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Molecular detection of algal prey in copepod guts and fecal pellets

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Abstract

The ability to obtain information about feeding selectivity and rates in situ for key organisms such as copepods and other zooplankton is vital for understanding the mechanisms structuring marine ecosystems. Copepods feed on a wide range of prey, and there are presently no methods available to directly quantify zooplankton feeding on all different prey types in situ. Therefore, the development of a new nonintrusive direct method is necessary to gain a better understanding of the trophic interactions in aquatic ecosystems. Molecular methods based on the polymerase chain reaction have recently become an important tool to study predation by arthropods, particularly insects. Here we present the first results of successful molecular detection of a specific prey consumed by calanoid copepods from gut and fecal material. Using the calanoid copepod species *Calanus finmarchicus* consuming the haptophyte alga *Emiliania huxleyi* as a model system, 18S ribosomal DNA originating from *E. huxleyi* was unambiguously detected in whole DNA extracts from copepods and from their fecal pellets. The results also suggest that prey DNA may be quantified for determination of prey-specific zooplankton feeding rates. However, significantly more research under controlled laboratory and field conditions will be required to achieve these objectives. We hypothesize that molecular methods will become an important tool with the potential to quantify undisturbed trophic interactions between individual predators and all their prey in the complex natural plankton.

Accurate quantitative understanding of the trophic roles of zooplankton is critical for understanding how the composition and behavior of planktonic organisms influence important oceanic processes including cycling of climate gasses, vertical flux of carbon and nutrients, and occurrence of harmful blooms, and for the development of reliable ecosystem models for prediction of marine fisheries (Verity et al. 2002).

Zooplankton play a central role in the marine food web as selective predators, selective nutrient regenerators, and as mediators of energy to higher trophic levels (Riegman et al. 1993; Banse 1995; Gismervik et al. 1996; Verity and Smetacek 1996). Microzooplankton are generally the major consumers of phytoplankton in the sea (Banse 1995), whereas mesozooplankton, in particular copepods, dominate biomass of marine plankton and are key prey for higher trophic levels (Cushing 1990; Planque et al. 1997; Tande et al. 2000). Copepods are often omnivorous and different species ingest prey ranging from small algae to fish larvae (Turner et al. 1985; Landry and Fagerness 1988; Hansen et al. 1994; Nejstgaard et al. 1995). However, copepods and other zooplankton may select between seemingly similar prey, even of the same species, on the basis of biochemical composition (Houde and Roman 1987; Wolfe 2000). Many copepods feed selectively on larger microzooplankton, and when only algal ingestion is measured, the data suggest that algal consumption alone is often insufficient to meet even the predators' basic metabolic costs, much less support growth or reproduction (see further discussions in Kleppel 1993; Ohman and Runge 1994; Atkinson 1996; Nejstgaard et al. 1997; Roman et al. 2000: this contains numerous other references.). Both carnivorous (Yen 1985; Olsen et al. 2000) and omnivorous copepods (Paffenhöfer and Knowles 1980; Sell et al. 2001) may be voracious predators of other copepods and larvae of higher organisms.

In addition, copepods explore minute food patches, perform daily and seasonal migrations, and they may show highly variable feeding behavior in time and space down to scales of centimeters and minutes, or less. Copepods also both produce

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fecal pellets that may sediment and are important consumers of sedimenting material (Turner 2002). Copepods may therefore act as key top-down regulators both of the marine plankton food web and of the vertical flux of materials (Verity and Smetacek 1996; Verity 1998; Calbet and Landry 1999; Svensen and Nejstgaard in press). In order to quantify the trophic interactions of copepods and other mesozooplankton in situ, it is not only necessary to assess all important prey, including heterotrophs, but it should also be done with an absolute minimum of handling and temporal and spatial confinement prior to collection and analysis of the predator.

Several methods have been developed to assess mesozooplankton grazing rates (Båmstedt et al. 2000), including the fast and coarse gut pigment method (Mackas and Bohrer 1976), isotope-based in vitro approaches (Roman and Rublee 1981), and laborious time-intensive microscopy-based studies (Verity and Paffenhöfer 1996; Nejstgaard et al. 2001). Each approach has its own experimental limitations. In vitro methods suffer from biased grazer behavior and food composition, complex food-web interactions, and cycling of isotopes in incubation bottles (Roman and Rublee 1981; Fuchs et al. 2000; Nejstgaard et al. 2001). The in situ gut pigment method is limited to herbivory, although attempts have been made to quantify carnivory by gut content (Ohman 1992; Juhl et al. 1996; Peterson and Dam 1996; Perissinotto et al. 2000), and it is also limited by variable breakdown of pigment during gut passage (McLeroy-Etheridge and McManus 1999; Pandolfini et al. 2000; both contain numerous additional references.). Thus, new direct methods are needed to assess in situ zooplankton feeding selection and rates.

