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Evaluation of DNA extraction and handling procedures for PCR-based copepod feeding studies

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Molecular methods are becoming increasingly common for taxonomic and ecological studies of marine and freshwater plankton. Recently, nucleic acids have been used as target molecules for identification and quantification of prey species in studies of trophic interactions. A critical step in the quantification of mesozooplankton feeding by molecular analysis is the isolation of microalgal DNA from predator guts and in the food environment. It is essential that total genomic DNA extraction provides maximum quantitative yield suitable for downstream analysis. In this study, we compared the efficacy and experimental variability of eight different protocols for total genomic DNA extraction from free-living microalgae and microalgae within the gut of copepods. We also developed and evaluated different sampling procedures for copepods prior to genomic extraction. The optimal protocol was evaluated using real time quantitative polymerase chain reaction (qPCR) and the integrity of the genomic DNA was determined by amplifying PCR targets of increasing size. Considerable variability was observed between purification protocols. Qiagen DNeasy® Blood & Tissue kit was the most efficient of the tested methods for genomic extraction from both free-living microalgae and microalgae inside copepod guts. Furthermore, the appropriate handling of predator copepods prior to genomic extraction was essential for quantitative gut content estimates.

INTRODUCTION

Copepods dominate the marine plankton biomass and they play a critical role in the marine food web as mediators of energy transfer to higher trophic levels (Verity and Smetacek, 1996). Thus, the ability to accurately estimate copepod feeding preferences and rates is vital for a quantitative understanding of trophic interactions and processes that structure marine ecosystems. However, because of methodological constraints, it remains a challenge to identify and quantify copepod feeding *in situ* without significant experimental bias (Båmstedt *et al.*, 2000; Nejstgaard *et al.*, 2003, 2008). Molecular techniques could potentially overcome this challenge. These techniques are increasingly being used in taxonomic and ecological studies of plankton in marine and fresh water systems. In particular, polymerase chain reaction (PCR) based techniques are becoming more and more common for the detection of prey species in trophic interactions in plankton and elsewhere (Nejstgaard *et al.*, 2003; Harwood and Obrycki, 2005; Jarman *et al.*, 2006 and recently reviewed by King *et al.*, 2008). There has been a promising development in PCR-based methods to detect prey-specific nucleic acid target molecules in the diet of copepods and other marine zooplankton. For example, Nejstgaard *et al.* (Nejstgaard *et al.*, 2003) demonstrated successful extraction of total genomic DNA and PCR amplification of the prey-specific 18S ribosomal RNA gene from the guts of calanoid copepods and from their fecal pellets. A major advantage of using nucleic acids for gut

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content analysis is that they allow precise identification of species-specific predator–prey relationships which is not possible to ascertain based on most other classes of biomarkers.

To further develop nucleic acid-based methods for gut content analysis, efforts have been undertaken to estimate feeding rates by quantifying prey DNA in predator guts using real time quantitative PCR (qPCR). Troedsson *et al.* (Troedsson *et al.*, 2007) and Nejstgaard *et al.* (Nejstgaard *et al.*, 2008) used this approach to estimate feeding rates of appendicularians and copepods, respectively. Although successful for appendicularians, the assay developed for copepods highlighted two main concerns. First, a critical step in the use of prey nucleic acids as biomarkers for quantitative estimates of trophic links is the use of *in situ* target cells as standards for the quantitative PCR assay. It is therefore necessary to use DNA purification methodologies that yield maximal efficiency on free-living prey and from within the gut of copepods. The second concern was that the prey ingestion rates obtained by qPCR were significantly underestimated compared with independent methods. Nejstgaard *et al.* (Nejstgaard *et al.*, 2008) hypothesized that qPCR-based gut content underestimates were due to rapid digestion of prey DNA inside copepod guts. Durbin *et al.* (Durbin *et al.*, 2008) reached a similar conclusion based on investigations of feeding by the carnivorous copepod *Centropages typicus* on another copepod, *Acartia tonsa*. In recent laboratory studies, Troedsson *et al.* (Troedsson *et al.*, 2009) demonstrated that algal prey DNA is rapidly digested after ingestion by the copepod *Calanus finmarchicus*. Thus, for the development of quantitative nucleic acid-based gut content assays for copepods, it is critical to optimize sampling and DNA purification protocols. These procedures must maximize prey DNA recovery, minimize experimental variability and prey DNA digestion.

In this study, we systematically investigated a variety of copepod sampling and handling procedures and DNA purification protocols to develop the most robust and reproducible approach for extracting and purifying prey DNA consumed by calanoid copepods suitable for quantitative PCR-based analyses.

METHOD

Algal cultures

Culture conditions and origins of the 10 algal species used in this study are reported in Table I. Algae were cultured in *f*/2 medium (Guillard, 1975) and in IMR medium (Eppley *et al.*, 1967). Cell concentrations were determined by light microscopy using a Fuchs-Rosenthal hemocytometer.

Genomic DNA purification from algal cultures

Each algal species (ca. 2×10^6 cells) was harvested during exponential growth phase in sterile 50 mL centrifuge tubes (BD Falcon, BD Biosciences). Cells were collected by centrifugation at 5450*g* for 30 min and cell pellets were stored at −80°C until further analysis. Total genomic DNA was extracted using six different methods. Four commercially available kits (Chelex® 100 Bio-Rad, Ultra clean™ soil DNA isolation MoBio, and DNeasy® plant mini kit Qiagen, DNeasy® blood and Tissue kit Qiagen) and two methods based on hexadecyl-trimethyl-ammonium bromide (CTAB) were compared.

