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Detecting copepod grazing on low-concentration populations of *Alexandrium fundyense* using PCR identification of ingested prey

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Zooplankton grazing is often a significant loss term for phytoplankton populations, including harmful algae, impacting the development and decline of blooms. However, detecting and quantifying predation on phytoplankton is often challenging, particularly during early bloom stages when phytoplankton cell concentrations are low. In this study, we used polymerase chain reaction (PCR) to detect ingestion of toxic dinoflagellates of the *Alexandrium tamarense* species complex by two copepods, *Acartia hudsonica* (laboratory population) and *Calanus finmarchicus* (field population). Recent ingestion of *Alexandrium fundyense* cells was indicated by positive amplification of an LSU rDNA fragment specific to *A. fundyense* from whole copepod extracts. In laboratory experiments, *A. fundyense* DNA was detectable for 2–4 h post-ingestion in *A. hudsonica* fed *A. fundyense*, but not detected in animals fed other phytoplankton, or starved. In field samples, ingestion of *A. fundyense* by *C. finmarchicus* was confirmed by PCR, including at four stations where the *A. fundyense* concentration was ≤ 14 cells L^{-1}. At these low prey concentrations, ingestion rates on *A. fundyense* may have been as low as 1 cell copepod^{-1} day^{-1}. Nevertheless, simulations of *A. fundyense* population growth suggest that a few predators L^{-1} have the potential to curb the early development of a slow-growing bloom, even if ingestion rates are extremely low. Low predation rates can still have a large impact when prey populations are small.

KEYWORDS: red tides; harmful algal blooms; trophic interactions; zooplankton; *Calanus finmarchicus*; *Acartia hudsonica*

INTRODUCTION

Phytoplankton blooms occur when the growth rate of a phytoplankton population exceeds its loss rate (Buskey *et al.*, 1997). Zooplankton grazing often dominates the loss terms for phytoplankton (Calbet *et al.*, 2003), exerting a significant impact on the development and decline of blooms. Specifically, copepods can be important grazers of phytoplankton, particularly when abundant and when the phytoplankton assemblage consists of larger cells (Bautista and Harris, 1992; Campbell *et al.*, 2005). Whereas factors such as advection, mixing, sinking and disease can all subtract from phytoplankton

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populations, mortality through grazing often keeps pace with their growth rates, such that grazing and growth are often nearly in balance (e.g. Juhl and Murrell, 2005). However, under favorable environmental conditions (e.g. nutrients, temperature and salinity) grazing may fail to keep up with increased phytoplankton growth, allowing a bloom to develop (Uye, 1986; Smayda, 2008). It has been suggested that the point at which grazing exerts the most control over the progression of a phytoplankton bloom is in its initial stages, when phytoplankton cell numbers are low (Uye, 1986).

Controlling a bloom at its onset requires that zooplankton grazers effectively feed on the proto-bloom phytoplankton species when they are at low concentration. In that context, it has been hypothesized that there are prey concentrations below which zooplankton (specifically copepods) greatly reduce or even stop their feeding (e.g. Wlodarczyk *et al.*, 1992; Frost, 1993; Pahlow and Prowe, 2010). It is also possible that at low concentrations of specific phytoplankton, zooplankton may switch to more abundant prey species (e.g. Landry, 1981). However, some copepod feeding experiments have demonstrated continued grazing at low prey concentrations, although with a concomitant reduction in feeding activity (e.g. Frost, 1975; Frost *et al.*, 1983). This work and others (e.g. Saage *et al.*, 2009; Schultz and Kiørboe, 2009) argue that the zooplankton–phytoplankton predator–prey relationship is best fitted with a Holling (1959) type III (or similar) curve with low, though theoretically measurable, ingestion at low prey concentration.

Although theoretically measurable, investigating *in situ* zooplankton feeding behavior at very low prey concentrations is challenging. Most current methods for measuring zooplankton grazing on individual phytoplankton species are based on the disappearance of prey cells over time during incubations with the grazer (e.g. Campbell *et al.*, 2005; Leising *et al.*, 2005; Turner and Borkman, 2005). Although this general approach is well accepted and reliable, it has several drawbacks. Because of limitations inherent in the precision and accuracy of cell counting, the technique is less effective when prey concentration is low and often requires cell amendments to grazer incubations (e.g. Turner and Anderson, 1983; Campbell *et al.*, 2005). In addition, the approach is highly labor intensive, usually limiting the spatial and temporal resolution of resultant data.