Ideally, it should be possible to directly quantify in situ ingestion of all prey of a single zooplankton by analysis of a freshly caught individual or its feces, without any prior interference with the food environment. For other small invertebrates, such as insects, the primary tools for analyzing predation in the field are now molecular gut content analysis, especially use of prey-specific antibodies (Zaidi et al. 1999; Symondson 2002). However, use of antibodies with omnivorous predators is impractical because such antibodies are time-consuming and costly to develop, require specialized culture facilities, and may yield problems with reproducibility, cross-reaction, and false positives (Chen et al. 2000; Symondson 2002). For these reasons, the use of antibodies in copepod fecal pellets was found to be problematic (Ohman 1992). However, in other marine organisms, the application of antibodies has been successful, e.g., bacterivory by heterotrophic flagellates (Christoffersen et al. 1997), predation by euphausiids on the early life stages of anchovy (Theilacker et al. 1993), identification of zooplankton prey in the guts of paralarval squid (Venter et al. 1999), and cod larvae in fish stomachs (Rosel and Kocher 2002).

Recently, genetic techniques based on polymerase chain reaction (PCR) amplification of DNA have been successfully applied in qualitative studies of carnivorous insects and other organisms (Symondson 2002). Compared to insects, copepods have a simpler gut structure, relatively neutral gut pH, and shorter digestion time, suggesting that these approaches will work as well in copepods as in insects. Furthermore, because DNA is both much more prey-specific and less easily oxidized than plant pigments, prey DNA should have a much larger potential as a quantitative prey tracer in zooplankton guts and fecal pellets than plant pigments.

By quantitative molecular analysis of the gut content, feeding rates may potentially be determined for any prey organism with possible precision down to a few prey cells. Such a method would have groundbreaking potential by providing the means to quantify virtually undisturbed trophic interactions between the individual prey and predator, on a short time scale. As an initial model for this approach, we apply PCR amplification to detect a genetic marker of a specific algal prey in a marine copepod and its feces under laboratory conditions.

Materials and procedures

Algal cultures—Three algal species were used in these studies: *Emiliania huxleyi* (Lohmann) Hay and Mohler, *Phaeocystis pouchetii* (Hariot) Lagerheim, and the cryptophyte *Rhodomonas baltica* Karsten *sensu* Zimmermann. *P. pouchetii* and *E. huxleyi* were isolated from the Raunefjord, Norway, in 1998 and 1994, respectively. *R. baltica* was obtained from Ifremer, Brest, France. The algae were cultured in semi-continuous batches, in f/2 media diluted 10 times (f/20; Guillard 1975), 14 : 10 hour light cycle and at 15°C, except for *P. pouchetii*, which was maintained at 5°C.

Collection of copepods—Eucalanus pileatus Giesbrecht were collected for sequencing purposes from the South Atlantic Bight, USA, using a 200-μm-mesh drifting plankton net as previously described (Verity and Paffenhöfer 1996), and were maintained in algae-free seawater for 48 h to evacuate gut contents prior to DNA extraction. For feeding studies, females of *Calanus finmarchicus* (Gunnerus) were collected from 0- to 30-m depth in the Raunefjord, Norway, using a 500-μm-mesh size 1-m diameter net, with a 14-L nonfiltering cod-end in March 2002. The copepods were sorted using wide-mouthed pipettes and acclimated to the experimental food concentration for 24 h before use in feeding experiments. Copepods were acclimated, fed, and handled in a walk-in cold room at in situ temperature (5°C) and dim light maintained in a 14 : 10 hour light cycle.

Copepod feeding studies—Approximately 90 females of *C. finmarchicus* were acclimated for 24 h in saturating food concentrations of *E. huxleyi* (ca. 1500 μg C L^{-1}). This concentration was similar to that described by Båmstedt et al. (1999). After acclimatization to *E. huxleyi* as a sole food source, animals were randomly split into seven groups and transferred to new saturating suspensions of *E. huxleyi* in 450-mL Perspex chambers with 500-μm false bottoms, and incubated for another 24 h. At the end of the incubation, the copepods were rinsed by dipping the Perspex chambers in four consecutive baths of 450 mL clean filtered (0.22 μm) seawater each. The rinsed animals were allowed to empty their guts in filtered seawater for various time intervals from ca. 2 min to 2 d before transfer onto a Petri dish

Table 1. 18S rRNA gene sequences used in this study for the design of Haptophyceae-specific PCR primers