The different extraction procedures were as follows. For the Chelex® 100 Bio-Rad method, hereafter referred to as "Chelex", a protocol adapted from Walsh *et al.* (Walsh *et al.*, 1991) was utilized. In this procedure, the algal pellet was dissolved in 200 μL of 10% (w/v) solution of Chelex and incubated at 95°C for 20 min with thorough mixing after 10 min. Following this

Table I: Origin and culture conditions of the algae used in this study

Algae species	Group	Origin	Culture medium	Dark:light cycle (h)	Temperature (°C)
Tetraselmis suecica	Chlorophyte	Raunefjord (Western Norway)	IMR	10:14	15.0
Skeletonema marinoi	Diatoms	Raunefjord (Western Norway)	*f*/2	10:14	9.0
Chaetoceros debilis			*f*/2	10:14	9.0
Pseudonitzschia longissima			*f*/2	10:14	9.0
Rhodomonas marina	Cryptophyte	INFREMER (Brest, France)	*f*/2	10:14	15.0
Prymnesium parvum	Haptophyte	Raunefjord (Western Norway)	*f*/2	10:14	15.0
Phaeocystis pouchetii			IMR	10:14	8.0
Emiliania huxleyi			IMR	10:14	15.0
Isochrysis spp.		BIO UiB algae collection (Bergen, Norway)	*f*/2	10:14	15.0
Chrysochromulina ericina		Raunefjord (Western Norway)	IMR	10:14	15.0

incubation, the tubes were centrifuged at 6000 *g* for 1 min and the supernatant containing genomic DNA was used directly as a template in downstream PCR analysis. For the Ultra clean™ soil DNA isolation MoBio, hereafter called "MoBio", we followed the procedure described by the manufacture for maximum yields. All samples were eluted in 60 μL of PCR grade nuclease-free water. For the DNeasy® plant mini kit Qiagen, hereafter reported as "Plant kit", we followed the procedure described by the manufacturer for total DNA from plant tissues. All samples were eluted in 2 × 50 μL of PCR grade nuclease-free water and the elutants were pooled. The DNeasy® Blood and Tissue kit Qiagen, abbreviated henceforth as the "Tissue kit", was used according to manufacturer's instructions for total DNA from animal tissues with an additional RNase A treatment as recommended by the manufacturer. All samples were eluted in 2 × 100 μL of PCR grade nuclease-free water and the elutants were pooled. In one of the approaches based on CTAB, genomic DNA was extracted using phenol/chloroform−ethanol as described by Sambrook *et al*. (Sambrook *et al*., 1989), and all samples were eluted in 38 μL of PCR grade nuclease-free water. This method is hereafter called "CTAB Phenol/Chloroform". In the second CTAB-based approach, called "CTAB DNeasy® mini spin column", samples were initially extracted in the presence of CTAB as described above and purified using DNeasy® mini spin columns (Qiagen). Briefly, algal pellets were initially dissolved in a 500 μL lysis solution (0.5% SDS, 20 μg/ml proteinase K) and incubated 60 min at 55°C. An extraction buffer containing 80 μL of 5 M NaCl and 100 μL pre-warmed CTAB solution (10% CTAB hexadecyltrimethyl ammonium bromide) in 0.7 M NaCl was added to each sample and incubated for an additional 10 min at 65°C. Genomic DNA was purified from these extracts using a DNeasy® mini spin column (Qiagen) with two subsequent washing steps provided in the kit. Samples were eluted in 2 × 50 μL of PCR grade nuclease-free water and the elutants were pooled.

PCR/qPCR amplification of free-living algae

To determine the best protocol for genomic DNA extraction from free living algae, Chelex, MoBio, Plant Kit, CTAB Phenol/Chloroform and CTAB DNeasy® mini spin column extraction protocols were evaluated using end-point PCR. The protocol resulting in the most efficient extraction was then further compared with the Tissue kit using both end-point PCR and qPCR.

In order to account for the different elution volumes produced from the extraction used here, genomic DNA concentrations for subsequent PCR and qPCR analyses were standardized to 1% of the final elution volume from each protocol. The actual amount of DNA was not routinely quantified.

End-point PCR and qPCR amplifications were performed using the 18S rRNA targeted oligonucleotide primers Univ F-1131 and Univ R-1629 (Tables II and III). As previously described by Troedsson *et al.* (Troedsson *et al.*, 2008), these universal primers generate an amplicon of ∼500 bp from most eukaryotic organisms including all the algal species utilized in this study and are therefore referred to as "universal" eukaryotic primers. End-point PCR reactions were performed in 25 μL reaction volumes using the TaKaRa Bio Inc Ex Taq™ (Mg^{2+} free Buffer) polymerase kit, 0.8 μM of each universal primer (Univ F-1131 and Univ R-1629), 10 mM dNTPs and 1 mM of Mg^{2+}. Amplification was performed using an iCycler™ standard thermocycler (BioRad) as follows: initial template denaturation (95°C for 5 min); 30 amplification cycles (94°C for 30 s; 51°C for 30 s; 72°C for 30 s); and a final extension step (72°C for 10 min). PCR products were visualized by electrophoresis on 1.5% SeaKem® LE Agarose gel and stained with a final concentration of 0.5 μg mL^{-1} ethidium bromide. Real time PCR reactions were performed using the same universal primers described

Table II: Name, sequence and source of the 18S rDNA primers used in this study

Primer (a)	Primer sequence (5′ to 3′) (b)	Source
Rhod 97F	CGT TTA TTT GAT GGT CCC TTA	Troedsson *et al.*, 2009
Rhod 150R	GTC GGA CCT TTG TGC ATG TAT	Troedsson *et al.*, 2009
Rhod 1030F	GCG ACT CCA TTG GCA CCT TGT	Troedsson *et al.*, 2009
Rhod 1450F	GCG CGC TAC ACT GAT GAA TGC	Troedsson *et al.*, 2009
Rhod 1662R	TTT CAC CGG ACC ATT CAA TCG	Troedsson *et al.*, 2009
Univ F-1131	AAA CTY AAA GRA ATT GAC GG	Troedsson *et al.*, 2008
Univ R-1629	GAC GGG CGG TGT GTRC	Troedsson *et al.*, 2008