As an alternative, DNA-based approaches may eventually offer a rapid and species-specific way to measure zooplankton grazing on phytoplankton, even when the prey of interest is at low abundance within a mixed community. Polymerase chain reaction (PCR) is already widely used to detect and quantify free-living phytoplankton by targeting unique DNA sequences (Dyhrman *et al.*, 2006, 2010; Demir *et al.*, 2008) and several studies have documented the use of PCR to identify and quantity specific phytoplankton species in zooplankton gut contents (Nejstgaard *et al.*, 2003, 2008; Troedsson *et al.*, 2009). Identifying and quantifying grazing on specific phytoplankton species is especially relevant within the context of harmful algal blooms (HABs). Although not intrinsically different from other kinds of phytoplankton blooms, HABs are distinguished by having negative public health, economic or ecological effects (Smayda, 1997), all caused by a single species. One of the difficulties in predicting HABs is that the harmful species responsible for a HAB event is often a minor component of the phytoplankton community during the formative stage of the bloom. In addition, some algae are considered harmful even without achieving high relative biomass (Anderson, 1997). Increasingly, molecular techniques have been used to identify phytoplankton species that are considered to be harmful [Scholin *et al.*, 1994; Dyhrman *et al.*, 2006; Lin, 2008 (and references therein)] and these approaches should be generally applicable to studies of zooplankton grazing on HAB species. Our focus in this study is copepod grazing on toxic dinoflagellates of the *Alexandrium tamarense* species complex.

Blooms of the dinoflagellates that make up the *A. tamarense* species complex (which includes *Alexandrium catenella*, *A. tamarense* and *Alexandrium fundyense*) cause serious threats to coastal ecosystems and human health (Anderson, 1997; Van Dolah, 2000) through the production of paralytic shellfish toxins (PSTs; Anderson *et al.*, 1990; Strichartz and Castle, 1990). Two closely related morphospecies, *A. tamarense* and *A. fundyense*, are frequent bloom species in the Gulf of Maine and often lead to PST closures (Anderson *et al.*, 1994). Within the eastern Gulf of Maine, all species examined to date have been *A. fundyense*, whereas both morphospecies have been found in western waters. Genetic (Scholin *et al.*, 1994; Lilly *et al.*, 2007) and mating studies (Brosnahan *et al.*, 2010) suggest that these are strains of a single species, rather than separate species. For convenience, the term *Alexandrium* will be used hereafter to refer to the regional populations.

In this study, we used PCR to detect *Alexandrium* in the gut contents of two calanoid copepods, *Acartia hudsonica* (laboratory population) and *Calanus finmarchicus* (Gulf of Maine field population) that co-occur with *Alexandrium* blooms. It is well accepted that zooplankton grazers ingest *Alexandrium* spp. (Turner *et al.*, 2000; Doucette *et al.*, 2005), including many copepod species (Turner and Anderson, 1983; Teegarden *et al.*, 2003). This study demonstrates the value of PCR-based assays

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to detect copepod grazing on harmful algae, particularly when the algae are at very low abundance. The results highlight the potential significance of predation on phytoplankton populations when cell concentrations are extremely low. Because low cell concentrations likely characterize the earliest phases of bloom development for most phytoplankton blooms, the approach and findings may be broadly applicable.

METHOD

Algal cultures

Alexandrium fundyense (strain GTCA28), isolated and maintained in the laboratory of D. M. Anderson (Woods Hole Oceanographic Institution) was used in all experiments. Cells were grown in f/2 medium at 15°C in cool white fluorescent light (~200 μmol quanta m^{-2} s^{-1}) on a 14 h light:10 h dark cycle. The f/2 medium was made with 0.2 μm filtered Vineyard Sound seawater (salinity ~32 psu) autoclaved with inorganic f/2 nutrients (Guillard, 1975). Filter-sterilized f/2 vitamins were added after autoclaving. *Alexandrium* cell concentration in stock cultures and experimental treatments was determined via microscopic counts of acid-Lugol's preserved subsamples.