Species	GenBank accession nr	Taxonomy
Emiliania huxleyi	M87327	Haptophyceae; Isochrysidales
Phaeocystis globosa	AJ278037	Haptophyceae; Prymnesiales
Phaeocystis pouchetii	AJ278036	Haptophyceae; Prymnesiales
Phaeocystis globosa	AJ278035	Haptophyceae; Prymnesiales
Phaeocystis sp.	PLY559	Haptophyceae; Prymnesiales
Phaeocystis sp.	AJ279499	Haptophyceae; Prymnesiales
Phaeocystis jahnii	AF163148	Haptophyceae; Prymnesiales
Phaeocystis cordata	AF163147	Haptophyceae; Prymnesiales
Phaeocystis pouchetii	X77475	Haptophyceae; Prymnesiales
Phaeocystis globosa	X77476	Haptophyceae; Prymnesiales
Phaeocystis antarctica	X77481	Haptophyceae; Prymnesiales
Phaeocystis antarctica	X77479	Haptophyceae; Prymnesiales
Phaeocystis antarctica	X77478	Haptophyceae; Prymnesiales
Phaeocystis antarctica	X77477	Haptophyceae; Prymnesiales
Phaeocystis antarctica	X77480	Haptophyceae; Prymnesiales
Rhodophysema elegans	U23817	Florideophyceae; Palmariales
Gracilariopsis sp.	M33639	Florideophyceae; Gracilariales
Papiliocellulus elegans	X85388	Coscinodiscophyceae; Cymatosiraceae
Pithophora sp.	AB066647	Ulvophyceae; Cladophorales
Ulva rigida	AJ005414	Ulvophyceae; Ulvales
Pfiesteria piscicida	AY033488	Dinophyceae; Unclassified Dinophyceae
Hematodinium sp.	AF286023	Dinophyceae; Syndiniales
Callinectes sapidus	M34360	Eucarida; Decapoda
Eucalanus pileatus	AY192563	Copepoda; Eucalanoida
Calanus finmarchicus	AF367719	Copepoda; Calanoida

using a wide-mouthed pipette and sampled by grabbing the antennule with a forceps. Care was taken to minimize the amount of water on the sampled copepod and that copepods did not defecate during the sampling process (ca. 20–40 s). Copepod or fecal pellets were sampled directly into extraction tubes. When more time is needed to sort samples of live animals after washing, they should be rapidly frozen before sorting as described by Båmstedt et al. (2000). Copepods were subfractioned for subsequent pigment and DNA analysis. Animals for pigment analysis were placed in 90% acetone and copepods for DNA analysis were immediately frozen at –80°C.

Estimation of copepod gut chlorophyll—Copepods reserved for gut pigment analyses were extracted for 12 h in 90% acetone at 4°C and analyzed on a Turner Designs Model 10-AU Fluorometer as previously described (Nejstgaard et al. 1995). Copepod gut pigment concentration and gut evacuation rates were calculated as described in Båmstedt et al. (1999), assuming an exponential decrease.

Extraction of total DNA from algal cultures, copepod guts, and fecal pellets—PCR-amenable genomic DNA from centrifuge-harvested cultures of *E. huxleyi* was purified using the Ultra Clean Soil DNA Isolation Kit (Mo Bio Laboratories) essentially following the manufacturer's instructions except that cells were initially lysed using a FastPrep FP120 bead beater (BIO 101). Cells (ca. 10^6 cells mL^{-1}) were collected (500 mL) by centrifugation at 8000 × *g* for 20 min at 4°C. Genomic DNA from whole copepods or copepod fecal pellets that had been washed in 0.2 μm filtered seawater were purified using the Ultra Clean Soil DNA Isolation Kit following exactly the protocol for maximizing yields as described by the manufacturer. Sufficient DNA for PCR amplification can be obtained from a single copepod or fecal pellet, but we generally pooled 3 to 5 copepods or 5 to 10 pellets prior to DNA purification. Copepods and fecal pellets were either extracted fresh or after a brief period of storage (up to 2 weeks) at –80°C. Purified DNA was stored in nuclease-free water at –20°C and remained amenable to PCR amplification for at least eight months under these conditions.