Table III: Product length, optimal annealing temperature and mean (± SD) qPCR efficiency of the 18S rDNA primer sets used in this study

Forward primer	Reverse primer	Product length (bp)	Annealing temperature (°C)	Mean ± SD qPCR efficiency (%)
Rhod 97F	Rhod 150R	74	55	84.2 ± 9.5
Rhod 1450F	Rhod 1662R	213	56	89.0 ± 7.5
Rhod 1030F	Rhod 1662R	616	63	84.5 ± 7.5
Univ F-1131	Univ R-1629	498	51–52	81.4 ± 6.5

earlier (Tables II and III). An Opticon2 MJ Research real time thermal cycler was used. Twenty-five microliter reaction volumes contained 10 μL of 2 X QuantiTech SYBR Green Master Mix (Qiagen) and 0.3 μM of each primer. Amplification was performed as follows: initial template denaturation (95°C for 15 min); 40 amplification cycles (94°C for 30 s; 52°C for 30 s; 72°C for 30 s; 78°C for 2 s) and a final extension step (72° for 10 min). Fluorescence was measured after both 72 and 78°C to investigate possible primer dimmer formation that would confound the results. Finally, each reaction was followed by a melt-curve thermal profile from 65° to 95°C to evaluate the specificity of the primers. PCR grade water was used as template for negative control. Each sample was run three times. Samples yielding amplification efficiencies lower than 70% were not used in the analysis. Mean primer efficiency in all qPCR reactions was $81.4 \pm 6.5\%$ (Table III).

Feeding experiments

The quantity and quality of prey genomic DNA recovery by the different extraction and purification protocols was determined in controlled gut filling experiments using the calanoid copepod *Calanus* spp. fed the cryptophyte alga *Rhodomonas marina* (Dangeard 1892) Lemmermann. All copepods were collected by gentle net tows from 0 to 30 m depth in the Raunefjord, western Norway (60°16′N, 05°14′E) at the end of November 2007. A 500 μm mesh size, 1 m diameter net with a 14 L non-filtering cod-end was used. Plankton tow samples were diluted in 40 L surface water and transferred within 30 min of collection to a walk-in cold room, maintained at constant temperature (12°C) with dim light on a 14:10 h light:dark cycle. Using a wide mouth pipette, stage CV or CVI females of *Calanus* spp. were sorted into 5 L beakers with food suspensions of *R. marina* (800–1600 μg C L^{-1}). These prey concentrations had previously been shown to yield saturated feeding rates by *C. finmarchicus* (Nejstgaard *et al*, 1995; Båmstedt *et al*., 1999). The food suspensions were replaced every 48 h. Copepods were acclimatized and maintained under these conditions for 3 weeks.

At the beginning of each feeding experiment, animals were starved for 3 h in filtered (GF/F) seawater to empty their guts. To allow each group of animals to fill their guts simultaneously, they were randomly split into 12 groups of ca. 20 individuals, transferred into acrylic Perspex chambers with 500 μm false bottoms containing 450 mL saturating food suspensions of *R. marina* and incubated for 20 min. This incubation time was chosen to maximize gut content and minimize defecation. After the incubation, copepods were removed and quickly rinsed by dipping the Perspex chambers in four consecutive baths of 450 mL filtered (GF/F) seawater. The 12 groups were further divided into three subgroups and processed according to the different sampling procedures (see below).

Sampling methods of copepods

To determine the effect of different sampling manipulation on the efficiency of prey genomic DNA recovery and its integrity, we compared three independent sampling and handling methods. In the first method, after feeding incubation, copepods were flash frozen by dipping the Perspex chambers in liquid nitrogen for 10–20 s to stop gut activity and prevent defecation before further processing. Within few minutes, the copepods were thawed in filtered (GF/F) seawater, collected onto a Petri-dish and randomly sorted under dim light for DNA or gut pigment analysis [as described for gut pigment analysis of copepods, (Båmstedt *et al*., 2000)]. In the second method, copepods were killed after the feeding incubation by dipping the Perspex chambers in 400 mL 80% ethanol. Animals were then collected onto a Petri dish and randomly sorted under dim light for DNA or gut pigment analysis. In the third method, copepods were sedated by dipping the Perspex chamber in 400 mL 0.37 mg/mL tricaine methane sulphonate (MS222) (Sigma). Animals were then collected onto a Petri dish containing 0.37 mg/mL MS222 and randomly sorted under dim light for DNA or gut pigment analysis.

Copepods for gut pigment analyses were extracted for 12 h in 90% acetone at 4°C and analyzed with a Turner Designs™ Model 10-AU Fluorometer as previously described in Nejstgaard *et al*. (Nejstgaard *et al*., 1995). Copepod gut pigment [chlorophyll *a* (Chl *a*) and derivates] concentrations were calculated as described in Båmstedt *et al*. (Båmstedt *et al*., 2000). To convert gut pigment concentration to algal cell number, the pigment concentration from 5, 1 and 0.1 mL of a *R. marina* culture was determined and correlated with cell counts by microscopy (1.4 pg Chl *a* $cell^{-1}$, $r^2 = 0.99$). *Rhodomonas marina* culture was obtained from the same strain, grown at the same conditions and harvested at the same growth phase as the one used in the feeding experiment. Chlorophyll *a* and cell counts were determined in triplicates.

