RESEARCH ARTICLE

Quantitative PCR to estimate copepod feeding

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Abstract Copepods play a central role in marine food
webs as grazers of plankton and as key prey for many pre-
dators. Therefore, quantifying their specific trophic interac-
tions is critical for understanding the role of copepods in
ocean processes. However, because of methodological con-
straints, it remains difficult to investigate in situ copepod
feeding without reliance on laborious intrusive and poten-
tially biased incubation approaches. Recent advances in
PCR-based methodologies have demonstrated the feasibil-
ity of directly identifying copepod diets based on prey
DNA sequences. Yet, obtaining quantitative information
from these approaches remains challenging. This study pre-
sents results of systematic efforts to develop a quantitative
PCR (qPCR) assay targeted to 18S rRNA gene fragments to
estimate copepod gut content of specific species of prey
algae. These results were first compared to gut content esti-
mates based on fluorescence in the copepod *Calanus finm-
archicus* fed monocultures of two different microalgae
species in controlled laboratory studies. In subsequent field
studies, we compared feeding rates obtained by microscopy
and qPCR for *Temora longicornis* and *Acartia clausi* feed-
ing on the haptophyte *Phaeocystis globosa* in natural
blooms. These investigations demonstrate a semi-quantita-
tive relationship between gut content estimates derived
from qPCR, gut pigment, and direct microscopy. However,
absolute estimates of gut content based on qPCR methodol-
ogy were consistently lower than expected. This did not
appear to be explained by the extraction methods used, or
interference by non-target (predator) DNA in the PCR reac-
tions, instead suggesting digestion of prey-specific nucleic
acids. Furthermore, the 18S rDNA target gene copy number
of the phytoplankton varied with growth phase. Nonethe-
less, when prey target gene copy number in the ambient
water is quantified, the qPCR-approach can be compared to
other methods, and then used to semi-quantitatively esti-
mate relative copepod grazing on specific prey in situ with-
out involving further incubations. A distinct advantage of a
DNA-based molecular approach compared to gut fluores-
cence and direct microscopic observation, is the ability to
detect non-pigmented and macerated prey. Future studies
should aim to correct for breakdown in prey DNA and per-
form extensive calibrations to other methods in order to
achieve a quantitative measure of feeding rates in situ.

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Introduction

The ability to accurately estimate feeding preferences and
rates for key zooplankton organisms is vital for under-
standing the processes that structure marine ecosystems.

Mesozooplankton, and in particular copepods, dominate the biomass of marine plankton and are key prey for larger organisms (e.g. Cushing 1990; Planque et al. 1997; Tande et al. 2000). Copepods are not indiscriminate feeders and it has been shown that among other factors, prey size, concentration, and motility influence ingestion (Frost 1972; Berggreen et al. 1988; Jakobsen et al. 2005). It has long been known that different copepod species and developmental stages may ingest a wide variety of prey, ranging from algae of a few micrometers to metazooplankton and fish larvae (Paffenhöfer and Knowles 1980; Turner et al. 1985; Yen 1985; Landry and Fagerness 1988; Nejstgaard et al. 1995; Sell et al. 2001). However, the mechanisms of prey selection by copepods are incompletely understood. Copepods can use mechanical and chemical cues (Woodson et al. 2007, and references therein), switch between suspension- and raptorial feeding, and even perceive individual cells and deliberately route them to mouthparts (Paffenhöfer and Lewis 1990). They may select between seemingly similar prey, even of the same species, clone or growth stage, on the basis of biochemical composition (Houde and Roman 1987; Wolfe 2000; Dutz and Koski 2006; Long and Hay 2006). Many copepods feed selectively on larger microzooplankton and, when only algal ingestion is measured, data often suggest that algal consumption is insufficient to meet even basic metabolic costs (Stoecker and Capuzzo 1990; Kleppel 1993; Ohman and Runge 1994; Atkinson 1996; Peterson and Dam 1996; Verity and Paffenhöfer 1996; Nejstgaard et al. 1997; Liu et al. 2005). In addition, copepods may perform diel and seasonal migrations over hundreds of meters, while they explore minute food patches in time and space. Such complex behavior highlights the need for methodologies for determining in situ copepod feeding and ingestion, ideally avoiding confinement to incubation bottles and minimizing all handling prior to analysis.

The need for new tools to investigate zooplankton trophic processes was clearly articulated by the science community at the Marine Zooplankton Colloquium 2 (2001) "It is not so much a lack of ideas but inadequate methodologies and instrumentation that limits the pace of advances in understanding marine zooplankton". Several methodological approaches have been developed to assess mesozooplankton grazing in situ including detailed HPLC analysis of gut pigments (Kleppel et al. 1988; Buffan-Dubau et al. 1996), direct microscopy approaches for analysis of water, gut, and fecal pellet contents (Øresland and Ward 1993; Båmstedt et al. 2000; Fleddum et al. 2001; Nejstgaard et al. 2001; Kaartvedt et al. 2002), automated image analysis of total gut contents in copepods (Billones et al. 1999), determination of zooplankton ingestion and assimilation rates with stable isotope tracers (e.g. Verschoor et al. 2005), use of amino acids to determine trophic niche (Guisande et al.

2002), and different molecular approaches including the use of immunological stains (Ohman 1992). However, to date the large majority of copepod feeding investigations continue to rely on analysis of bulk gut pigments (chlorophyll a, and derivates), despite the recognition of serious limitations of this approach (McLeroy-Etheridge and McManus 1999; Pandolfini et al. 2000; Bustillos-Guzman et al. 2002; Froneman 2004), largely because it is straightforward, fast and inexpensive. Some zooplankton gut studies have utilized more laborious microscopic and HPLC approaches, but these methods are especially sensitive to differential pigment breakdown (see references above), and pigments thought to be specific to particular prey types are often not specific (e.g. Antajan et al. 2004; Irigoien et al. 2004). Furthermore, pigment based methods are limited to exploration of pigmented prey types only and do not yield data for heterotrophic prey. Microscopic analysis of gut content is extraordinarily laborious and even a trained microscopist can find it difficult to definitively identify copepod gut contents. For example, during the recent CYCLOPS study in the Eastern Mediterranean, Pasternak et al. (2005) attempted to characterize the contents of herbivorous and omnivorous copepods by direct microscopy and reported that 60–82% of gut contents consisted of "unidentified mass" that could have originated from flagellates, aloricate ciliates, athecate dinoflagellates or detritus. Also, different microscopy methods yield different results (Gowing and Wishner 1992).