Laboratory grazing studies

Acartia hudsonica were collected from Great Pond, a small estuary near Falmouth, Massachusetts, USA, with a 153-μm mesh plankton net fitted with a solid cod end. Copepods were immediately transported to the laboratory and isolated from the sample using a dissecting microscope and reared as cohorts according to the procedures of Feinberg and Dam (1998). Copepods were maintained at 10°C on a 14 h light: 10 h dark cycle and received a mixed diet of *Isochrysis galbana*, *Rhodomonas lens* and *Tetraselmis chui* at a concentration of ~400–500 μg C L^{-1}. Healthy and intact adult and late copepodites were used for the experiments. To assess the efficacy of PCR to distinguish between copepods fed *Alexandrium* from those not fed *Alexandrium*, 10 copepods were transferred to each of three different treatments (a–c) in 100-mL tri-corner beakers fitted with a 100-μm Nitex mesh bottom and nested in a second beaker: (a) 100 mL filtered (0.2 μm) seawater amended with 100 *Alexandrium* cells mL^{-1}, (b) 100 mL filtered seawater (FSW) amended with a mixed diet of *I. galbana*, *R. lens* and *T. chui* (at maintenance concentration) and (c) 100 mL FSW. The copepods were incubated for 20 h at 10°C. After 20 h, the copepods were washed by transferring the mesh-bottom beaker to a second beaker with FSW. The transfer step was rapidly repeated two additional times, for a total of three rinses with FSW to exclude carry-over of un-ingested *Alexandrium* cells. After the final rinse, the mesh-bottom beaker with the copepods was dipped into liquid nitrogen. The mesh was removed, placed in a cryovial and stored at −80°C for later DNA extraction.

The experimental design to measure the persistence of the *A. fundyense* DNA signal within copepod guts was similar. Briefly, 10 *A. hudsonica* each were incubated in mesh-bottom beakers suspended in FSW amended with *A. fundyense* (100 cells mL^{-1}). The copepods were incubated for 20 h at 10°C to allow feeding. After 20 h, the copepods were rinsed three times, as described above. After the final rinse, the mesh-bottom beakers with copepods were suspended in FSW and incubated for the requisite amount of time (0, 1, 2, 4, 6, 12 and 24 h). At the end of each time point, the mesh-bottom beaker with the copepods was removed from the FSW and processed as above. The experiment was repeated three times, although the full set of time points was not included in each experiment. Because gut passage times of starved copepods are generally longer than for those feeding continuously (e.g. Kiørboe and Tiselius, 1987), suspending the copepods in FSW after feeding resulted in the longest possible detection period for the ingested *Alexandrium* DNA signal.

To begin DNA extractions for all laboratory grazing experiments, *A. hudsonica* individuals were hand picked from the Nitex mesh on a cold (<0°C) dissecting microscope stage, inspected to ensure that no *Alexandrium* cells were stuck to appendages, and transferred to a screw-cap tube containing 0.4 mL Buffer ATL (Qiagen, Valencia, CA, USA). The cold stage prevented full thawing of the frozen copepods during handling. Copepods in buffer were stored at −20°C until proceeding with subsequent DNA extraction steps.

Gulf of Maine sample collection

Zooplankton samples were collected from the Gulf of Maine and from several points south (e.g. Georges Bank and Nantucket Sound) during three *Alexandrium* survey cruises conducted by the ECOHAB-Gulf of Maine (May 2004; R/V Oceanus, #OC402) and GOMTOX (May–June 2007; R/V Endeavor, #EN435 and EN437) programs. Zooplankton was collected by sieving pumped near-surface seawater (~ 2 m) through a 200-μm mesh. The sample was backwashed off the sieve with FSW and filtered onto a 5-μm Durapore filter (25 mm). The filter was transferred to a cryovial and stored in liquid nitrogen. Corresponding samples

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were collected at each station for counting *Alexandrium* sp. in the water using two complementary methods: live microscopic counts and whole-cell (WC) oligonucleotide labeling (Anderson *et al.*, 1999, 2005a). For the live counts, 10 L of surface seawater (1 m) was filtered through 20-μm Nitex mesh, and the concentrated material was resuspended to 14 mL with FSW. Using a ship-board light microscope, counts of live *Alexandrium* sp. cells were made by loading a Sedgewick-Rafter counting slide with 1 mL of the concentrated cell suspension and examining two transects at 200× (0.1 mL of the 1-mL suspension). An independent sample for *Alexandrium* detection and quantification via the WC labeling procedure, which is based on epifluorescence microscopy of preserved samples, was collected and processed following Anderson *et al.* (1999, 2005a). Detection limits for the live count and WC methods are ~14 and 1 cell L^{-1}, respectively. In the laboratory, the filtered zooplankton samples were inspected on a cold dissecting microscope stage. Sixteen of the samples had at least five individual copepods of the same species (*C. finmarchicus*). For these 16 samples, 5 intact copepods were hand picked from the filter, inspected to ensure that no *Alexandrium* cells were stuck to appendages, and transferred to a screw-cap tube containing 0.4 mL Buffer ATL. Copepods in buffer were stored at −20°C until proceeding with subsequent DNA extraction steps.