Prey DNA isolation from predators gut

The small size of the copepods (<2.5 mm long) makes dissection of their stomachs impractical; therefore DNA was extracted from whole animals. The efficiency of DNA recovery from algal prey inside the copepods was

compared using the four commercial DNA purification kits described earlier (Chelex, Plant kit, Tissue kit and MoBio). Total genomic DNA from *Calanus* fed algae was extracted using, (i) the protocol recommended by the manufacturer and (ii), according to a modified protocol in which copepods were sorted in pre-heated homogenization buffer and subsequently extracted following the recommended protocol. In the modified Chelex protocol, copepods were sorted into 1.5 mL Eppendorf™ microtubes containing 100 μL of 10% Bio-Rad Chelex at 95°C. For the modified Plant kit protocol, copepods were sorted into 400 μL of buffer AP1 and 4 μL of RNase A at 65°C. In the modified Tissue Kit, prey DNA was extracted by sorting copepods into 180 μL of buffer ATL and 20 μL of Proteinase K at 56°C while for the modified MoBio kit copepods were sorted in 60 μL of solution S1 and 200 μL of Solution IRS (Inhibitor Removal Solution) at 60°C.

qPCR amplification of algae inside copepod guts

To investigate the integrity of prey DNA extracted from copepod guts, three *Rhodomonas* spp.-specific 18S rDNA targeted PCR primer sets (Tables II and III) that amplify different length fragments ranging from 74 to 616 bp (Table III) were used. The level of DNA degradation was determined by observing the decrease in estimated cell number using amplicons of increasing size. As explained in Deagle *et al.* (Deagle *et al.*, 2006), the ability to recover large fragments via PCR/qPCR indicates low level of DNA degradation.

Real time qPCR was performed under the same conditions as reported earlier for free living algae, with the exception of the annealing temperature which differed for the three *Rhodomonas* primer sets (Table III). For qPCR standards, algal cells were enumerated in the cultures by light microscopy using Fuchs-Rosenthal haemocytometer and harvested by centrifugation (7000 g) for 15 min. Cell loss during harvesting was corrected by counting and subtracting the number of cells found in the supernatant. Genomic DNA was purified from harvested cells using the Plant kit as described earlier. Four-point standard curves were made by diluting the extracted DNA in nuclease-free water which amounted to 1.6×10^4, 1.6×10^3, 1.6×10^2 and 1.6×10^1 cells. All standards were run in triplicate and the correlation coefficients (r^2) ranged from 0.993 to 0.998. Each of the triplicate copepod samples for qPCR was run in two independent reactions and the mean of these estimates was used for statistical comparisons. Reactions with amplification efficiencies lower than 70% were not used in the analysis. Mean reaction efficiencies were $84.2 \pm 9.5\%$ for the 74 bp amplicon, $89.0 \pm 7.5\%$ for the 213 bp amplicon and $84.5 \pm 7.5\%$ for the 616 bp amplicon (Table III).

Statistical analysis

All statistical analysis was carried out using SPSS software version 3.5 (SigmaStat, Chicago, IL, USA). Data are expressed as mean ± standard deviation. Comparison between two groups was made by paired *t* tests, while comparison between three groups was first done by a one-way ANOVA, followed by a *post hoc* Holm−Sidak test. The normal distribution of the data was automatically tested by the software.

RESULTS

Extraction and PCR/qPCR amplification of free living algae

As an initial qualitative indicator of the efficacy of the different methods to extract DNA from free-living algae, end-point PCR targeting the 18S rRNA gene was utilized. The Plant kit provided the best overall purification method for genomic DNA, yielding PCR amplifiable DNA from all 10 microalgal species tested as shown by gel visualization of PCR products (Fig. 1A and Supplementary, Fig. 3). The MoBio kit appeared to be the second best followed by the Chelex method.

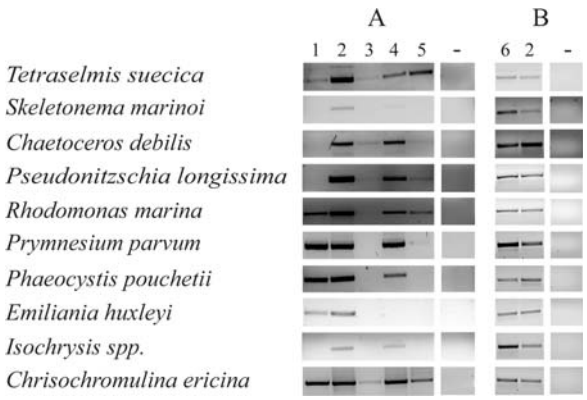

Fig. 1. First (**A**) and second (**B**) experiments to determine the optimal method for genomic DNA extraction from free-living algae. PCR products, visualized by gel electrophoresis, obtained from the DNA isolated with different extraction methods. (1) Chelex® 100, Bio-Rad; (2) DNeasy® plant kit, Qiagen; (3) CTAB DNeasy® mini spin column (Qiagen); (4) Ultra clean™ soil DNA isolation, MoBio; (5) CTAB Phenol/Chloroform; (6) DNeasy® Blood & Tissue kit, Qiagen; (−) negative control. The pictures of the whole gels are available as Supplementary material, Fig. 3.

Protocols based on CTAB generated little or no PCR amplicons (Fig. 1A). In general, there was not a clear relationship between the type of algae and the effectiveness of different DNA purification protocols, but our results indicate considerable variability in overall efficiency between the different protocols.

In a second experiment, we qualitatively compared the effectiveness of genomic DNA extraction from independent algal cultures using the Plant kit and the Tissue kit. Both protocols yielded PCR amplifiable DNA from all 10 microalgae (Fig. 1B and Supplementary Fig. 3). The intensity of the bands, used as a proxy to estimate the amount of extracted DNA, was similar for the two extraction protocols. Only from three algae, *Skeletonema marinoi*, *Prymnesium parvum* and *Isochrysis* spp., did the extraction from the Tissue kit appear to be more efficient (Fig. 1B). To further investigate these methods, we compared the extraction efficiency using a real time qPCR analysis. The cycle threshold *C*(*t*) values obtained were compared for each algal species. Significantly lower *C*(*t*) values for the Tissue kit (Table IV) showed that this kit extracted more genomic DNA than Plant kit from the tested algae (t-test, $P = 0.031$).