Some studies have successfully utilized immunological approaches in marine organisms, e.g. bacterivory by heterotrophic flagellates (Christoffersen et al. 1997), predation by euphausiids on early life stages of anchovy (Theilacker et al. 1993), and identification of zooplankton prey in the guts of paralarval squid (Venter et al. 1999) and of cod larvae in fish stomachs (Rosel and Kocher 2002). However, these methods are both tedious and subject to a significant amount of uncertainty, especially in copepods (Ohman 1992) and other omnivores (Symondson 2002; Harwood and Obrycki 2005).

A promising strategy for assessing feeding in small invertebrates is the use of prey-specific nucleic acid molecules as biomarkers of trophic interactions (Sheppard and Harwood 2005). Genetic techniques based on PCR amplification of DNA have been successfully applied in qualitative studies of carnivorous insects and other organisms (Symondson 2002; Harper et al. 2005; Harwood and Obrycki 2005; Sheppard and Harwood 2005) including marine vertebrates (Jarman et al. 2002; Jarman et al. 2006) and invertebrates (Blankenship and Yayanos 2005; Galluzzi et al. 2005; Vestheim et al. 2005; Martin et al. 2006). Previously, we reported the development of a DNA-based method to detect specific feeding in copepods and conducted a series of studies that provided proof of concept

and feasibility for development of a quantitative PCR-based approach for investigations of in situ gut content of calanoid copepods (Nejstgaard et al. 2003). In these studies successful extraction and PCR amplification of algal 18S ribosomal DNA from prey inside calanoid copepods and their fecal pellets was demonstrated. These results suggested that it should be possible to quantify prey items to estimate specific feeding rates. More recently we developed a real time quantitative PCR (qPCR) assay for investigation of trophic interactions in the filter feeding appendicularian *Oikopleura dioica* (Troedsson et al. 2007). To our knowledge this is the first successful report of the use of a quantitative PCR-based approach for investigating feeding in a marine zooplankton species.

In this study, we present the results of systematic efforts to develop a qPCR assay targeted to 18S rRNA gene fragments to estimate copepod feeding on different algal species. To develop the assay, we conducted controlled laboratory feeding experiments based on gut fluorescence. To evaluate the field applicability with natural plankton, classical mesozooplankton bottle incubation feeding assays were utilized.

Materials and methods

Algal cultures

Three algal species were cultured in these studies. *Emiliania huxleyi* (Lohmann) Hay and Mohler, *Phaeocystis pouchetii* (Hariot) Lagerheim, and the cryptophyte *Rhodomonas marina* (Dangeard) Lemmermann. *P. pouchetii* and *E. huxleyi* were isolated from the Raunefjord, Western Norway (60°16′N, 05°14′E), in 1998 and 1994, respectively. *R. marina* was obtained from IFREMER, Brest, France. The algae were cultured in semi-continuous batches, in *f*/2 media diluted ten times (*f*/20) (Guillard 1975), 14:10 hours light cycle and at 15°C, except for *P. pouchetii* which was maintained at 5°C. Algae used in the experiments were in exponential growth. To determine whether the physiological condition of a culture affected rRNA gene copy number and therefore quantitative estimates based on qPCR, rRNA cell content of a 1–week-old (exponential phase) and 2–week-old (plateau phase) culture of *E. huxleyi* were compared.

Feeding studies

Copepod feeding studies in the laboratory

Copepods for the laboratory feeding studies were collected by gentle tows from 0 to 30 m depth in the Raunefjord using a 500-μm mesh size, 1 m diameter net with a 14 l non-filtering cod-end. The samples were diluted with 40 l surface water and brought to a walk-in cold room at in situ temperature (5–10°C) and dim light at a 14:10 hours light:dark cycle. Stage CV or CVI females of *Calanus finmarchicus* (Gunnerus) were sorted into 5 l beakers using a wide mouth pipette and acclimated to the experimental food concentrations of either *E. huxleyi* (ca. 400 μg C l^{-1}) or *R. marina* (ca. 800–1,600 μg C l^{-1}) for 24 h before use in feeding experiments. Such high concentrations do not reflect natural food concentrations, but were used to ensure saturated feeding conditions, as has been shown for these copepods and prey in previous studies (Båmstedt et al. 1999, their Fig. 5. Note that the strain of cf. *R. baltica* used there is the same as used here, but is now determined to *R. marina*). In contrast, natural food concentrations were used in the subsequent field studies (see below). After acclimatization to the specific alga as a sole food source, animals were starved for 3 h in filtered (0.22 μm) seawater to allow complete gut evacuation prior to gut filling experiments. After starvation, animals were randomly split into four or five groups of 18–20 individuals, transferred into new saturating suspensions of the same prey type in 450 ml Perspex chambers with 500 μm false bottoms, incubated for 10, 20, or 40 min in experiments with *R. marina* and 5, 10, 20 or 30 min with *E. huxleyi*. In each experiment one group was sampled initially (0 min) as a starved control. After the incubation, copepods were removed and quickly rinsed by dipping the Perspex chambers in four consecutive baths of 450 ml filtered (0.22 μm) seawater and flash frozen in liquid nitrogen within 10–20 s to stop gut activity and prevent defecation before sampling. Within a few minutes, the copepods were thawed in filtered (0.22 μm) seawater, collected onto a Petri-dish and randomly sorted under dim light into 2–3 Eppendorf™ 1.5 ml-microtubes for DNA analysis, and another 2–3 glass tubes for gut pigment analysis. Each of these micro- and glass tubes contained 3–5 individuals. For the further analysis, each of these tubes was treated as a replicated sample. To minimize the risk of inclusion of prey algae in the water on the outside of the copepods, they were sorted by grabbing the base of the antennule with a forceps and dip-washing each copepod in droplets of prey-free filtered seawater before transfer to the tubes. Care was taken to minimize the amount of water in the tubes and to re-freeze the sorted copepods in liquid nitrogen within minutes. Samples were then stored at −80°C until analysis.