Primer design—An 18S rRNA targeted PCR primer pair specific for *E. huxleyi* was designed in this study. A database of 24 aligned complete and nearly complete 18S rRNA gene sequences from available haptophyte species, representative green and red algae, diatoms, dinoflagellates, and crustacean species were assembled using the Genetic Database Editor (GDE) (Smith et al. 1992). In addition, the 18S rRNA gene from the copepod *E. pileatus* was sequenced in this study and included in this database. Sequencing in both the forward and reverse direction was accomplished as previously described (Gruebl et al. 2002). All representative organisms including the newly sequenced *E. pileatus* 18S rRNA gene and Genbank sequence accession numbers are provided in Table 1. Sequence strings of 15 to 25 bp, unique to *E. huxleyi*,

Table 2. PCR primers and PCR reaction conditions used in this study*

Specificity	Forward primer (5′→3′)	Reverse primer (5′→3′)	Product size
E. huxleyi†	EHuxF-745 (TCA AGC AGG CAG TCG)	EHuxR-803 (CAC CAG AGT CCT ATT TCA)	58 bp
Universal 18S rDNA‡	UnivF-15 (CTC CCA GTA GTCATA TGC)	UnivR-1765S (ACC TTG TTA CGA CTT)	1759 bp

*PCR reaction mix: 4 to 10 μg purified DNA; 30 ng each forward and reverse primers (primer stock concentration 100 ng μL^{-1}); Taq Hot Start Master Mix (Qiagen); 2.0 mM $MgCl_2$ final concentration.
†*E. huxleyi* amplification conditions: initial template denaturation (94°C for 15 min); 35 amplification cycles (94°C for 30 s; 48°C for 30 s; 72°C for 60 s). Following the completion of amplification cycles, a final extension step (72°C for 10 min) was completed and samples were then stored at 4°C.
‡Universal 18S rDNA amplification conditions: initial template denaturation (94°C for 3 min); 30 amplification cycles (94°C for 15s; 45°C for 15s; 72°C for 30s). Following the completion of amplification cycles, a final extension step (72°C for 10 min) was completed and samples were then stored at 4°C.

were identified using the *Find Variable Regions* algorithm available in the GDE loaded with the aligned sequence database. Optimal probe target sites were initially identified based on the criteria of exhibiting at least two nucleotide differences from other aligned sequences. Following the initial identification of suitable sequences, PCR primers sets were designed that would amplify fragments less than 250 bp, exhibit a minimum propensity for the formation of primer dimers and self-hybridization, and place nucleotide positions unique to the target organism in optimal locations for enhancing primer specificity. Primer design tools available in the Primer Premier Version 5.00 software package (Premier Biosoft International) were used to facilitate optimal primer design. Oligonucleotides were synthesized either by Integrated DNA Technologies or by the Molecular Genetics Instrumentation Facility at the University of Georgia.

Empiric testing of primer specificity—Based on comparison of sequence alignments of the 18S rRNA gene from *E. huxleyi*, closely related prymnesiophyte species, other algal species, and representative copepod and other crustacean species, it was possible to identify several short sequence stretches that were sufficiently unique to *E. huxley*, which could then be targeted as *E. huxleyi*–specific oligonucleotide PCR primers. Initially, 10 potential primer pairs were identified on the basis of these sequence comparisons, each of which exhibited at least three nucleotide mismatches with the 18S rRNA gene sequence of the copepod *C. finmarchicus* and at least one nucleotide mismatch with the other available sequences. From this collection of potential primer target sites, one primer set conforming to all primer design criteria was identified, synthesized, and tested for its specificity and sensitivity in a PCR format. This primer set consists of forward primer EHuxF-745 and reverse primer EHuxR-803 (Table 2). Comparison of these primer sequences with all 18S rRNA sequences available in the Ribosomal Data Base Project (Maidak et al. 2001) indicated that these primers would also amplify one Isochrysidales species (*Isochrysis galbana*) but not others.

The specificity of this primer set for *E. huxleyi* was confirmed by amplifying genomic DNA purified from a culture of *E. huxleyi*, a closely related prymnesiophyte algae *Phaeocystis pouchetii*, and DNA from the cryptophyte alga *Rhodomonas baltica*. PCR amplification of 18S rRNA gene fragments from three algal species (*E. huxleyi*, *P. pouchetii*, and *R. baltica*) was attempted, using the generic eukaryote-specific primer set UnivF-15 and UnivR-1765S (Table 2; Frischer et al. 2000, 2002) and the *E. huxleyi*–specific primer set primer EHuxF-745 and EHuxR-803 designed in this study. The *E. huxleyi*–specific primer set amplified the expected 58-bp product only from *E. huxleyi* and did not produce product from *Phaeocystis* or *Rhodomonas* (Fig. 1). These results experimentally confirm the specificity determined by sequence comparison. The amenability of each genomic