Sampling, and extraction of prey algae in copepod guts

Eight genomic DNA extraction methods and three different sampling procedures were compared to determine the best combination to quantitatively extract and purify algal prey DNA (*R. marina*) from predator guts (*Calanus* spp.). Extraction efficiency was determined by comparing the amount of DNA recovered by each protocol expressed as the number of cells in predator guts (cells cop^{-1}). Moreover, the level of degradation of the recovered prey DNA was determined by observing the decrease in amplified target DNA by increasing sized amplicons (Fig. 2 and Table V).

Table IV: Mean (± SD) cycle threshold C(t) values obtained by qPCR amplification of the DNA extracted with Tissue kit and Plant kit for each algal species

Algae species	Tissue kit *C*(*t*)	Plant kit *C*(*t*)
Tetraselmis suecica	13.5 ± 0.2	15.0 ± 0.3
Skeletonema marinoi	15.9 ± 0.2	17.9 ± 0.1
Chaetoceros debilis	10.9 ± 0.0	11.7 ± 0.2
Pseudonitzschia longissima	11.6 ± 0.3	14.3 ± 0.1
Rhodomonas marina	11.5 ± 0.2	13.3 ± 0.1
Prymnesium parvum	11.1 ± 0.1	14.7 ± 0.1
Phaeocystis pouchetii	13.8 ± 0.1	11.3 ± 0.2
Emiliania huxleyi	13.7 ± 0.1	14.9 ± 0.1
Isochrysis spp.	12.5 ± 0.4	16.6 ± 0.2
Chrysochromulina ericina	12.8 ± 0.3	15.6 ± 0.2

Note that lower *C*(*t*) values indicate more template molecules.

The Plant kit and MoBio appeared to be the least efficient methods to extract *R. marina* DNA from copepod guts. Both kits yielded low amounts of prey DNA compared with the other extraction protocols in all of the sampling procedures used (Fig. 2 and Table V).

Although Chelex yielded somewhat higher DNA extractions than the Plant and MoBio kits, especially at room temperature when copepods were either flash frozen in liquid nitrogen or killed in 80% ethanol, the extracted DNA was heavily degraded as indicated by the estimated cell number difference using the 74 bp versus the 616 bp amplicons (174 ± 79 vs. 17 ± 17 cells cop^{-1} respectively when flash frozen, and 127 ± 82 vs. 4 ± 4 in 80% ethanol, Fig. 2A and E and Table V). Further, at 95°C, the DNA yield from the Chelex was even lower than at RT, and comparable with the Plant and MoBio kits, independently of the sampling method (Fig. 2A and Table V). Copepods sedated in MS222 yielded the lowest cell estimates for Chelex in both RT and at 95° (Fig. 2I and Table V).

The Tissue kit combined with MS222 sampling showed the highest extraction efficiency combined with the lowest degradation (Fig. 2J and Table V). When the Tissue kit was combined with the other sampling methods, it also showed high extraction efficiency but higher DNA degradation. When copepods were flash frozen in liquid nitrogen and extracted with the Tissue kit at RT and at 56° C the number of prey cells estimated with the 74 bp amplicon was 99 ± 42 and 103 ± 43 cells cop^{-1}, respectively, while the longer amplicon (616 bp) showed only 30 ± 10 and 29 ± 16 cells cop^{-1}, respectively, indicating degraded DNA (Fig. 2B and Table V). DNA recovered from copepods sampled in 80% ethanol and purified using the Tissue kit at RT resulted in high, but variable, prey cell numbers based on the 74 bp amplicon (252 ± 256 cells cop^{-1}), and because there was a large difference in the estimated gene copy number based on the 616 bp amplicon (48 ± 12 cells cop^{-1}, Fig. 2F and Table V), we conclude that the DNA recovered by this combination of sampling and purification protocols yielded highly degraded material. Combining the sampling in 80% ethanol and the Tissue kit at 56° C may have yielded a lower amount of prey cells with the shortest (74 bp) amplicon (84 ± 5 cells cop^{-1}). However, the similar or higher cell numbers estimated with the two longer amplicons (119 ± 97 and 60 ± 25 cells cop^{-1}, respectively, for the 213 and 616 bp amplicons, Fig. 2F and Table V) indicate that prey DNA was less degraded in the 56°C Tissue kit when using ethanol sampling.