Copepod feeding studies in the field

Copepod feeding studies with natural plankton were conducted during two cruises in the English Channel off Wimereux (50°48′N–1°34′E) and Somme (50°14.645′N–1°26.794′E) estuaries, northwest France in May 2003 and

April 2004 on board R/V Côtes de la Manche (INSU) as a part of the French National and Regional research programs "PNEC Chantier Manche Orientale - Sud Mer du Nord" and "CPER bloom de *Phaeocystis*". Three feeding experiments (one in 2003 and two in 2004) were conducted by incubating 2.3 l polycarbonate bottles containing natural seawater with females of the copepods *Temora longicornis* (25 per bottle) or *Acartia clausi* (40 per bottle) 24 h on deck (at in situ temperatures). Each experiment was run with two replicate bottles per copepod species, except in the first experiment where only a single bottle with *A. clausi* was used. Specific feeding rates on the haptophyte algae *Phaeocystis globosa* were quantified by microscopy as prey disappearance, corrected for microbial food web interactions as previously described (Nejstgaard et al. 2001). Although, the PCR assay was developed for *P. pouchetii*, this primer also amplifies the expected 18S rDNA gene fragment from *P. globosa* (not shown). When the incubations were terminated, all the copepods in each bottle were collected on a 300-µm mesh, immediately rinsed in filtered seawater, flash-frozen in liquid nitrogen, and sorted into an Eppendorf™ microtube for DNA analysis (one bottle = one replicate in the further analysis). Instantaneous gut contents of prey DNA were converted into feeding rates based on general gut evacuation rates corrected for temperature (Irigoien 1998).

Assay development

Estimation of copepod gut pigments

Copepods 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 (chlorophyll *a* and derivatives) concentration and gut evacuation rates were calculated as described in Båmstedt et al. (2000) and as recently reported by Nejstgaard et al. (2003) assuming an exponential decrease.

Extraction of gut DNA

Several methodological approaches were evaluated to extract and purify total DNA from copepods (DNA derived from the copepod and gut contents), using a number of algae species including *E. huxleyi*, *Phaeocystis* spp, and *R. marina* (Simonelli et al. in preparation). Two manual DNA extraction and purification methods were tested by dissolving copepods in 500 µl GTC (4 M guanidium thiocyanate, 1 M trisodium citrate pH 7.0, 10% sarkosyl) or alternatively in 500 µl EGTA lysis buffer (0.2 M EGTA, 1 mM calcium chloride, 10 mM Tris–HCl pH 7.5). DNA was ethanol precipitated (2× volume of 100% EtOH), and

the pellet was dissolved in a 500 µl lysis solution (100 mM NaCl, 25 mM EDTA, 0.5% SDS) together with 5 µg ml^{-1} proteinase K, and incubated over night at 55°C. The genomic DNA was further purified using the phenol/chloroform–ethanol method (Sambrook et al. 1989) with an additional TE/RNase A digestion (10 mM Tris–HCl pH 7.5, 1 mM EDTA pH 8.0, 50 µg ml^{-1} RNase A) for 15 min at 37°C followed by a phenol/chloroform–ethanol extraction. The genomic DNA was finally dissolved in 50 µl TE buffer (pH 8.0). Also, two commercially available kits were tested including Mo-Bio's Ultra Clean Soil DNA™ purification kit (Mo-Bio Laboratories, Inc.) and Qiagen's DNeasy® tissue extraction kit (Qiagen, Inc.). In addition to following manufacture instructions and published protocols, we also evaluated whether digestion with proteinase K, chitinase, and RNase A individually or in combination with each other, improved DNA extraction recovery or purity for subsequent PCR amplification. After completion of these optimization studies, it was concluded that there were no clear systematical differences in DNA yield and purity, between the different methods, but the most robust method, based on total reproducibility and methodological ease, was the Qiagen DNeasy® extraction method (Simonelli et al. in preparation). Thus, after initial optimization studies, copepod and algal DNA was extracted routinely using the DNeasy® protocol as described by the manufacture's instructions for total DNA from animal tissues with an RNase A treatment as recommended by the manufacturer. All samples were eluted in 200 µl of PCR grade nuclease-free water.

Algal-specific qPCR primers

We designed an 18S rRNA gene-targeted PCR primer pair specific for the cryoptophyte genus *Rhodomonas* (RhodoF-624 and RhodoR-660) and haptophyte genus *Phaeocystis* sp. (PhaeoF-489 and PhaeoR-683), respectively. In addition, a previously designed 18S rRNA gene targeted PCR primer pair specific for the haptophyte algal species *E. huxleyi* were used (Nejstgaard et al. 2003). All primers are shown in Table 1. The design of *Rhodomonas*- and *Phaeocystis*-specific primers were facilitated using the BioEdit (version 7.0.5.2) sequence database editor (Hall 1999). Species specificity was evaluated in silico by comparison against sequences in the Ribosomal Database Project II (release 8) and against sequences in GenBank using the Blastn utility for short sequences. The specificity of the primers were empirically tested in standard PCR assays using genomic DNA purified from several other algal species including the cryptophyte *Hemiselmis* sp., the bacillariophytes *Chaetoceros muelleri*, *Coscinodiscus wailesii*, *Pseudo-nitzschia pseudodelicatissima*, *Skeletonema* sp., *Thalassiosira weissflogii*, the chlorophytes *Brachiomonas*

Table 1 PCR primers and reaction conditions used in this study

Specificity	Forward primer	Reverse primer	Expected amplicon size (bp)
Emiliania huxleyi	EhuxF-745 5′ TCA AGC AGG CAG TCG	EhuxR-803 5′ CAC GAG AGT CCT ATT TCA	59
Phaeocystis sp.	PhaeoF-489 5′ GGC TAC TTC TAG TCT TGT AAT GGG A	PhaeoR-683 5′ AAA GAA GGC CGC GCC	209
Rhodomonas sp.	RhodoF-624 5′ AGC TGT CGG CCT TTG GTC	RhodoR-660 5′ TAG GAT CCC CAG GCA GAA	58

qPCR reaction mix: 20 μl reaction volume; 0.01–100 ng purified DNA (one case up to 800 ng); 2.5 mM MgCl$_2$ final concentration; 0.3 μM of each primer in QuantiTech® SYBR Green (Qiagen) or 0.5 μM of each primer in DyNAmo HS®

Emiliania huxleyi 95°C, 15 min; 40 amplification cycles (94°C, 15 s; 48°C, 30 s; 72°C, 60 s), 72°C, 10 min

Phaeocystis sp. 95°C, 15 min; 36 amplification cycles (94°C, 30 s; 55°C, 30 s; 72°C, 30 s), 72°C, 10 min

Rhodomonas sp. 95°C, 15 min; 36 amplification cycles (94°C, 30 s; 62.3°C, 30 s; 72°C, 30 s), 72°C, 10 min

submarina, *Dunaliella* sp., *Nannochloris* sp., the dinophyte *Gymnodinium nelsoni*, the haptophytes *Chrysochromulina ericina*, *E. huxleyi*, *Isochrysis galbana*, *Pavlova lutheri*, *Phaeocystis pouchetii*, *P. globosa*, *Prymnesium parvum*, and the prasinophytes *Micromonas pusilla*, *Pyramimonas orientalis* and *Tetraselmis* sp.. The potential for non-specific amplification by these primer sets of metazoan and prokaryotic genes was also evaluated. Genomic DNA from several metazoan species including the copepods *C. finmarchicus* and *Eucalanus pileatus*, the blue crab (*Callinectes sapidus*), the bay scallop (*Argopecten irradians*), herring sperm and genomic DNA purified from two bacterial species (*Escherichia coli* and *Bacillus subtilis*) were also tested with these primer sets.

