into the body of the predator, and can be measured to assess *in-situ* feeding on a variety of different prey. Stable isotopes were used on *Euphausia superba* to assess feeding on different prey in the West Antarctic Peninsula region (Schmidt et al 2003, Schmidt et al 2006). Carbon isotopes are used as an indicator of the source of original autotrophic production leading to a predator, and Nitrogen isotopes are used as a marker of trophic level, with $\delta^{15}\text{N}$ increasing by $\sim 3\%$ with each increasing trophic level (Schmidt et al 2003). The use of stable isotopes allowed for distinguishing between otherwise indistinguishable prey, notable the same species of diatom growing as ice algae or freely floating plankton (Schmidt et al 2003). However, the resolution of this technique can often be fairly low, with difficulties differentiating between different prey organisms of similar trophic level.

Fatty acids have been used to look at diet in *Euphausia superba* and *Meganyctiphanes norvegica*. Fatty acids have the additional benefit of being meaningful in terms of energy transfer, as lipids are important nutritional and storage molecules (Virtue et al 2000). In *E. superba* fatty acid analysis allowed for further information than was obtainable from stable isotopes or gut contents microscopy alone, and elucidated the role of weekly silicified diatoms and athecate heterotrophic dinoflagellates in the diet of Antarctic krill (Schmidt et al 2006). Two studies have looked at fatty acids in *M. norvegica* as a trophic marker. Rossi et al investigated planktonic trophic webs in the Gulf of Maine and Georges Bank region, and sampled

M. norvegica in Oceanographer Canyon (Rossi et al 2008). They found conflicting results, sums of fatty acids suggesting krill ate Bacillariophyceae, Dinophyceae and Prymnesiophyceae, but fatty acid ratios did not consistently support these interpretations (Rossi et al. 2008). High levels of the fatty acid 22:1 (n-11) were suggested to indicate krill feeding on *C. finmarchicus* (Rossi et al 2008). Fatty acids have been used to investigate regional differences in *M. norvegica* diet in the Eastern Atlantic, comparing krill from the Clyde Sea, Kattegat Sea, and Ligurian (a deep basin in the Mediterranean) (Virtue et al 2000). Similar to results from Oceanographer canyon, krill were found to rely heavily on copepod prey, as indicated by high levels of 22:1 and 20:1 fatty acids (Virtue et al 2000). One issue with fatty acids that may confuse interpretation is the ability of some higher trophic level organisms to synthesize fatty acids de-novo (Rossi et al 2008). Another potential drawback of the fatty acid analysis technique is the difficulty in determining the linkages of the markers. For example in the Clyde and Kattegat diatom fatty acid markers were found in krill, but it is unclear whether these diatoms were consumed directly by the krill, or whether these diatoms were consumed by copepods, and these copepods then consumed by the krill (Virtue et al 2000).

Biomarkers, both stable isotopes and fatty acids, integrate feeding over time. This can be seen as an advantage as it allows for a more average diet, and potentially more representative. However, this time integration may be a disadvantage in a spatially or temporally heterogeneous environment, such as areas with high seasonality or small scale spatial patchiness. The stable isotope baseline, the signature of the lowest trophic level, was found to be quite variable, with differences up to 10

‰ within only a few weeks time, as the seasonal bloom progressed (Schmidt et al 2003). While these temporal changes in the stable isotope signature were reflected in copepod stable isotopes, the krill, which integrate biomarker signals over longer time periods, did not show similar changes, and this baseline variability may have confused interpretation of krill diet (Schmidt et al 2003). Fatty acid profiles of potential prey can also vary spatially and temporally (Virtue et al 2000). For relatively large and mobile zooplankton such as krill, this may make it difficult to resolve what krill are consuming, and may mask any seasonal or day/night changes in diet.

Another potential difficulty in the use of biomarkers is the low prey type resolution, and complex interpretation of results in species rich ecosystems. Stable isotopes differentiate mainly the original source of carbon fixation and the trophic level of the organism. This may leave many unanswered questions in diverse and complex ecosystems where there may be a variety of different potential prey of roughly the same trophic level and original carbon source, but different behaviors and morphologies. This same relatively poor resolution affects fatty acid analysis, with resolution only to the level of diatoms vs. dinoflagellates (Rossi et al 2008). Interpretation of biomarkers may be particularly challenging in organisms such as krill which potentially feed at multiple trophic levels.