DNA extractions

All algal and zooplankton samples were extracted using the DNeasy Blood and Tissue kit (Qiagen) with a minor modification in the lysis procedure. Lysis was performed by adding ~250 μL zirconium/silica beads (0.5 mm diameter) to the thawed volume of Buffer ATL and vortexing (250 rpm) for 1 min. The resulting cell lysate was digested with proteinase K for 3.5 h at 55° with rotation in a hybridization oven. The resulting lysate was transferred to a clean tube and processed as per the remaining steps of the DNeasy protocol. The eluted DNA was stored at −20°C for later PCR analysis. The DNeasy-based protocol was found to provide higher quality and greater recovery of *Alexandrium* DNA than two other commercially available DNA extraction kits: Generation® Capture Column DNA Purification kit (Qiagen) and UltraClean™ Soil DNA Isolation kit (MO-BIO Laboratories, Carlsbad, CA, USA) (data not shown).

Polymerase chain reaction

Ingested *Alexandrium* DNA was detected using PCR. PCR primers were designed to amplify a 183-bp sequence of the ribosomal large subunit (LSU) gene. The forward primer (5′-GCAAGTGCAACACTCCCA CCAAGCAA-3′) was designed from an alignment of publicly available *Alexandrium* strain sequences. The reverse primer (5′-GCAAGTGCAACACTCCCACCAA GCAA-3′) was modified from a previously designed oligonucleotide (NA1; Anderson *et al.*, 1999). The NA1 oligonucleotide is specific for toxic North American ribotypes of the *A. fundyense/tamarense/catenella* species complex (Scholin *et al.*, 1994; Dyhrman *et al.*, 2006). PCR amplification was performed using 5 μL gDNA in a final reaction mixture (25 μL) containing 10× PCR buffer (Bio-Rad), 0.25 mM deoxynucleoside triphosphates (Bio-Rad), 1.5 mM $MgCl_2$, 150 nM of each forward and reverse primer, and one unit of Taq DNA polymerase (Bio-Rad). Reactions were cycled with an iCycler (Bio-Rad) using a temperature profile of 98°C for 5 min, 60°C for 1 min (1×), 96°C for 30 s, 63°C for 30 s and 72°C for 30 s (45×), and a final extension of 72°C for 7 min (1×). PCR products were resolved on a 2% agarose gel, stained with ethidium bromide, and imaged with a Gel Logic 440 imaging system (Kodak, Rochester, NY, USA). Positive (*Alexandrium* genomic DNA from strain GTCA28) and negative (no template) controls were included in each PCR assay. Quantitative PCR (qPCR) was not used in this study because we anticipated that the *Alexandrium* signal in some samples would be too low for proper application of the method. Thus, our PCR results only indicate whether or not sampled copepods had recently ingested *Alexandrium*, but not how many cells they had ingested.

Sequencing of selected PCR products

To confirm the identity of the PCR products, the putative *Alexandrium* LSU rDNA amplicon was identified on an agarose gel from two field samples (OC402-243 and OC402-244; see Gulf of Maine samples subsection in Results) and the excised bands were gel purified using the QIAquick gel extraction kit (Qiagen). Sequencing of PCR products was performed at the University of Maine DNA Sequencing Facility (Orono, ME, USA). All sequence data were analyzed manually using the programs Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA) and BLASTN (http://www.ncbi.nlm.nih.gov/BLAST).