Each alga-specific primer set was further quantitatively evaluated in a real time qPCR assay using pure cultures of the target algal species. Defined volumes of exponential growth phase culture were filtered onto 0.8 μm Supor® filters (Pall Life Sciences) so that individual filters contained from 1 to 7,800 cells per filter. Filters of *P. pouchetii* contained ca. 2, 20, 200, and 1,000 cells per filter, filters of *E. huxleyi* contained ca. 1, 10, 70, 350, 700, and 1,400 cells per filter, filters of *R. marina* contained ca. 15, 75, 160, 800, 1,500, and 7,800 cells per filter. Each filter was extracted using the DNeasy® extraction (Qiagen) as described above.

Quantitative PCR

Quantitative real time PCR assays targeted to the algal prey species *E. huxleyi*, *R. marina*, and *P. pouchetii* were developed and validated in this study. All real time PCR reactions were performed in 20 μl reaction volumes using the alga-specific primer sets (Table 1). Initial real time qPCR assay development using the *E. huxleyi* and *P. pouchetii* genomic DNA were performed using a Bio-Rad iCyclerIQ Real-Time Detection System (Bio-Rad Laboratories, Inc.). Reactions were performed in 96-well plates with 10 μl of

2× QuantiTech SYBR Green Master Mix (Qiagen), 0.3 μM of primers, and 0.01–100 ng extracted algae or copepod DNA. All subsequent assay development and field studies utilized a MJResearch Opticon® 2 real-time thermal cycler, similarly performed in 96-well plates, with each reaction well containing 10 μl of 2× Dynamo HS™ SYBR Green Master Mix (Finzymes Oy, Finland), 0.5 μM of each primer, and 0.8–100 ng DNA extracted algae or copepods (except in one case when testing for interference of copepod DNA). The appropriate amount of template DNA in all assays was generally achieved using 1–5 μl of either undiluted or a ten-fold dilution (in water) of the final elution from the DNeasy purification eluted in 200 μl of PCR grade water. Amplification cycle conditions are reported in Table 1. Each reaction was followed by a melt-curve thermal profile from 65 to 95°C to evaluate the specificity of the primers. For each qPCR run, a dilution series of extracted genomic DNA from algae in culture or present in the water (100 ml) from field samples, were run as standards together with the copepod samples. PCR grade water was used as template for negative control.

Interference of copepod DNA and other non-target material in the qPCR reactions

To investigate the influence of copepod material on quantification of specific algal prey, independent of potential digestion processes inside the copepod guts, two sets of experiments were conducted. The algae used in both experiments were *R. marina* in exponential growth.

First, qPCR reactions were conducted with series of increasing amount of genomic algae DNA (0.01, 0.1, 1, 10 and 100 ng DNA per reaction) in the presence of increasing amounts of genomic DNA purified from starved adult female *C. finmarchicus* (4, 10, 40, 100, 400 and 800 ng DNA per reaction), The efficiency of the qPCR assay was estimated based on the slope of the log cell number versus

the Cycle Threshold (C_t) curve. Efficiency was calculated as $[(10^{\text{slope}})\text{-}1] \times 100$ with a slope of -0.301 representing 100% qPCR efficiency (Wong and Medrano 2005).

Secondly, to determine whether quantification of algal prey DNA was affected by presence of co-purifying copepod material, relatively low concentrations of *R. marina* (200 or 2,000 cells per sample) were extracted either alone (triplicates) or in the presence of starved *C. finmarchicus* with empty guts (either 2, 3 or 8 copepods per single extractions), where after the algae cells were quantified by qPCR.

Both real time thermal cyclers used in this study, the BioRad iCycler and the MJResearch Opticon® 2, appear to perform equivalently, and since we used dilution series of extracted cells as standards in all quantification assays, this should correct for differences between machines as well as between separate runs on the same machine.

Statistical analysis

Each sample for qPCR (standards, samples and controls) was run in at least three PCR reactions. A mean of these runs for each sample was used for the further analysis. Thus only true replicated samples (not the individual qPCR runs) are used to evaluate the reproducibility of the results. Basic statistical analyses were performed using Microsoft® Exel®. Statistical comparisons of linear regressions (elevations and intercepts) were facilitated using Sigma Stat® v 3.0.