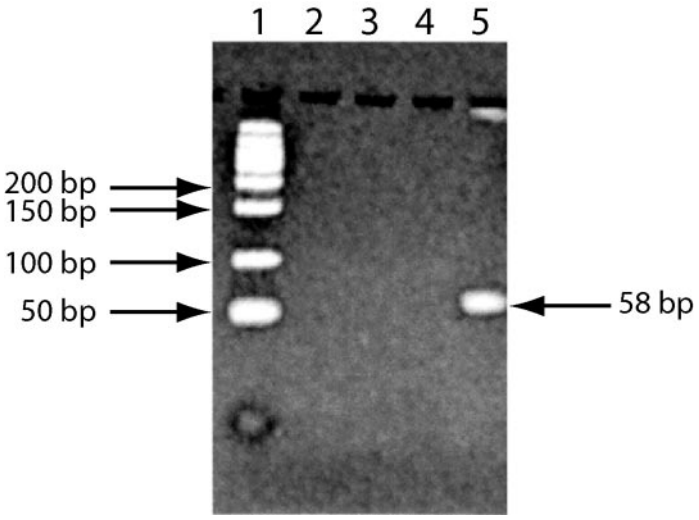

Fig. 1. Specificity of the 18S rDNA targeted *E. huxleyi*–specific PCR primer set EHuxF-745 and EHuxR-803. Lane numbers are indicated at the top. Molecular weights are indicated on the left and the size of the PCR–amplified product is shown on the right. DNA purified from cultures of *E. huxleyi* (lane 5), *Phaeocystis pouchetii* (lane 4), and *Rhodomonas baltica* (lane 3) were used as DNA template for PCR amplification with the *E. huxleyi*–specific primers as described in Table 1. Lane 2: 'No DNA' negative control. The expected 58-bp 18S rDNA fragment was amplified from *E. huxleyi* but not the other algal species. A 50-bp molecular weight marker is shown in lane 1. PCR products were visualized and sized on a 2% agarose gel.

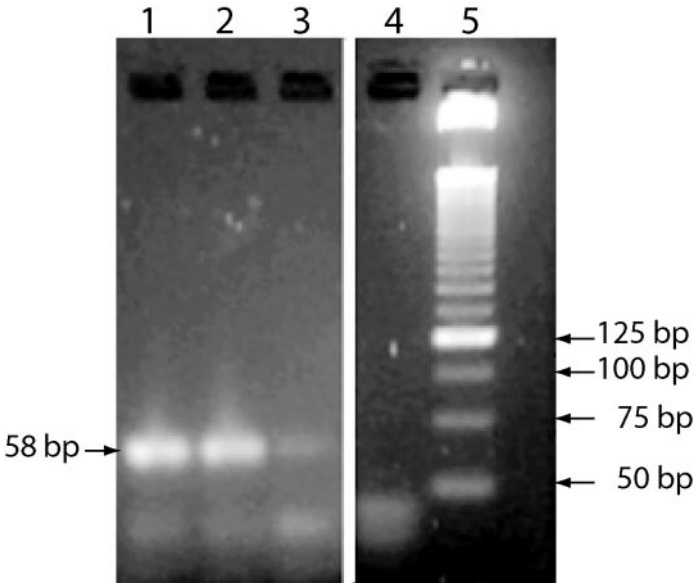

Fig. 2. Detection of the 58-bp *E. huxleyi*–specific18S rDNA PCR amplicon in the copepod *Calanus finmarchicus* after feeding on *E. huxleyi* cells. Lane numbers are indicated at the top. Molecular weights are indicated on the left and the size of the PCR–amplified product is shown on the right. Detection of the *E. huxleyi*–specific 18S rDNA amplicons 2 min (lane 1), 14 min (lane 2), and 6 h (lane 3) after feeding was terminated. Lane 4: 'No DNA' negative control. PCR product size and molecular weight markers (lane 5) are shown. PCR products were visualized and sized electrophoretically on a 2% agarose gel.

DNA preparation to PCR was confirmed by amplifying each DNA template with the generic 18S rRNA targeted primer set UnivF-15 and UnivR-1765S. These primers produced the expected 1759-bp product, indicating that each DNA preparation used was amenable to PCR amplification and contained sufficient high molecular weight DNA for efficient PCR amplification (data not shown).

PCR amplification was facilitated using the Taq Hot Start Master Mix (Qiagen) and a Perkin Elmer 2400 or 9600 thermal cycler in 25-μL reaction volumes. Primer sequences and PCR reaction conditions are described in Table 2. Each set of reactions included a series of amplification controls including experimental blanks consisting of DNA extracted from the algae-free seawater used to rinse the copepods, a "no DNA template" negative control, and a positive control containing DNA purified from a culture of *E. huxleyi*. We visualized PCR amplicons by gel electrophoresis on a 2% agarose gel buffered in 1X TAE (0.04M Tris-Acetate, 0.001M EDTA, pH 8.0).