Simulating *Alexandrium* growth in the early bloom phase

Grazing could have a large impact on the development of low-concentration *Alexandrium* populations, even with very low predator ingestion rates, because every cell

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ingested would represent a large proportional loss to the local population. To highlight this argument, growth of *Alexandrium* within a representative volume (1 L) was simulated with and without low-level grazing. The *Alexandrium* concentration (N_t) at each time step was equal to the daily growth minus the daily loss to grazing as:

$$N_t = \text{growth} - \text{grazing loss} = (N_0\, e^{rt}) - (D \times I),$$

daily growth was exponential with an intrinsic rate of growth, r, of 0.3 day^{-1} (MacIntyre *et al.*, 1997; Etheridge and Roesler, 2005) and a time interval, t, of 1 day. The number of cells lost each day to grazing was estimated as ($D \times I$) where D was the predator concentration (constant at 0, 1, 2 and 3 L^{-1} in different runs), and I was the specific ingestion rate of each predator (constant at either 0, 0.5, 1 and 2 cells predator^{-1} day^{-1} in different trials). This predation parameterization is different from those commonly used in more complicated models of phytoplankton blooms which typically have losses to the population parameterized through a mortality function that is proportional to prey concentration (e.g. He *et al.*, 2008 and references therein). Here, the ingestion rate was kept low and constant, independent of the prey concentration, to highlight the impact of the low ingestion rates on eventual *Alexandrium* yield. Using an initial *Alexandrium* concentration (N_0) of 10 cells L^{-1}, the *Alexandrium* concentration was plotted as a function of time for 14 days. Running the calculations with zero predators and zero ingestion, equivalent to purely exponential growth, was considered the baseline for comparison to results with predation.

RESULTS

Laboratory grazing studies

Survivorship for copepods fed *Alexandrium* was between 90–100% for all experiments. Ingestion of prey diets by *A. hudsonica* was confirmed qualitatively by visually noting pigmented guts and the presence of fecal pellets. A subsample of the food suspension showed that *Alexandrium* cells were still present at the end of the incubations. Using PCR, the presence or absence of the LSU rDNA gene specific for *Alexandrium* was examined in copepods fed either *A. fundyense*, a mixed algal diet, or starved for 20 h. The LSU rDNA gene was detected in copepods that fed on *Alexandrium*, but not in copepods given a non-*Alexandrium* diet, or starved (Fig. 1). Negative (no template) controls were always negative for amplification.

The persistence of the ingested *Alexandrium* DNA signal was measured in copepods fed *Alexandrium*, then starved for different durations. In repeated experiments, an *Alexandrium* rDNA signal was always detected in copepod guts immediately after the copepods (0 h) were removed from the *Alexandrium* food source (Fig. 2 and Table I). The signal was also always present for at least 1 h after the copepods had been removed from *Alexandrium*. In two experiments, *Alexandrium* DNA was no longer detectable 2 h after starvation began, and in the third experiment the signal was retained at least 4 h (Table I). The *Alexandrium* DNA signal was not found in copepods that had been starved for 6 h or longer (Fig. 2

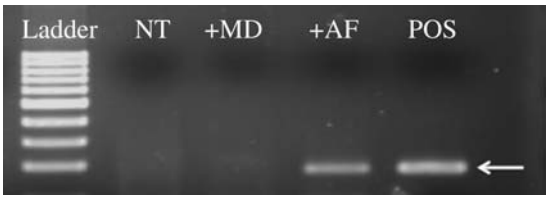

Fig. 1. DNA ladder and PCR products from *A. hudsonica* copepods fed different diets. Lane designations are as follows: Ladder, 100 bp ladder starting at 200 bp; NT, Negative control, no DNA; +MD, *A. hudsonica* fed a mixed diet of *T. chui*, *I. galbana* and *R. lens*; +AF, *A. hudsonica* fed *A. fundyense* GTCA28; and POS, Positive control, *A. fundyense* GTCA28 genomic DNA. The arrow indicates the *A. fundyense* GTCA28 183 bp LSU rDNA amplicon.

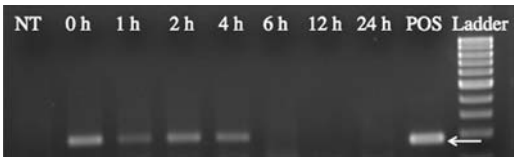

Fig. 2. Persistence of ingested *A. fundyense* DNA in *A. hudsonica* after removal from *Alexandrium* diet. Results of second experiment are shown (see Table I for summary of results from this and other experiments). Lane designations are as follows: NT, Negative control, no DNA; X h, Time from removal from *Alexandrium* diet; POS, Positive control, *A. fundyense* GTCA28 genomic DNA; Ladder, 100 bp ladder starting at 200 bp. The arrow indicates the *A. fundyense* GTCA28 183 bp LSU rDNA amplicon.