Results

Assay development

Specific qPCR primers

The specificity of the primers for each of the algae taxa *E. huxleyi*, *Phaeocystis* sp. and *Rhodomonas* sp. were empirically tested in standard PCR assays using genomic DNA purified from several other algae, metazoan and pro-karyotic species. As predicted by in silico sequence analyses, amplification products were only produced from genomic DNA of the target algae (data not shown). Each alga-specific primer set was further quantitatively evaluated in a real time qPCR assay using mono cultures of *P. pouchetii* (Fig. 1a), *E. huxleyi* (Fig. 1b) and *R. marina* (Fig. 1c), respectively. Highly significant linear relations between cell numbers and qPCR threshold cycle (C_t) were achieved with each primer set ($P \leq 1 \times 10^{-6}$, $r^2 = 0.81$–0.95). The regression coefficients represent variability associated with both the extraction protocol and triplicate qPCR reactions.
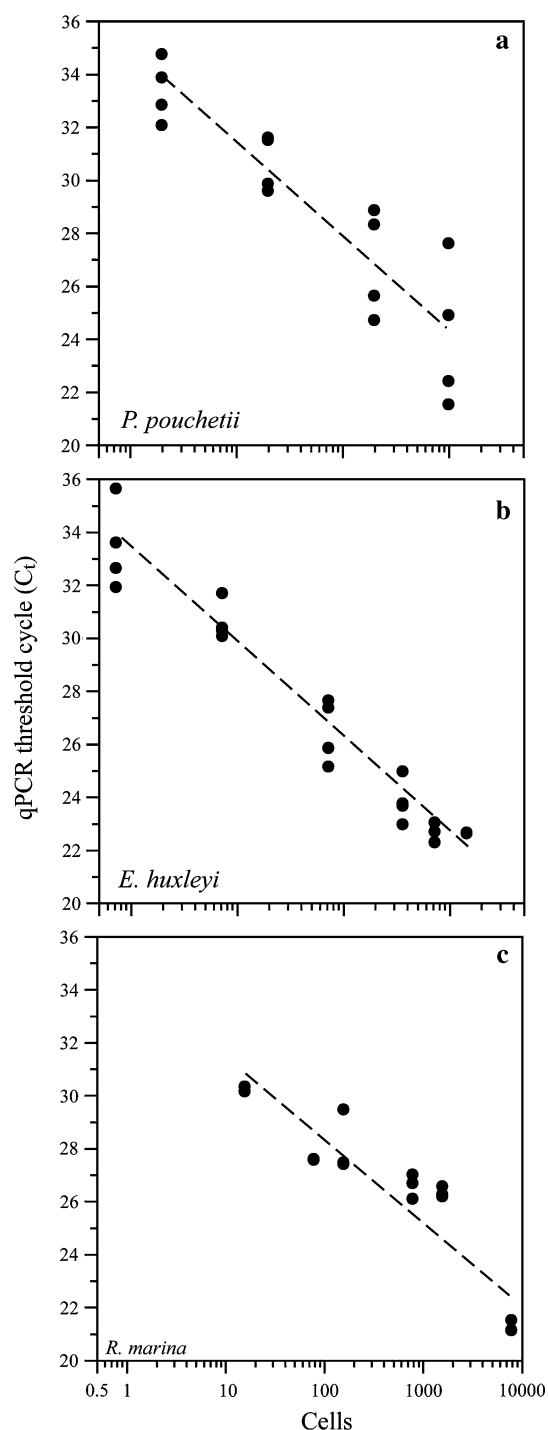
Fig. 1 Quantification of free-living microalgae. **a** *Phaeocystis pouchetii* ($f(x) = -1.50 \times \ln(x) + 34.70$, $r^2 = 0.820$, $P = 1 \times 10^{-6}$, $n = 16$), **b** *Emiliania huxleyi* ($f(x) = -1.56 \times \ln(x) + 33.21$, $r^2 = 0.951$, $P = 2 \times 10^{-14}$, $n = 22$), and (**c**) *Rhodomonas marina* ($f(x) = -1.25 \times \ln(x) + 34.04$, $r^2 = 0.812$, $P = 8 \times 10^{-7}$, $n = 17$) by real time quantitative PCR. Algal cultures are used as quantitative standards for estimating copepod gut content in units of algal cells per individual. All results are from single runs with 2–4 replicates per dilution (note that the highest concentration of *E. huxleyi* were run as duplicates but these differ <1% and appear as one). Cell numbers <1 represents diluted samples

The quantification of cells was linear over the range of cells tested (3–4 orders of magnitude) with a detection limit of as little as a single cell.

Quantitative standards

In similar experiments, the effect of growth condition of algal strains on quantification of 18S rRNA gene fragments by qPCR was investigated. In these studies, quantification by qPCR of a 1-week-old (exponential phase) and a 2-week-old (plateau phase) culture of *E. huxleyi* was compared (Fig. 2). Although both cultures yielded quantitative linearity with slopes not significantly different from each other (using log-transformed *x*-axis (cell) numbers: slope = -3.49 ± 0.10; $P = 0.781$), the intercepts were significantly different from each other (1 week = 38.31 ± 0.22; 2 weeks = 43.65 ± 0.58; $P < 0.001$). These results suggest that per cell 18S rDNA copy number differs with respect to the physiological condition of the algae. This has large implications for the choice of algal standards for quantification of prey-specific gut content.

Interference of copepod DNA and other non-target material

Considering that even with a full gut the biomass of prey inside a copepod represents a minority fraction of the total copepod biomass, it is likely that total DNA extracts from a copepod will be dominated by copepod DNA rather than DNA derived from prey cells. Thus, although the primers used in these studies to detect prey cells are prey specific and will not amplify the host (copepod) DNA, it is possible
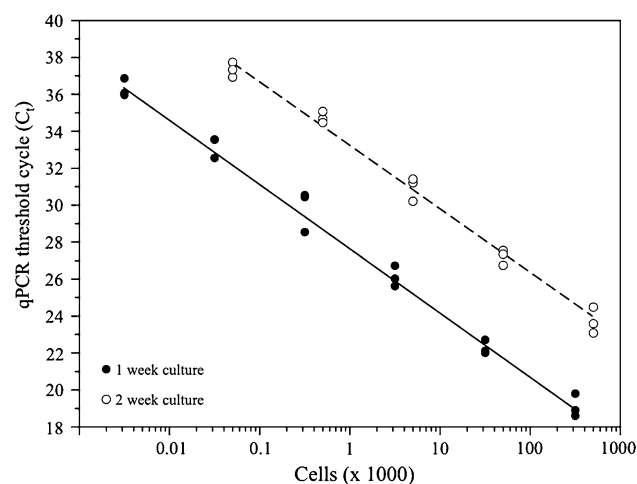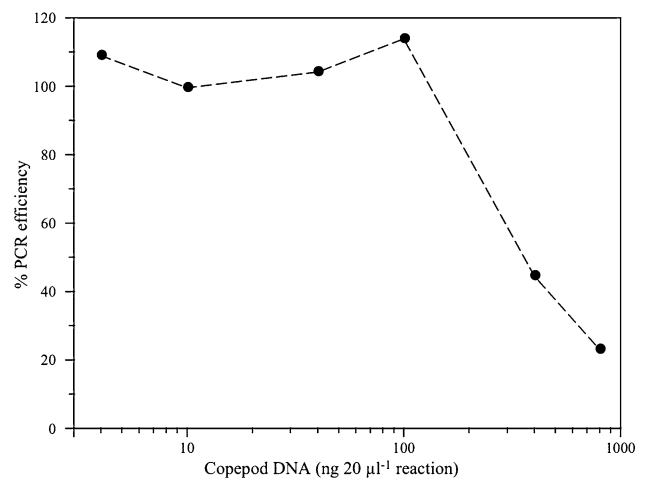
that the large amount of non-target DNA or the presence of co-purifying material from the host copepod may interfere with quantification.