Assessment

Specific detection of E. huxleyi DNA in copepod gut and fecal pellets—DNA from *E. huxleyi* cells consumed by *C. finmarchicus* was routinely detected by PCR amplification using the *E. huxleyi*–specific primer set of extracted DNA from whole copepods and from fecal pellets. As described above, amplification was facilitated using the Taq Hot Start Master Mix (Qiagen) in 25-μL reaction volumes. PCR reaction conditions are described in Table 2.

18S rRNA gene fragments using the primer set EHuxF-745 and EHuxR-803 were successfully amplified from total DNA extracts from whole copepods (*Calanus finmarchicus*) fed *E. huxleyi* cells (Fig. 2). *E. huxleyi* rDNA could be detected up to 6 h after feeding had been terminated, although only a very faint PCR product was detected after this period. *E. huxleyi* rDNA was also successfully amplified from fecal pellets produced by these animals (Fig. 3). Amplified 18S rDNA was detected in pellets collected 1 h after feeding, but not in pellets collected 6 h after feeding had been terminated.

Comparison of gut pigment and PCR methods—Copepod gut content of *E. Huxleyi* cells was independently determined and compared by extimating gut chlorophyll and by specific PCR in replicate copepods of animals produced from the copepod feeding studies. Based on the decline of gut chlorophyll content after feeding was terminated, the estimated time for 50% gut evacuation was 14.4 min, with chlorophyll concentrations approaching undetectable concentrations after 60 min (Fig. 4). Decline in gut pigment reflects both defecation and breakdown of pigment during gut passage (McLeroy-Etheridge and McManus 1999; Pandolfini et al. 2000). The gut evacuation time in Fig. 4 should thus be interpreted as a minimum value. However, all copepods were acclimated to, and fed, saturating food concentrations of an algae monoculture. Thus, the pigment breakdown should be reduced to a minimum, and the relative decrease in pigment over time should reflect the gut evacuation (McLeroy-Etheridge and McManus 1999). In order to validate whether prey DNA could serve as a tracer for quantitative feeding studies, gut content of prey pigment was compared with a semiquantitative estimate of *E. huxleyi* DNA determined from densitometric quantification of PCR–amplified product in copepods produced in the feeding experiments described above. Total DNA was extracted from whole copepods and fecal pellets as described above. Densito-

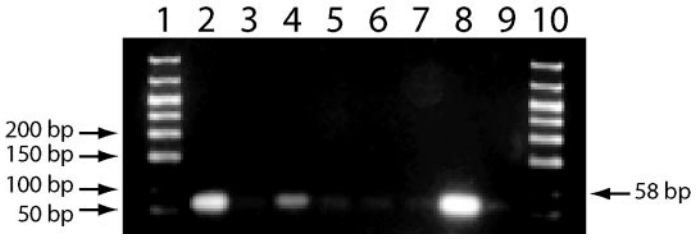

Fig. 3. Detection of *Emiliania huxleyi* 18S rDNA in faecal pellets produced by *Calanus finmarchicus* after feeding on *E. huxleyi* cells. Lane numbers are indicated at the top. Molecular weights are indicated on the left and the size of the PCR–amplified product is shown on the right. Detection of *E. huxleyi*–specific 18S rDNA amplicons in faecal pellets produced after 45 min (lane 2), 60 min (lane 4), 6 h (lane 6), and feeding was terminated. Matching seawater blanks are shown in lanes 3, 5, and 7, respectively. Lane 8: Positive control. Lane 9: 'No DNA' negative control. Molecular weight markers are shown in lanes 1 and 10. PCR products were visualized and sized electrophoretically on a 2% agarose gel.

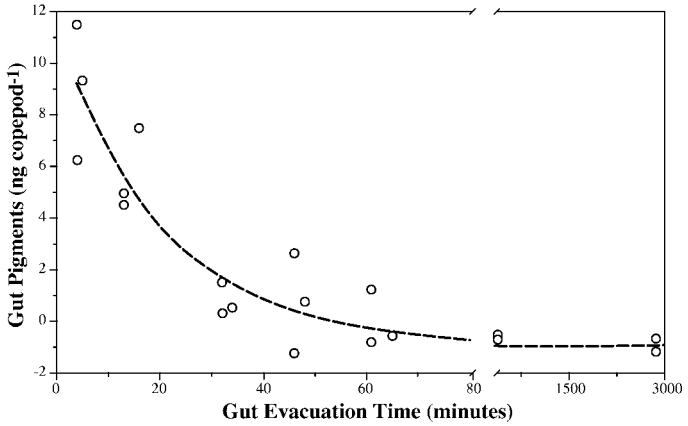

Fig. 4. Evacuation of gut pigments in individual Calanus finmarchicus fed with saturating concentrations of *E. huxleyi*. The X-axis shows the time elapsed after feeding was terminated. Gut pigments (Y-axis) are expressed in terms of chlorophyll *a* weight equivalents per individual. Estimated time for 50% gut evacuation was 14.4 min at 5°C; $P(x) = P_0 \times \exp^{(-k \times x)}$, P_0 = 11.4; SE = 1.1; $P < 10^{-4}$; k = 0.048; SE = 0.008; $P < 10^{-4}$; r^2 = 0.86; df = 18.