Table I: Alexandrium DNA amplicon detection in A. hudsonica extracts at different time points post-feeding in three independent experiments

Duration (h)	Experiment 1	Experiment 2	Experiment 3
0	+	+	+
1	+	+	+
2	–	+	–
4	ND	+	–
6	–	–	–
12	–	–	–
24	ND	–	–

ND, No data.

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and Table I). Negative (no template) controls were always negative for amplification.

Gulf of Maine samples

The dominant copepod in all samples was *C. finmarchicus*. Six of the 16 stations had detectable free-living *Alexandrium* cells present at concentrations well above the detection limits for both of the counting methods (Table II). Ingestion of *Alexandrium* by *C. finmarchicus* was confirmed by PCR in all of these samples. Ten stations had *Alexandrium* concentrations at or below the detection limit for at least one counting method. Even at these very low concentrations, ingestion of *Alexandrium* by *Calanus* was confirmed by PCR at four stations (Table II). Negative (no template) controls were always negative for amplification.

Sequence alignments of the LSU rDNA gene fragment from PCR products of the field samples analyzed confirmed high sequence similarity to *Alexandrium* sp. Sequence identity across the 183-bp amplicon ranged from 93 to 98%, with a 96% identity to four New England *Alexandrium* isolates (CCMP1980, GTPP01, GT429 and GTCA28). No significant alignments were generated for any other organisms.

Simulating *Alexandrium* growth in the early bloom phase

Figure 3 shows the growth trajectory over 14 days of exponentially growing *Alexandrium* populations with different levels of predation. The same initial cell concentration was used in all calculations (10 cell L^{-1}). The baseline of exponential growth without predation is shown as the solid black curve while the dashed curves each represent *Alexandrium* yield under a different specific ingestion rate of the predators. Although the predator concentrations and ingestion rates were maintained at very low levels throughout, the impact of predation on *Alexandrium* yield was dramatic. For example, with only 1 predator L^{-1}, an ingestion rate of only 2 cells day^{-1} decreased the *Alexandrium* yield over the 2-week span by more than 50%. Higher predator concentrations at the same low ingestion rate led to local extinction of the *Alexandrium* population within a few days.

DISCUSSION

PCR is a robust technique for detecting ingested *Alexandrium* in copepods, even several hours after ingestion and at low *in situ* cell numbers. In laboratory grazing experiments, the PCR assay detected a prey rDNA signal in whole copepod extracts only from animals that ate *Alexandrium*, and this signal could be resolved for ~2–4 h after the animals stopped feeding. Attenuation of the signal after about 4 h suggests degradation and loss, probably through digestion and defecation, of prey DNA. This is a relatively long detection period compared with *Acartia* sp. gut passage times for ingested phytoplankton pigments, typically 1 h or less (although several-hour gut passage times for pigments

Table II: Presence or absence of Alexandrium DNA in extracts of field-collected C. finmarchicus (far right column)

Cruise-Station	Lat./Long.	Temp. (°C)	Salinity (psu)	*Alexandrium* cells L^{-1} (Live)	*Alexandrium* cells L^{-1} (WC)	*Alexandrium* DNA present in *C. finmarchicus*?
EN435-68	43.6065/68.1805	7.4	32.3	<14*	<1*	No
EN435-54	43.2875/68.393	6.7	32.0	<14*	<1*	No
OC402-222	43.7005/68.2562	6.7	32.0	<14*	<1*	No
OC402-243	43.4628/69.7545	8.9	31.6	<14*	<1*	Yes
OC402-244	43.4112/69.7227	8.7	31.8	<14*	<1*	Yes
EN435-86	43.6775/67.3571	7.0	32.6	<14*	2	No
EN435-77A	44.0693/68.1968	5.5	32.0	14	1	Yes
EN437-70	41.5118/69.6585	13.5	31.2	14	3	Yes
EN435-45A	43.6627/69.8677	7.0	29.8	14	22	No
EN435-117	44.9815/66.8243	5.2	30.9	14	15	No
OC402-242	43.5132/69.7808	9.1	31.8	66	67	Yes
OC402-245	43.6332/70.0658	8.8	31.3	100	ND	Yes
EN435-205	41.1547/70.4675	12.6	31.8	490	60	Yes
EN435-51/52	41.0383/66.9062	12.4	32.7	952	738	Yes
EN437-66A	41.6067/66.0307	9.9	32.8	1274	758	Yes
EN435-184A	40.6542/68.4372	10.3	33.0	3318	1806	Yes