To investigate the influence of naked copepod genomic DNA on quantification of specific algal prey, qPCR reactions were conducted in the presence of increasing amounts of genomic DNA purified separately from the prey alga *R. marina* (ranging from 0.01 to 100 ng DNA per reaction) and starved adult female *C. finmarchicus* (ranging from 4.0 to 800 ng DNA per reaction, Fig. 3). In these experiments the addition of as much as 100 ng of non-specific *C. finmarchicus* DNA per 20 μl qPCR reaction (5 ng μl^{-1}) did not appear to affect PCR efficiency with an average efficiency of $106 \pm 6\%$ (Fig. 3). However, at concentrations of 20 ng μl^{-1} (400 ng per 20 μl^{-1}) and 40 ng μl^{-1} (800 ng per 20 μl^{-1}) of *C. finmarchicus* genomic DNA, qPCR efficiency declined to 45 and 23%, respectively. Further, excluding reactions based on 400 and 800 ng total copepod DNA, yielded a standard curve with a very high fit $r^2 = 0.991$, $n = 20$, and very low variation also at the lowest algae DNA concentrations (0.01 ng DNA per reaction, not shown). Thus there was no difference between the detection efficiency at predator-to-prey DNA-ratios up to 10,000:1.

To determine whether quantification of algal prey DNA was affected by the presence of co-purifying copepod material, two relatively low concentrations of *R. marina* cells (200 or 2,000 cells per sample) were extracted in the presence of increasing numbers (0–8) of starved *C. finmarchicus* with empty guts, whereafter prey cells were quantified by qPCR (Fig. 4). The quantification of prey algae did not vary significantly between treatments with or without

Fig. 2 Comparison of qPCR quantification from different aged cultures of *Emiliania huxleyi*. Quantification of rDNA cycle threshold number (C_t) in 1 week ($f(x) = -1.52 \ln(x) + 38.31$, $r^2 = 0.991$, $P = 1 \times 10^{-17}$) and 2 weeks ($f(x) = -1.51 \ln(x) + 43.65$, $r^2 = 0.989$, $P = 4 \times 10^{-14}$) old algal cultures

Fig. 3 Effect of increasing amount of copepod (*Calanus finmarchicus*) genomic DNA on qPCR amplification efficiency of a 58 bp *Rhodomonas marina*-specific rRNA gene fragment. Amplification efficiency was obtained from standard curves using 0.01, 0.1, 1.0, 10 and 100 ng genomic *R. marina*-DNA, treated with series of added copepod DNA (4, 10, 40, 100, 400 and 800 ng) per 20 μl qPCR reaction
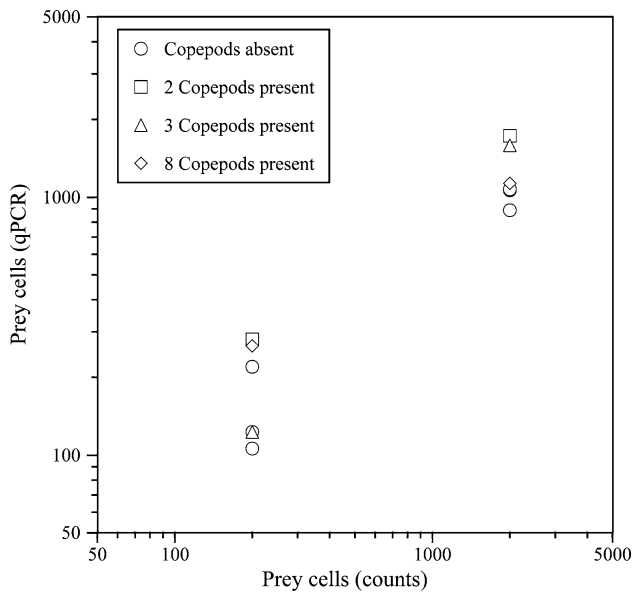
Fig. 4 qPCR amplification of *R. marina*-specific rRNA gene in the absence and presence of co-extracted starved copepods (2, 3 or 8 females of *Calanus finmarchicus*, per extraction tube). The correlations between cell counts based on qPCR (DNA) and direct microscopy cell counts are shown. Note that two of the amplifications at 2,000 cells of *R. marina* in the absence of copepods differ <3% and appear as one

copepods ($P = 0.30$, $n = 3$ for the 200 cells treatment and $P = 0.07$, $n = 3$ for the 2,000 cells treatment, paired two-tailed t test). Further, since most of the values with co-purifying copepod material appeared to be slightly higher than the values with algae alone (in the 200 cells treatment: averages ± SD without copepods 150 ± 61 and with 2–8 copepods 223 ± 87; and the 2,000 cells treatment: averages ± SD without copepods $1{,}009 \pm 103$ and with 2–8 copepods $1{,}483 \pm 313$), there was no indication of lower algae cell detection in presence of 2–8 copepods per extraction.

These studies indicate that neither extraction nor amplification of algal DNA was limited by presence of copepod DNA in concentrations up to 5 ng μl^{-1} or at predator:prey DNA-ratios up to 10,000:1 in the qPCR reactions. This suggests that the presence of specific algal DNA may be effectively quantified in copepods also at low feeding rates of single prey types, providing that the concentration of copepod DNA is kept below this value in the qPCR reactions.

Feeding studies

Quantification of algae consumed by copepods was assessed in both laboratory and field studies. In the laboratory setting, feeding (gut filling) experiments were conducted with females of *C. finmarchicus* and two model algal prey species *R. marina* and *E. huxleyi*. The amount of

algae ingested per copepod was estimated by real time qPCR and total gut fluorescence. In the laboratory experiments, a quantitative relationship ($r^2 = 0.95$–0.97) between gut contents assessed by fluorescence and by real time PCR was observed for both algae (Fig. 5). However, the estimated absolute number of algal cells in the guts, based on DNA, was systematically lower (2–32%) than the number of prey cells estimated by gut pigment (chlorophyll *a*, and derivates) analysis.
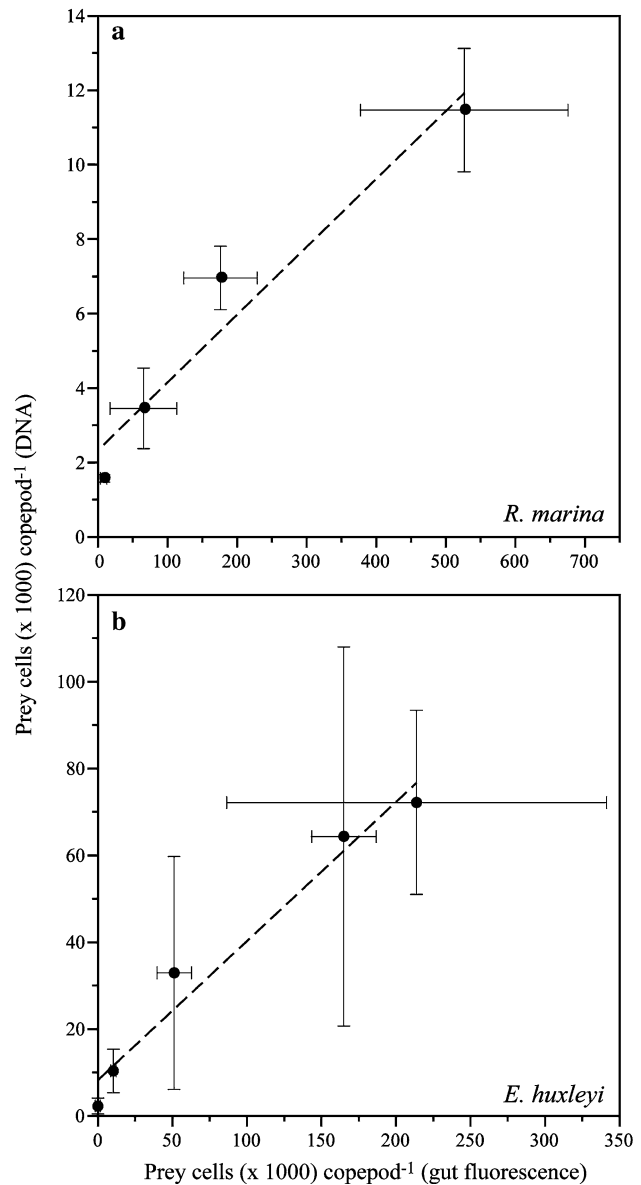