metric quantification of PCR product visualized on a 2% agarose gel containing 1X GelStar® nucleic acid stain (BMA) was facilitated using the Quantity One software package (Bio-Rad) after digitally capturing gel images using a EDAS 290 gel documentation system (Eastman Kodak). The brightness of each PCR product compared with background levels was determined. Relative band intensity was quantified in arbitrary pixel units. These units do not directly relate to the absolute starting concentration of target algal cells in the original sample, but they do provide a relative estimate of target abundance in different copepod samples.

Similar to the decline in gut chlorophyll content observed in the feeding experiment (Fig. 4), the intensity of the *E. huxleyi*–specific 18S rDNA PCR product produced from DNA that was extracted from replicate copepods using the standard PCR conditions, declined with evacuation time and was barely detectable after 6 h (Fig. 2). Gut chlorophyll concentration was significantly correlated with PCR band intensity (r^2 = 0.95), suggesting that PCR detection of copepod prey items can be quantified by PCR amplification (Fig. 5).

Discussion

Recently, genetic techniques based on PCR amplification of DNA have been successfully applied in qualitative studies of carnivorous insects and other organisms (Symondson 2002). In these studies, prey DNA was shown to be inversely correlated to digestion time and DNA amplicon fragment size. Half-lives of detection ranged from 4 to 9 h for smaller sized amplicons similar in size to those used in this study (Chen et al. 2000). Compared to terrestrial insects, copepods have a relatively simple gut structure (Brunet et al. 1994), relatively neutral gut pH (Pond et al. 1995), shorter digestion time (Irigoien 1998), and food remains are often identifiable in guts and fecal pellets of copepods (Turner 2002). These observations suggest that the PCR approach should be effective in copepods. In these studies, we demonstrated that DNA from the algal species *Emiliania huxleyi* could be detected by PCR in the guts and fecal pellets of the copepod *Calanus finmarchicus* after the copepod had ingested this algal species. Prey specific 18S rDNA could be detected in both copepod guts and fecal pellets up to 6 h after ingestion. The rate of disappearance of a PCR signal in copepod guts was well correlated with estimates of gut evacuation rates determined by gut pigment content. Combined, these results suggest the applicability of a DNA-based approach for identifying ingestion of specific prey types and perhaps feeding rates by copepods in situ that does not require experimental manipulation. However, because these studies were conducted as simple prey-predator laboratory experiments using only one prey type (*E. huxleyi*), further testing under more complex laboratory and field settings are required to determine if these methods are appropriate under natural conditions. That rDNA was detected up to 6 h after feeding in this study is consistent with the hypothesis that prey DNA may be more stable than prey pigment in copepods and that DNA is an appropriate tracer for ascertaining feeding activity in marine copepods.

A distinct advantage of a DNA-based approach for assessing in situ feeding by marine copepods is that it is not limited to the detection of feeding on pigmented organisms. Because nonpigmented ciliates and heterotrophic dinoflagellates are generally available, and even preferred, as prey by copepods (Stoecker and Capuzzo 1990; Kleppel 1993; Fessenden and Cowles 1994; Suzuki et al. 1999; Levinsen et al. 2000; Nejstgaard et al. 2001), the ability to directly investigate feeding on nonpigmented organisms by copepods is of considerable importance. Yet, current methodological limitations hamper our ability to quantify

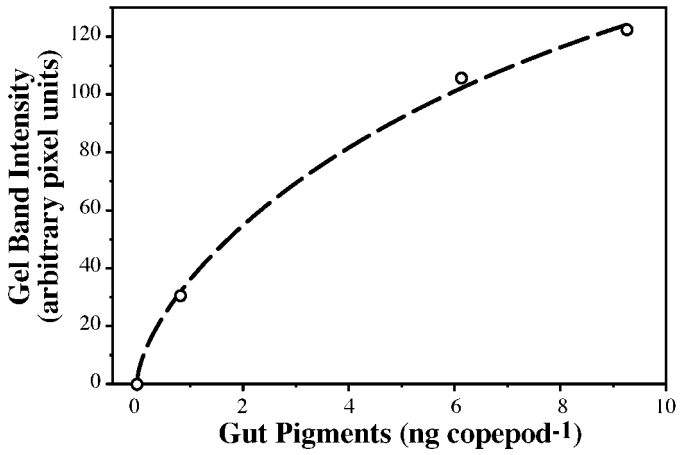

Fig. 5. Comparison of pigment gut evacuation and densitometric quantification of PCR amplified *E. huxleyi*–specific DNA in *Calanus finmarchicus* after feeding on *E. huxleyi*. A significant correlation (r^2 = 0.99) between gut pigments and DNA detection was observed. $F(x) = a \times x^b$; a = 37.1; SE = 4.6; P = 0.015; b = 0.55; SE = 0.06; P = 0.012; df = 2.