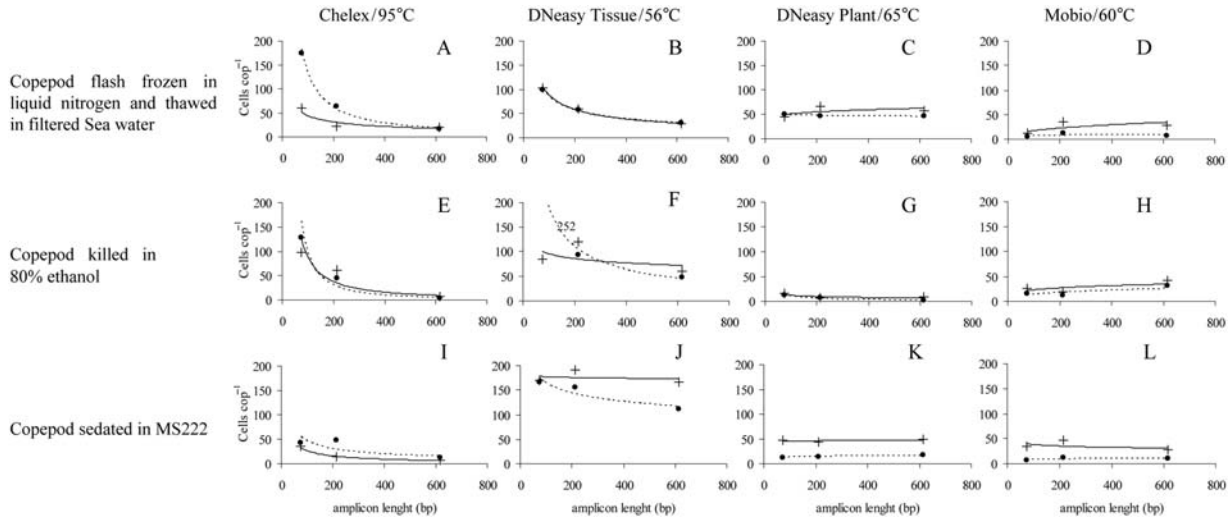

Fig. 2. Number of *R. marina* cells ingested by *Calanus* spp. determined by qPCR using three different length amplicons (74, 213 and 616 bp). Data reported for the three different sampling procedures and for the four different DNA extraction protocols. Dotted lines represent the standard protocols. Plain lines represent the modified protocol in which copepods were sorted after feeding directly into a pre-warmed homogenization buffer.

This was also found when Tissue kit 56°C was combined with the two other sampling methods. Overall, the combination of sedation of copepods in MS222 and sampling them directly into the Tissue kit homogenization buffer at 56°C appeared to be the most effective procedure for purifying algal DNA consumed by *Calanus* spp. (Fig. 2J and Table V). In particular, sorting the copepods directly into a pre-warmed homogenization buffer provide the best combination between total DNA yield and integrity (171 ± 84 and 166 ± 79 cells cop^{-1}, respectively, for the 74 and 616 bp amplicons) (Fig. 2J and Table V).

The comparison between the three different copepod-sampling procedures was based on the

Table V: Mean (± SD) of the number of Rhodomonas marina cells ingested per copepod (cell cop^{-1}) estimated by qPCR using different amplicons length and by gut fluorescence analysis

Post sampling manipulation	DNA extraction method	74 bp (Cells cop^{-1})	213 bp (Cells cop^{-1})	616 bp (Cells cop^{-1})	Chl *a* (Cells cop^{-1})
Copepod flash frozen in liquid nitrogen and thawed in filtered Sea water	Chelex	174 ± 79	64 ± 45	17 ± 17	3534 ± 853
	Chelex 95°C	61 ± 68	21 ± 87	20 ± 10	
	Tissue kit	99 ± 42	57 ± 18	30 ± 10	
	Tissue kit 56°C	103 ± 43	59 ± 12	29 ± 16	
	Plant kit	50 ± 26	46 ± 27	46 ± 22	
	Plant kit 56°C	45 ± 20	66 ± 27	57 ± 20	
	MoBio	5 ± 5	12 ± 6	8 ± 11	
	MoBio 60°C	13 ± 5	36 ± 38	28 ± 8	
Copepod killed in 80% ethanol	Chelex	127 ± 82	45 ± 15	4 ± 4	6084 ± 1147
	Chelex 95°C	97 ± 27	62 ± 22	7 ± 7	
	Tissue kit	252 ± 256	93 ± 64	48 ± 12	
	Tissue kit 56°C	84 ± 5	119 ± 97	60 ± 25	
	Plant kit	12 ± 18	7 ± 3	2 ± 2	
	Plant kit 56°C	16 ± 10	6 ± 5	8 ± 9	
	MoBio	15 ± 8	12 ± 9	31 ± 44	
	MoBio 60°C	27 ± 11	19 ± 11	42 ± 27	
Copepod sedated in MS222	Chelex	44 ± 22	48 ± 11	12 ± 5	4269 ± 831
	Chelex 95°C	35 ± 17	14 ± 1	7 ± 10	
	Tissue kit	166 ± 69	155 ± 131	112 ± 68	
	Tissue kit 56°C	171 ± 84	192 ± 92	166 ± 79	
	Plant kit	13 ± 10	14 ± 8	17 ± 9	
	Plant kit 56°C	47 ± 33	43 ± 21	50 ± 13	
	MoBio	7 ± 6	12 ± 10	10 ± 9	
	MoBio 60°C	35 ± 20	46 ± 5	27 ± 11	

assumption that the copepods contained the same amount of prey cells in all experiments. Although the independent copepod gut fluorescence analysis (Table V) indicated some differences within the three experiments (ANOVA, $P = 0.02$), the only significant difference in Chl *a* gut content was between the amount of prey consumed by copepod killed in ethanol and the flash frozen copepods (Holm–Sidak, $P = 0.007$, adjusted critical level=0.017). Thus, the result that the Tissue kit combined with MS222 sampling yields the highest extraction efficiency and lowest prey degradation cannot be explained by differences in gut contents, because the copepods sedated in MS222 did not contain significantly more prey (4269 ± 831 cells cop^{-1}) than the ones flash frozen in liquid nitrogen (3534 ± 853 cells cop^{-1}, Holm–Sidak, $P = 0.295$, adjusted critical level=0.050) or the ones that had been killed in ethanol (6084 ± 1147 cells cop^{-1}, Holm–Sidak, $P = 0.034$, adjusted critical level=0.025).

DISCUSSION

In this study, we systematically investigated several different DNA extraction, sampling and handling protocols to identify an optimal procedure for quantitatively determining the amount of prey DNA present in copepod guts using a qPCR approach. This study and our previous investigations (Nejstgaard *et al.*, 2008) suggest that quantitative estimates of copepod gut content based on nucleic acid biomarkers is susceptible to experimental artifacts associated with prey DNA extraction procedures and with the animal sampling and handling approaches. Therefore, if quantification of copepod feeding rates is to be determined based on qPCR estimates of prey DNA extracted from copepods, it is critical that these factors are considered and minimized.