Fig. 5 Comparison of estimated copepod (*Calanus finmarchicus* females) gut content of (**a**) *Rhodomonas marina* and (**b**) *Emiliania huxleyi* by qPCR and gut fluorescence (chlorophyll *a*). Error bars represent the standard deviation of the mean ($n = 2$–3). The regressions are described by the equations: **a** ($f(x) = 0.018x + 232$, $r^2 = 0.95$, $P = 0.027$), and **b** ($f(x) = 0.320x + 821$, $r^2 = 0.97$, $P = 0.0028$)

Comparable quantitative results were also observed in the field (Fig. 6). In three different experiments during bloom and post bloom conditions in the English Channel, the specific consumption rate of the alga *P. globosa* by two different copepod species, *Acartia clausii* and *T. longicornis*, was investigated by bottle incubation grazing studies and DNA-based gut analyses. Similar to the controlled laboratory experiments, there was a strong quantitative relationship between DNA-based and independently determined feeding rates on the algae *P. globosa*. Note that the value with the greatest deviation from the common regression is based on a single cell count measurement, and thus represents the most uncertain value. This indicates a significant relationship between feeding estimates for the other five replicated measurements ($r^2 = 0.998$, $P = 0.00004$). Error estimates (similar for both qPCR and gut pigment) are at least partly due to the low replication ($n = 2$–3, in most cases, Figs. 5, 6), and may thus be reduced with higher, but still feasible replication ($n = 4$–5) in future field studies.

As was observed in the laboratory studies, feeding estimates based on gut DNA were consistently lower than
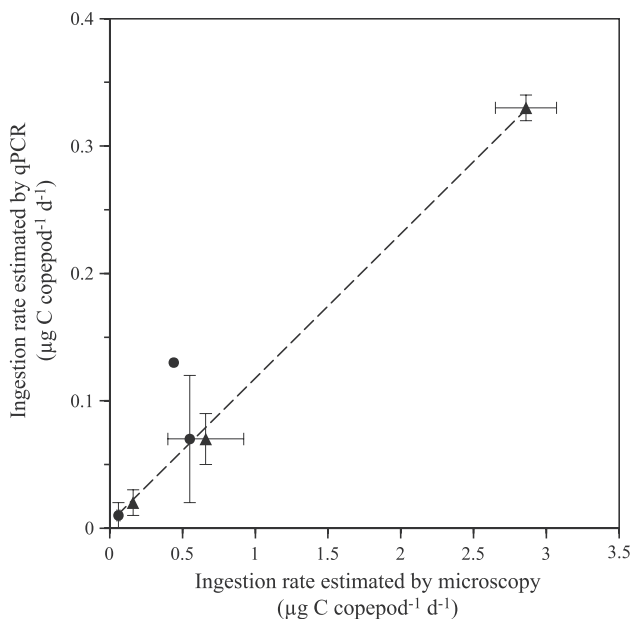
Fig. 6 Comparison of estimated selective ingestion rates by qPCR and direct microscospy of female copepodite *Temora longicornis* (*triangles*) and *Acartia clausii* (*circles*) on *Phaeocystis globosa* in English Channel water during May 2003 and April 2004. qPCR and microscopy were conducted with animals incubated for 24 h in natural water containing *P. globosa*. Linear regressions based on all data points (shown): $f(x) = 0.109x + 0.018$, $r^2 = 0.931$, $P = 0.0018$, $n = 6$, and excluding the possible high outlier of *A. clausii f(x)* = $0.114x + 0.002$, $r^2 = 0.998$, $P = 0.00004$, $n = 5$), are both highly significant. Error bars for each data point represent the standard deviation of the mean for two replicated samples ($n = 2$) for each copepod treatment (except for the possible high outlier of *A. clausii* where $n = 1$)

those determined independently by classical microscopy based approaches. Ingestion rates based on qPCR gut DNA measurements for both the copepods were ca. 11–20% of rates derived from incubation experiments analyzed for particle removal by microscopy for that alga.

Discussion

During the past decade qPCR has begun to be applied widely in ecological studies including the quantification of algal species in marine planktonic and sediment environments and for investigations of protist parasites and pathogens of marine metazoans (Zhu et al. 2005; Dyhrman et al. 2006; Frischer et al. 2006; Lyons et al. 2006; Moorthi et al. 2006). PCR-based assays are now becoming routine in marine ecology studies, especially to detect free-living organisms. However, quantitative estimates of target species associated with larger host organisms presents a unique set of methodological challenges including the development of efficient quantitative DNA extraction and purification protocols, minimization of PCR artifacts associated with the detection of the target organism in the environment of a host organism, and importantly, the use of appropriate quantitative calibration standards. In this study, DNA purification and qPCR amplification conditions were investigated using several different algal prey species consumed by copepods (*C. finmarchicus*) to identify the most efficient approach for extracting prey organisms (algae) after ingestion by copepods. Relative extraction efficiency obtained using several different extraction and purification chemistries was assessed by qPCR. Two commercially available DNA extraction kits with several different modifications and two phenol/chloroform based extraction protocols were evaluated over the course of these studies. There were no obvious quantitative advantages between the protocols that were evaluated in this study. However, we adopted the Qiagen's DNeasy® tissue extraction kit with the recommended proteinase K and RNaseA digestions (Qiagen Inc.), because it is methodologically simpler and reliable.