copepod feeding on these prey types directly. Ultimately, the promise of DNA-based molecular techniques is that they will provide the means to overcome these constraints once further development and testing of the method under more complex laboratory and field studies are completed.

The question of who eats what, why, and at what rates has been at the forefront of plankton ecology for over a century (Smetacek et al. 2001). Whereas many advances in our understanding have been driven by technological breakthroughs, it remains difficult to identify and quantify in situ feeding by zooplankton. The ability to obtain accurate data on trophic interactions for key organisms, such as copepods and other zooplankton, is essential for understanding the mechanisms that structure pelagic ecosystems. In this study an initial method and important 'proof of concept' validation is provided for a DNA-based approach for studying copepod feeding. Ultimately, by collection and analysis of individual copepods directly in situ or collection of freshly produced fecal material from such copepods, it should be possible to directly determine zooplankton feeding patterns in nature. Development of such molecular methods could become a very important tool to quantify undisturbed trophic interactions between individual predators and all their potential prey in the complex natural plankton.

Comments and recommendations

Common problems—One of the most common and significant problems associated with this and all PCR-based assays is the possibility of false positive amplification caused by contamination. Because of the exquisite sensitivity of PCR, contamination of PCR reactions with even a few molecules of target can result in false amplification (Hiney and Smith 1998). For this reason, it is extremely important to include a series of negative (no DNA) controls with every PCR reaction. If amplification is detected in the negative control reactions, all other amplification results should be disregarded. To avoid contamination problems, it is helpful to aliquot all reagents including primers and Taq master mix into small single-use volumes in sterile disposable labware. All pipetting should be conducted in a sterile laminar flow hood or in a clean hood specifically designed for setting up PCR reactions. If contamination problems persist, and they will occur from time to time, it is usually most efficient to repeat efforts with all new reagents after thoroughly sterilizing all pipettes and work areas rather than spending large amounts of time and effort required to systematically identify the specific contamination source.

General method applicability—The method presented in this study targeted the detection of 18S rDNA specific to the alga *Emiliania huxleyi* consumed by the copepod *Calanus finmarchicus*. However, one of the advantages of a genetic sequence-based approach compared with other approaches (e.g., pigments or specific antigens) is the ease with which alternative genetic markers can be targeted. For example, a PCR primer set that specifically amplifies a small (209 bp) fragment of the 18S rRNA gene unique to the prymnesiophyte genus *Phaeocystis* has also been developed (data not shown), and future research will focus on the consumption of these algae by zooplankton. Similarly, other genes should be amenable to targeting by this approach. However, it should be cautioned that genetic sequence databases of all possible copepod prey species are not currently available so the specificity of all primer sets must be empirically confirmed under realistic conditions for their intended use. Ideally the target gene sequences of all potential prey should be known, but at present this is a distant goal and it continues to be important to expand genetic sequence databases of marine microorganisms. Theoretically, the more complete sequences databases are, the better the primer design will be. However, if primer sets are to be applied under conditions where all prey types are either not sequenced or unknown, it is advisable that representative PCR products produced from field samples be sequenced to confirm their identity as the expected PCR product. With respect to eventual field use of this method, one of the advantages of a PCR approach compared with other techniques (that is, antibodies) is that it is possible, with relatively little effort, to sequence PCR amplicons derived from the field samples and compare them to the known target sequence. This provides an unambiguous and independent means of verifying the predicted specificity of PCR primer sets used in field situations where all possible target organisms are not known.

Quantification by real time PCR—Increasingly, real-time quantitative PCR approaches are being utilized to quantify genetic targets in nature, including the marine environment (Warick et al. 2002; Dyer et al. 2001; Dhar et al. 2001; Overturf et al. 2001; Bowers et al. 2000). Preliminary studies using a general SYBR Green-based quantitative PCR assay (Witter et al. 1997) with the primers developed in this study suggest that it will be possible to quantify prey consumption by copepods using this approach. However, significantly more research under controlled laboratory and field conditions will be required to achieve these objectives.

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