For free-living microalgae, the Qiagen kits, especially the Tissue kit, were the most effective for extracting genomic DNA from all the algal species examined. In comparison, the MoBio kit appeared to be second best, but was less robust not yielding clear bands for all tested species (Fig. 1A). Chelex did not effectively extract DNA from any of the four diatoms, and was effective only on four naked flagellate algae species (Fig. 1A). Chelex is a chelating agent that is presumed to prevent the degradation of DNA by chelating metal ions that act as catalysts in the breakdown of DNA (Singer-Sam *et al.*, 1989; Walsh *et al.*, 1991). Cell lysis is achieved by heating the samples to 95°C in a 10% Chelex solution. We suggest that the Chelex treatment is too mild for efficient lysis of the cell wall in the tested diatoms. However, Richlen and Barber (Richlen and Barber, 2005) obtained DNA suitable for PCR from the marine dinoflagellates *Gambierdiscus toxicus* using Chelex. The thecal plates of this microorganism are thick (Adachi and Fukuyu, 1979) and highly resistant to freezing and sonication (Vernoux and Lejeune, 1994). While these results appear contrasting, we hypothesize that the cell walls of these dinoflagellates may be more vulnerable to lysis under higher temperatures than the silicate thecae of diatoms and possibly also the calcareous coccolith covered *E. huxleyi* cells tested here. However, this needs further investigation. Both of the CTAB-based extraction methods tested in this study were not effective. This was unexpected since the CTAB method was developed for routine genomic DNA extraction of a range of organisms including dinoflagellates (Kamikawa *et al.*, 2006), bacteria from environmental samples (Thakuria *et al.*, 2008) and higher plants (Murray and Thompson, 1980; Saghai Maroof *et al.*, 1984; Doyle and Doyle, 1990; Porebski *et al.*, 1997). Although it is possible that the method performs better on bacteria or dinoflagellates, it does not seem to be an adequate method for the quantitative extraction of DNA from a large diversity of potential copepod prey organisms.

When we tested the effectiveness of the different methods to extract prey DNA from copepod guts, we observed that the Plant and MoBio kits were not effective at purifying DNA from the cryptophyte *R. marina* after it had been fed as prey to *Calanus* spp. (Table V), despite the fact that both these kits were able to effectively extract genomic DNA from the same algal species when free living (Fig. 1A). For the Plant Kit, this was probably due to the chitinous exoskeleton of copepods acting as a barrier resistant to the lysis process of this protocol. The poor performance of the MoBio kit is more difficult to understand. This kit is designed to extract microbial organisms DNA in soil using a combination of heat, detergent and vortexing with beads to lyse the samples. This relatively vigorous lysis process should be sufficient to lyse the copepod exoskeleton. However, because the procedure is so vigorous, genomic DNA is sheared, which reduces the amount of available PCR amplifiable DNA recovered, even when short amplicons are targeted. Overall, the Tissue kit appeared to be best of the tested methods for extracting prey DNA from *Calanus* spp. guts.

Three different handling protocols for copepods were evaluated. The protocol that involved sedating the animals immediately upon harvesting and transferring them live directly into the pre-warmed Tissue kit lysis buffer, produced PCR amplifiable prey DNA with a

minimum amount of degradation (Fig. 2J and Table V). Following the same procedure, Troedsson *et al.* (Troedsson *et al.*, 2007) reported quantitative ingestion rates and house trapping rates using DNA based gut content methods from the gelatinous zooplankton *Oikopleura dioica* which compared well with independent literature data using classical methods. We suggest that this protocol minimizes *post mortem* digestion of prey DNA by reducing the release and activity of native copepod nucleases. Degradation of prey DNA by predatory associated nucleases has also been suggested by Passmore *et al.* (Passmore *et al.*, 2006) as an explanation of poor PCR amplification yields from gut content analysis of *Euphausia superba* preserved by freezing. These investigators hypothesized that prey DNA was degraded by nuclease enzyme during the defrosting process (Passmore *et al.*, 2006). High levels of prey DNA degradation in copepods were also hypothesized by Nejstgaard *et al.* (Nejstgaard *et al.*, 2008) as the cause of the consistent underestimation of the number of algal cells in copepod guts based on DNA, compared with number estimated by gut pigment analysis. The results obtained here suggest that such degradation may be due to a combination of physiological DNA digestion related to the food assimilation process and degradation depending on the sampling and handling of the predators and on the prey DNA extraction. Here we have optimized the protocol for minimizing the *post mortem* prey DNA degradation.

In addition to the contribution of experimental procedures to prey DNA digestion associated with copepods, a host of physical, biological and physiological factors associated with the living animals are also thought to influence prey DNA digestion. For example, temperature, predator species, prey species and recent feeding history may all influence the digestion of prey DNA. However, assuming that it is possible to minimize *post mortem* prey DNA digestion due to handling and DNA extraction procedures, it should be possible to identify and quantify these factors which, unlike experimental artifacts, should be non-random and predictable. A full understanding of the processes that result in copepod prey DNA digestion will therefore enable the determination of robust correction factors that will allow the estimation *in situ* copepod feeding rates based on the quantitative determination of prey DNA in copepod guts by qPCR.

SUPPLEMENTARY DATA

Supplementary data can be found online at http://plankt.oxfordjournals.org.

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REFERENCES

Adachi, R. and Fukuyu, K. (1979) The thecal structure of a marine toxic dinoflagellate *Gambierdiscus toxicus* gen. et sp. nov. collected in a ciguatera-endemic area. *Bull. Jap. Soc. Sci. Fish.*, **45**, 67–71.

Båmstedt, U., Nejstgaard, J. C. and Solberg, P. T. (1999) Utilisation of small-sized food algae by *Calanus finmarchicus* (Copepoda: Calanoida) and adaptation responses in its life-cycle. *Sarsia*, **84**, 19–38.