Gut contents analysis is one of the more direct approaches to understanding zooplankton feeding. Gut contents analysis represents a snap shot in time, what the krill consumed in the minutes or hours before it was caught. Several techniques have been applied to analyzing krill gut contents, including microscopic examination, gut pigments, lipids, antibodies, and most recently DNA.

The most commonly used of these techniques has been microscopic examination of gut contents, which involves dissecting the krill, and removing its gut contents, which are stained, and affixed to microscope slides and identifiable prey remains categorized and enumerated. After maceration and partial digestion by the krill, often the only identifiable gut contents are hard parts, such as copepod mandibles and diatom frustules. Microscopic examination of gut contents has been used to investigate feeding by *Meganyctiphanes norvegica* in the Marginal ice zone in the Barents Sea, in the Skagerrak (Norway), and in the Clyde and Kattegat Seas (Dalpadado et al 2008, Båmstedt and Karlson 1998, Lass et al 2001). Microscopy of gut contents offers some advantages over other techniques, most notably it can offer information about the size or life stage of prey taken, as opposed to simply the species or type, and may have resolution down to species level in low diversity environments (Båmstedt and Karlson 1998, Dalpadado et al 2008). Gut contents microscopy requires few *a-priori* assumptions about the type of prey taken, assuming mainly that the important prey items contain hard parts and are within the size range to be visible under compound light microscopy.

Potential drawbacks of gut contents microscopy include biases from krill consuming partial prey, difficulties detecting soft bodied or non-descript prey, biases from differences in prey digestibility, and difficulties identifying macerated prey. If prey enumeration is based on specific hard parts, such as copepod mandibles, and krill preferentially eat only the back end of copepods, or suck out the insides of copepods predation will be underestimated, whereas if krill preferentially decapitate their prey and consume only heads, mandible enumeration will over-estimate copepod predation

(Båmstedt and Karlson 1998). Different prey will have different detectibilities in gut contents microscopy, with organisms featuring many distinctive hard parts most identifiable and identifiable after the longest digestion time, and organisms with few or no hard parts difficult to detect (Dalpadado et al 2008, Haberman et al 2002). This may lead to overestimation of the importance of hard part containing species, and missing soft bodied prey, such as salps, naked pteropods, or athecate dinoflagellates. Identifying macerated and partially digested prey is one of the challenges of this technique, and often results in a significant proportion of the gut contents being classified simply as unidentifiable, green fluff, digested green or similar (Dalpadado et al 2008, Lass et al 2001, Båmstedt and Karlson 1998). In gut contents microscopy of *M. norvegica* in Scandinavian waters Lass et al found that “in most cases the major part (80 – 90% in the Clyde and 70% in the Kattegat in summer) of the stomach contents was unidentifiable”, and SEM imagery suggested this unidentifiable stuff was mainly fragments of centric diatoms (Lass et al 2001).

Gut fluorescence has been applied to detecting krill feeding on photosynthetic prey. In essence, the krill is dissected and the gut placed in an organic solvent, typically acetone, to extract pigments, and this extract is read on a fluorometer. Kaartvedt et al (2002) used gut fluorescence to quantify the relative contributions of photosynthetic and heterotrophic prey for *Meganyctiphanes norvegica* in Oslofjorden. The advantages are relative simplicity, and low cost. Potential disadvantages include, a limited range of detectible prey, limited prey type resolution, and high pigment destruction in the guts of krill. Pigment destruction in krill guts is especially high, among the highest recorded for zooplankton, and has been measured

at between 58.1% and 98.1%, so much of the prey signal may be completely lost due to digestion (Perissinotto and Pakhomov 1996).