Three SYBR Green Real Time PCR assays targeted to the haptophyte algae *Phaeocystis* sp. and *E huxleyi* and the cryptophyte *Rhodomonas* sp. 18S rRNA genes were developed and successfully used to quantify these algae in culture and water samples (Fig. 1). The same assay was also used to quantify these algal species from copepods after they had been consumed. However, when absolute estimates of prey algae recovered from copepods based on DNA were compared to independent estimates of ingested algae, they suggested that algal consumption was underestimated by the DNA based qPCR assays (Figs. 5, 6).

At least two possible explanations other than low prey genomic DNA extraction yields from copepods might

explain these observations. First, the presence of large amounts of non-target genomic DNA may interfere with PCR amplification (Kanagawa 2003), and second, materials other than DNA that co-purify with DNA in the extraction procedure might inhibit amplification. However the results in Figs. 3 and 4 suggested that DNA extraction and PCR amplification was not influenced by the presence of non-specific copepod DNA or the presence of PCR inhibitors derived from copepods when performing the reactions as described here. Neither the amount of DNA nor the presence of inhibitors from copepods could account for the observed underestimation of algal prey consumed by copepods.

A remaining hypothesis that might help to explain the low detection of ingested algae is that they are rapidly digested during gut transit. For insects, spiders and other predators, it has been shown that the detection of DNA by PCR decreases with digestion time and PCR amplicon target length (Symondson 2002; Sheppard and Harwood 2005; Deagle et al. 2006; Jarman et al. 2006). Relatively little is known about the digestion of prey-associated DNA during copepod gut transit. Compared to insects, copepods have a simpler gut structure (Brunet et al. 1994), relatively neutral gut pH (Pond et al. 1995), and shorter digestion time (compare Irigoien 1998 for copepods; and Hoogendoorn and Heimpel 2001 for insects), suggesting that the use of DNA as feeding tracers should work at least as well in copepods as in insects. Recently, electron microscopy (Jansen and Bathmann 2007) and cultivation approaches (Montresor et al. 2003) demonstrated that significant fractions of diatoms and dinoflagellates consumed by different copepods remained intact in ejected fecal pellets and live cells could be isolated from them. Similar results have also been observed for at least one of our model algae, *P. pouchetii* (Nejstgaard et al. 2007). These observations suggest that digestion may be partial and that a significant fraction of prey associated macromolecules might survive gut transit in copepods. Further, since DNA is a chemically stable molecule in living cells, it is likely that DNA should be more resistant to breakdown in the gut compared to the less stable photosynthetic pigments. However, we are not aware of any quantitative studies of DNA gut passage time or breakdown in copepods and it is well known that gut transit time and digestion vary with respect to temperature and feeding rate, copepod species and developmental stage, feeding history, acclimation and physiological status of the copepod, and probably gut environment (e.g. pH and gut microflora) (see Mauchline 1998, for a thorough review). Yet another possible source of variable gut DNA degradation could be the freeze-thawing regime when sampling the copepods. Thus, the fate of prey DNA ingested by copepods needs to be further assessed before this approach can be used independent of other methods. Meanwhile, this method can be used to quantify ingestion of specific prey when calibrated as discussed below.

One of the most important elements of a real time qPCR assay is the choice of quantification reference standards. Most frequently, cloned target genes are used as calibration standards and quantification is reported as the number of gene copies present (Wong and Medrano 2005). The use of gene copy standards would require reliable conversion factors between ribosomal gene copies and cell number. In this study the variability of ribosomal copy number per cell as a function of cell growth condition was investigated by comparing qPCR detection of rRNA genes from 1 to 2-week-old cultures of *E. huxleyi*. From these studies it was clear that ribosomal gene copy number varied depending on the physiological condition of the algae (Fig. 2). Other studies have also shown that the number of rRNA genes per cell can vary greatly (Tourova 2003). Thus, it is recommended that rather than utilizing cloned target genes contained in plasmids as reference standards, in situ target cell numbers is a more appropriate quantification standard. Additionally, by extraction of genomic DNA from prey cells in parallel with extraction of DNA from copepods, variability associated with DNA extraction can be accounted for in the quantification standard curve. Furthermore, the use of prey organisms rather than cloned genes as reference standards is critical for quantification of prey species that contain nucleomorphic rRNA genes (Liaud et al. 1997) or that are known to possess large and variable numbers of duplicated ribosomal and other genes (Prescott 1994).

Both laboratory and field studies demonstrated strong quantitative relationships between gut DNA content and independently obtained gut content or feeding rate estimates for the specific prey. Indeed, the field data from incubation experiments with natural plankton collected at three different occasions showed a strikingly strong correlation between feeding rates estimated from gut DNA content and gut passage time, versus feeding rates estimated for *Phaeocystis* by overnight bottle incubation experiments analyzed by microscopy (Fig. 6).

Development of a correctly calibrated qPCR approach to estimate gut content of copepods will have a distinct advantage for assessing feeding in natural plankton because it would provide the possibility to directly identify and quantify in situ ingestion by an individual or group of copepods without incubations. Further, when calibrated, the qPCR based analysis allows a much larger number of animals to be analyzed than when only using the very time consuming incubation experiments analyzed by microscopy (Verity and Paffenhöfer 1996; Nejstgaard et al. 2001). Lastly, since all living prey contain DNA, targeting genes as feeding biomarkers does not put any theoretical constraint on the type of prey that can be analyzed. Since non-pigmented prey including ciliates and heterotrophic dinoflagellates are

generally available, and even preferred, as prey for copepods (Stoecker and Capuzzo 1990; Kleppel 1993; Fessenden and Cowles 1994; e.g. Atkinson 1996; Nejstgaard et al. 1997; Levinsen et al. 2000; Calbet and Saiz 2005), the ability to directly investigate feeding on non-pigmented organisms by copepods is of considerable importance. Further, specific genetic markers are becoming increasingly available in the literature and elsewhere; this can be used to boost the development and use of this technique.

In conclusion, when calibrated for prey target gene copy number, copepod ingestion of prey can be semi-quantitatively estimated using a qPCR approach. Although, presently only a relative quantification is achieved, this method can be applied in the field to approach questions of ecological relevance with high resolution, and with less disturbance than has been possible using more time-consuming classical approaches alone, especially for non-pigmented and macerated prey. However, future studies should aim to achieve a quantitative measure of feeding rates in situ, correcting for breakdown in prey DNA, in order to make this approach independent of calibrations to other methods.

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