Båmstedt, U., Gifford, D., Irigoien, X. *et al.* (2000) Feeding. In Harris, R., Wiebe, P., Lenz, J., Skjoldal, H., Huntley, M. *et al.* (eds), *ICES Zooplankton Methodology Manual*. Academic Press, London, pp. 297–399.

Deagle, B. E., Eveson, J. P. and Jarman, S. N. (2006) Quantification of damage in DNA recovered from highly degraded samples—a case study on DNA in faeces. *Front. Zool.*, **3**, 11.

Doyle, J. J. and Doyle, J. L. F. (1990) Isolation of plant DNA from fresh tissue. *Focus*, **12**, 13–15.

Durbin, E. G., Casas, M. C., Rynearson, T. A. *et al.* (2008) Measurement of copepod predation on nauplii using qPCR of the cytochrome oxidase I gene. *Mar. Biol.*, **153**, 699–707.

Eppley, R. W., Holmes, R. W. and Strickland, J. D. H. (1967) Sinking rates of marine phytoplankton measured with a fluorometer. *J. Exp. Mar. Biol. Ecol.*, **1**, 191–208.

Guillard, R. R. L. (1975) Culture of phytoplankton for feeding marine invertebrates. In Smith, W. L. and Chanley, M. H. (eds), *Culture of Marine Invertebrate Animals*. Plenum Press, New York, pp. 29–60.

Harwood, J. D. and Obrycki, J. J. (2005) Quantifyong aphid predation rates of generalist predators in the field. *Eur. J. Entomol.*, **102**, 335–350.

Jarman, S. N., Redd, K. S. and Gales, N. J. (2006) Group-specific primers for amplifying DNA sequences that identify Amphipoda, Cephalopoda, Echinodermata, Gastropoda, Isopoda, Ostracoda and Thoracica. *Mol. Ecol. Notes*, **6**, 268–271.

Kamikawa, R., Asai, J., Miyahara, T. *et al.* (2006) Application of a Real-time PCR Assay to a Comprehensive Method of Monitoring Harmful Algae. *Microb. Environ.*, **21**, 163–173.

King, R. A., Read, D. S., Traugott, M. *et al.* (2008) Molecular analysis of predation: a review of best practice for DNA-based approaches. *Mol. Ecol.*, **17**, 947–963.

Murray, M. G. and Thompson, W. F. (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.*, **8**, 4321–4326.

Nejstgaard, J. C., Båmstedt, U., Bagøien, E. *et al.* (1995) Algal constraints on copepod grazing. Growth state, toxicity, cell size, and season as regulating factors. *ICES J. Mar. Sci.*, **52**, 347–356.

Nejstgaard, J. C., Frischer, M. E., Raule, C. L. *et al.* (2003) Molecular detection of algal prey in copepod guts and fecal pellets. *Limnol. Oceanogr. Meth.*, **1**, 28–39.

Nejstgaard, J. C., Frischer, M. E., Simonelli, P. *et al.* (2008) Quantitative PCR to estimate copepod feeding. *Mar. Biol.*, **153**, 567–577.

Passmore, A. J., Jarman, S. N., Swadling, K. M. *et al.* (2006) DNA as a dietary biomarker in antarctic krill, *Euphausia superba*. *Mar. Biotechnol.*, **8**, 686–696.

Porebski, S., Bailey, L. G. and Baum, B. R. (1997) Modification of a CTAB DNA Extraction Protocol for Plants Containing High Polysaccharide and Polyphenol Components. *Plant Mol. Biol. Rep.*, **15**, 8–15.

Richlen, M. and Barber, H. (2005) A technique for the rapid extraction of microalgal DNA from single live and preserved cells. *Mol. Ecol. Notes*, **5**, 688–691.

Saghai Maroof, M. A., Soliman, K. M., Jorgensen, R. A. *et al.* (1984) Ribosomal DNA spacer length polymorphism in barley: Mendelian inheritance, chromosomal location and population dynamics. *Proc. Natl Acad. Sci. USA*, **81**, 8014–8018.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (eds) (1989) *Molecular Cloning A Laboratory Manual*. Harbor Laboratory Press, Cold Spring.

Singer-Sam, J., Tanguay, R. L. and Riggs, A. D. (1989) Use of Chelex to improve The PCR signal from a small number of cells. *Amplification: A forum for PCR Users*, **3**, 11.

Thakuria, D., Schmidt, O., Mac Siurtain, M. *et al.* (2008) Importance of DNA quality in comparative soil microbial community structure analyses. *Soil Biol. Biochem.*, **40**, 1390–1403.

Troedsson, C., Frischer, M. E., Nejstgaard, J. C. *et al.* (2007) Molecular quantification of differential ingestion and particle trapping rates by the appendicularian *Oikopleura dioica* as a function of prey size and shape. *Limnol. Oceanogr.*, **52**, 416–427.

Troedsson, C., Lee, R. F., Stokes, V. *et al.* (2008) Development of a denaturing high-performance liquid chromatography (DHPLC) method for detection of protist parasites of metazoans. *Appl. Environ. Microbiol.*, **74**, 4336–4335.

Troedsson, C., Simonelli, P., Naegele, V. *et al.* (2009) Quantification of copepod gut content by differential length amplification quantitative PCR (dla-qPCR). *Mar. Biol.*, **156**, 253–259.

Verity, P. G. and Smetacek, V. (1996) Organism life cycles, predation and the structure of marine pelagic ecosystems. *Mar. Ecol. Prog. Ser.*, **130**, 277–293.

Vernoux, J. P. and Lejeune, J. (1994) Ciguatera in the French West Indies. *Mem. Queensl. Mus.*, **34**, 631–638.

Walsh, P. S., Metzger, D. A. and Higuchi, R. (1991) Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques*, **10**, 506–513.