For each sample the cruise and station ID (EN = R/V Endeavor, OC = R/V Oceanus), location, temperature and salinity are shown with the local *Alexandrium* concentration in the water from both live counts (Live), and WC oligonucleotide labeling epifluorescence counts. ND, no data; *, detection limit for respective counting method.

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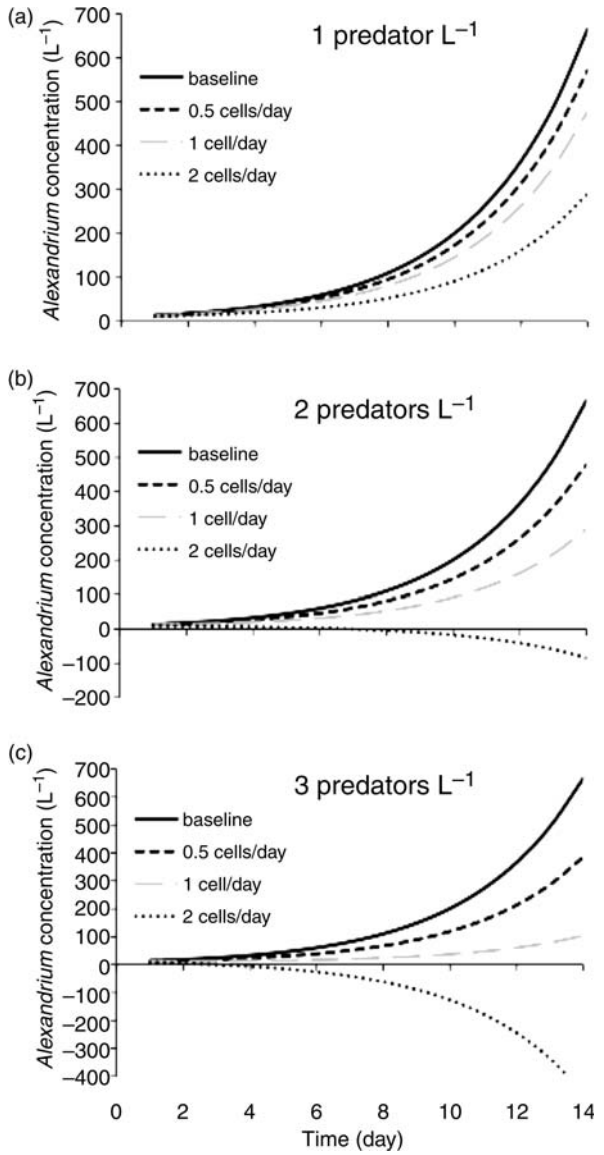

Fig. 3. Comparison of grazing impact by zooplankton predators on local *Alexandrium* yield for three different predator concentrations: (**a**) 1, (**b**) 2 and (**c**) 3 predators L^{-1}. In each plot, the *Alexandrium* growth trajectory was simulated for four predator ingestion rates: 0 (no predation baseline, *Alexandrium* growth strictly exponential at $\mu = 0.3$ d^{-1}), 0.5, 1 and 2 cells ingested per predator per day. Initial *Alexandrium* cell concentration was 10 cells L^{-1} in each case.

may occur when prey concentrations are low, e.g. Besiktepe and Dam, 2002; Liu *et al.*, 2010). The relatively long duration of the *Alexandrium* DNA signal in these copepods may be partly attributed to the very high sensitivity of the PCR assay (compared with pigment quantification methods), and also because the copepods in these experiments were starved after feeding on *Alexandrium*. Starved copepods may have longer gut passage times than copepods that continue to feed (Kiørboe and Tiselius, 1987). Thus, these results probably represent the maximum duration for detection of ingested prey using PCR.