Two other methods which have been applied infrequently to analyzing krill gut contents are antibodies and gut content lipids. Gut content lipid measures were combined with microscopy to investigate feeding in *Meganyctiphanes norvegica*: stomachs were dissected, lipids were extracted, and total lipids, sterols and fatty acid methyl esters were measured by gas chromatography (Lass et al 2001). While this method does not have particularly high prey type resolution, it does offer insight into some prey items which may not be detectable by microscopic examination of gut contents, such as bacteria or detritus (Lass et al 2001). An enzyme linked immunosorbent assay (ELISA) was used to detect *Euphausia superba* feeding on *Phaeocystis antarctica* (Haberman et al 2002). Antibodies were developed in rabbits specifically for *P. antarctica*, tested on lab reared krill, and then used to assess *in-situ* feeding of *E. superba* on *P. antarctica*, (Haberman et al 2002). Antibodies are very sensitive and able to detect even low abundance, and visually inconspicuous prey in krill guts. However, antibodies are time consuming and expensive to design, and results are difficult to replicate in different labs. Additionally, an antibody approach must necessarily include *a-priori* assumptions about what species, or groups the krill are consuming, and are likely to be limited to a small number of potential prey targets in any given study.

Dioxyribose nucleic acid (DNA) offers many advantages as a gut contents marker, and has been used in a few recent studies of feeding in *Euphausia superba* krill. As a universal information carrying molecule DNA can be used to detect any

living, or recently living, prey in krill guts. Interspecies variability in nucleotide sequence can be used to identify the species source of the DNA in krill guts.

Passmore et al investigated *Euphausia superba* feeding on diatoms using DNA from krill guts (Passmore et al 2006). 18S genes from all diatom DNA present in extracts from krill guts were amplified using a polymerase chain reaction (PCR) and diatom group specific primers (Passmore et al 2006). Primers can be designed to amplify species, or groups, with different levels of phylogenetic relatedness by taking advantage of the different regions of the DNA which show different levels of variation. Comparing the abundance and diversity of diatom species found by sequencing of gut contents DNA clone libraries, to that found by gut contents microscopy, showed similar species compositions, but in different proportions (Passmore et al 2006).

Martin et al investigated *Euphausia superba* feeding on a range of prey items around Anvers Island (Martin et al 2006). Krill gut contents DNA was amplified using a PCR reacting with universal eukaryote 18S primers designed to amplify any eukaryotic organism and then run through a denaturing gradient gel electrophoresis (DGGE) (Martin et al 2006) DGGE is a gel electrophoresis containing a gradient of formamide and urea used to separate DNA amplicons based on length and sequence (Martin et al 2006). They identified 26 different prey phylotypes, and sequenced many of these to determine their identity. However, issues with this technique included the necessity to dissect away all krill tissue before DNA extraction, co-migration of some bands, and multiple bands derived from the same prey sequence (Martin et al 2006).

The major technical difficulty in using DNA as a gut contents marker is the overwhelming quantity of predator DNA. There are a variety of possible techniques for approaching this problem and preventing the predator DNA from swamping any signal from the prey. Martin et al took the straightforward approach of dissecting away all of the krill tissue to extract DNA only from the gut contents (Martin et al 2006). However, this method is laborious, and may not quantitatively transfer all of the gut contents to the DNA extraction. Passmore et al used group specific primers which effectively ignored the krill DNA, as they are designed only to be complimentary to diatom 18S (Passmore et al 2006). An ideal technique would allow for the detection of all prey species, without in-depth dissection. One such technique is the use of Peptide Nucleic Acid (PNA).

Appendix B: Ethanol Precipitation Protocol

1. Place 25 μ l of PCR reaction (30 – 5 for gel) in a 1.5 ml labeled microcentrifuge tube
2. Add 2.5 μ l 3M Sodium Acetate and vortex
3. Add 55 μ l ice cold pure (~95%) ethanol and vortex
4. Store on ice for 10 minutes, or if more convenient in the freezer for up to 12 hours
5. Centrifuge for 10 minutes at top speed (1350) in microcentrifuge with hinge facing out
6. Carefully decant supernatant, use a sterile toothpick to release surface tension and drain last droplets, tap upside down on paper towel
7. Add 500 μ l 70% ethanol and rock once gently
8. Centrifuge for an additional 5 minutes at top speed again hinges out
9. Remove supernatant as above
10. Leave open in the clean hood for 20 min to 45 minutes to evaporate last traces of ethanol, check by sniffing
11. Dissolve the DNA in 50 μ l pure H₂O by running along the side by the hinge, vortex

Appendix C: Schematic of PNA-PCR reaction

Appendix D: Krill Length-weight relationship

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