The results of the PCR assay on field-collected copepods indicate that *C. finmarchicus* will ingest *Alexandrium* cells when they are present, and apparently even when concentrations are as low as ~14 cells L^{-1}. The presence of *Alexandrium* DNA in field-collected copepods was definitively confirmed by sequencing of excised PCR product in several of the positive field samples. Although we cannot exclude the possibility that those copepods with ingested *Alexandrium* DNA had fed in a higher concentration patch before they were captured, the putative detection of *in situ* ingestion of *Alexandrium* cells by *C. finmarchicus* at such low prey concentrations is interesting, as it is unlikely that ingestion at these low prey concentrations could be detected with other methods.

Such low prey concentrations must occur during the early development of all *Alexandrium* blooms, and may represent a key point where grazing could inhibit further bloom development. Extrapolating from the signal degradation rate in our laboratory studies with *A. hudsonica*, we can estimate that at least one of the five *Calanus* individuals in each DNA extract with a positive *Alexandrium* signal had ingested at least one *Alexandrium* cell ~2–4 h before collection in the field. This translates to a minimum ingestion rate of ~1 cell copepod^{-1} day^{-1} (1 cell × 5 copepods^{-1} 6^{-h} × 24 h day^{-1} = 0.8 cells copepod^{-1} day^{-1}). These data demonstrate that *Calanus* can graze *Alexandrium* cells even when the *Alexandrium* are at extremely low concentration, suggesting that there is no threshold where feeding on *Alexandrium* stops completely. However, even for the lowest *Alexandrium* concentrations, the data do not require selective feeding on *Alexandrium* (Turner and Anderson, 1983; Teegarden *et al.*, 2001). Clearance rates for *C. finmarchicus* of several hundred mL day^{-1} (Koski, 2007; Teegarden *et al.*, 2008) would allow for sufficient incidental encounters with *Alexandrium* cells, even for the lowest cell concentrations measured, to support an ingestion rate of 1 cell copepod^{-1} day^{-1}.

These very low ingestion rates may, nevertheless, be ecologically important with respect to early bloom development. For example, the simple growth simulations presented here are relevant to *Alexandrium* populations in the Gulf of Maine. When *Alexandrium* populations emerge in spring from cyst seedbeds in the Gulf of Maine, cell abundance will be low with growth rate limited by cold temperatures (Love *et al.*, 2005; McGillicuddy *et al.*, 2005). The results of the simulations demonstrate that when *Alexandrium* cells are at low concentration, even low levels of predation can have a

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dramatic impact on population development, because each ingested cell represents a proportionally large loss to a small local population. It is important to note that the effects of such low predation rates were minimal when the simulations were repeated with higher initial cell concentrations of 100 cells L^{-1} (not shown). Thus, periods of low cell concentration during early bloom development may provide a window of opportunity for predation to inhibit further growth. The results highlight the importance of predator concentration as a key aspect controlling early *Alexandrium* bloom dynamics (Campbell *et al.*, 2005; Turner and Borkman, 2005). Low predator abundance may be necessary for bloom formation (Buskey *et al.*, 1997), as a few predators L^{-1} have the potential to retard the early development of a slow-growing bloom, even if ingestion rates are extremely low. An important consideration for *Alexandrium* that could allow a population to persist and flourish despite grazing losses during the pre-bloom phase, is the continued gradual release of germinated cells from benthic cyst beds (Anderson *et al.*, 2006).

The general concept, that low predation rates can have a large impact on small prey populations, is potentially important for modeling other types of prey populations. Models of phytoplankton blooms generally recognize phytoplankton mortality as a key parameter that is admittedly oversimplified (e.g. Stock *et al.*, 2005; He *et al.*, 2008). Specifically, models that parameterize predation loss as proportional to population size will not show the sensitivity to predation at low population size demonstrated to occur here. This work may therefore be used to improve how this important parameter is represented.

CONCLUSIONS

Copepod ingestion of *Alexandrium* was detected using PCR in laboratory and field samples. In *A. hudsonica*, the DNA signal of ingested *Alexandrium* persisted 2–4 h after ingestion. This approach was used to confirm ingestion of *Alexandrium* by a field population of *C. finmarchicus*, even at low *Alexandrium* concentrations representative of incipient blooms. Detecting ingestion at these low prey concentrations would be extremely challenging without the sensitivity provided by the PCR-based method. Further, ingestion rates of *Alexandrium* at the lowest concentrations were consistent with opportunistic or incidental, non-selective ingestion and the lack of a lower feeding threshold. Our results highlight the impact of even incidental levels of predation when prey concentrations are low.

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