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Use of species-specific primers and PCR to measure the distributions of planktonic ciliates in coastal waters

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Abstract

We developed a method to extract environmental DNA and amplify target portions of the internal transcribed spacer region (ITS) of the ribosomal gene (ITS1-5.8S-ITS2) from individual species of oligotrich and choreotrich ciliate microzooplankton. To date, we have lab- and field-tested primers specific to the tintinnid *Favella ehrenbergii*, the oligotrich *Laboea strobila*, and the choreotrich *Strombidinopsis* sp. For all three species, the primers were both species-specific (not producing PCR product from non-target DNA) and comprehensive (able to amplify from different populations of the target species). The method is both time-efficient and sensitive, compared with microscopy. In seawater samples amended with both target and non-target DNA, we were able to detect the targets at < 1 cell L^{-1}. Some difficulties we encountered resulted from PCR-inhibitory compounds that co-extracted with the environmental DNA, and the rarity of the target DNA within natural plankton assemblages. Comparisons with microscopic counts were qualitatively similar to PCR (presence/absence of the species in different amounts of extract). We are evaluating ways to make the method fully quantitative by investigating the degree to which copy number for this gene may vary among individuals.

Ciliates are ubiquitous members of the marine plankton, playing important roles as grazers of phytoplankton and heterotrophic microbes and as links to higher trophic levels (Capriulo and Carpenter 1983; Sanders 1987; Klaas 1997; Turner and Tester 1997; Capriulo et al. 2002; Strom 2002). Current methods for identifying and quantifying ciliates in plankton include settling and observation by light microscopy (Utermohl method), filtration followed by scanning electron microscopy (SEM), or staining with silver proteinate (protargol). These methods share several drawbacks. All of them require preservation of samples with chemical fixatives, which can damage individual species to different degrees (Gifford and Caron 2000); all require hours of sample preparation and microscopy; and all suffer from the requirement that only a small sample can be analyzed. The latter is typically 50-500 mL, so the ability to record the presence of an individual species is limited to those present in concentrations well above 1 L^{-1}. In addition, identifying individuals to genus or species is often difficult, requiring substantial taxonomic expertise and near-perfect sample preparation. The most commonly used method, and the one that suffers least from preservative-induced destruction of species, is settling of Lugol's-preserved samples with subsequent observation by light microscopy (Gifford and Caron 2000). The great majority of ciliates are not recognizable to species level with this method, so inferences about distributions, life histories, behavior, etc., are often not possible for individual species of ciliates the way they usually are for copepods or other mesozooplankters. An additional problem with current methods is that taxonomic descriptions of ciliates sometimes have been based on variable morphological traits, making identifications doubtful. In the tintinnids, for example, taxonomy is based on size and shape of the lorica, an external sheath constructed by the organism. This character has been shown to be highly variable in both cultures and field samples of individual species (e.g., Bakker and Phaff 1976; Laval-Peuto 1981).

Identification of planktonic organisms using species-specific primers or probes has been used with a number of methods, including PCR, fluorescence in-situ hybridization, dot- and slot-blotting, and microarray analysis (Brinkmeyer et al. 2002; Connell 2002; Snoeyenbos-West et al. 2002; Hide et al. 2003;

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Petroni et al. 2003; Stine et al. 2003; Agatha et al. 2004; Hosoi-Tanabe and Sako 2005). Recent progress in cultivation of oligotrich and choreotrich ciliates, the most abundant ciliate groups in the plankton, has led to a growing database of DNA sequences from the ribosomal and other genes. To date, these have been used principally to address questions of local and global diversity (Snoeyenbos-West et al. 2002). This study reports on the use of DNA sequences to design species-specific primers for amplification of ciliate DNA from environmental samples. Preliminary data show this method is reliable, sensitive, and time-efficient, compared with microscope techniques. It thus has the ability to provide information on distribution and abundance of individual species on spatial and temporal scales commensurate with ciliate movement and growth (meters and hours, respectively).

Materials and procedures

Development of primers—Snoeyenbos-West et al. (2002) identified the internal transcribed spacer region (ITS) of the ribosomal gene (ITS1-5.8S rDNA-ITS2) as an area of the ciliate genome that appears to be highly conserved across populations within a species. This was the area we focused on for primer design. Our initial target species, *Laboea strobila* and *Favella ehrenbergii*, were chosen for three reasons: (1) we had ITS sequence data from multiple populations (isolated from different locations and times), (2) both species are readily identifiable in Lugol's preserved samples, allowing us to use microscopy to compare with the DNA-based method, and (3) both have been cultured in our lab for extended periods, allowing us to test the method extensively before working with field samples. A third target represents one species of the genus *Strombidinopsis*, frequently isolated from Long Island Sound (LIS), which we have not identified to species level using traditional methodology. All three target species were isolated from LIS and grown in 6-well plates, using various phytoplankton species as food.

The initial step in primer development was performing a multiple alignment of all available sequences for each target species (collected from different locations and times), using Clustal X (http://www.ebi.ac.uk/clustalw/). The sequences were reviewed to identify areas of the ITS that are conserved across all available populations. A second sequence alignment, using all of our oligotrich and choreotrich sequences, was then used to identify those regions within the target species' conserved areas that differed from all other species within our database. These sites were identified as potential target primer sites. Potential primer sites were evaluated using Beacon Designer 3.0 software (PREMIER Biosoft International) to find a primer pair where at least one was species-specific, produced a product at least 250 base pairs in length, minimized cross-dimer, and had a low self-dimer potential. The candidate primers were then compared with all oligotrich and choreotrich sequences within our database, including some sequences not yet published on GenBank (> 150 sequences in total). This step was intended to discover any potential conflicts with other species. They were then compared with all published sequences using the National Center for Biotechnology Information (NCBI) database Basic Local Alignment Search Tool (BLAST). If BLAST indicated a 100% match to a sequence from any other known ciliate, the potential primer was eliminated. We used a criterion of > 75% match to identify non-target sequences that might be amplified with a potential primer. The only marine planktonic organism that exceeded that level with any of our potential primers was the tintinnid *Metacylis angulata*, which was a potential match for *F. ehrenbergii*. As a result, *M. angulata* DNA from our library was added to our testing protocol.

Testing primers—Primers were tested to verify that they amplified the target species, did not amplify DNA from non-target ciliates, and did not amplify other planktonic DNA. This included testing them against DNA from multiple population isolates of each target species, including both cultures and ciliates picked from natural populations. Next, the primers were tested on DNA from other ciliate species, including testing the *F. ehrenbergii* primers on DNA from *M. angulata*. Then we tested with DNA from common phytoplankton species, including isolates of *Tetraselmis* sp., *Rhodomonas* sp., *Gymnodinium* sp., *Cyclotella cryptica*, *Ditylum* sp., *Scrippsiella* sp., *Prorocentrum minimum*, and *Akashiwo* sp. Finally, we tested the primers on DNA from mesozooplankton commonly found in LIS, including a mixed sample from a 200 μm net tow in LIS (September 2004), and from cultures of *Acartia tonsa* and *A. hudsonica*. Only those primer sets that passed these specificity tests were moved to the next phase of testing.

DNA amplification can be inhibited in samples from chemically complex natural waters (Toranzos 1997). Thus, we also tested the primers on DNA extracted from a natural seawater sample amended by the addition of various cultured planktonic organisms, including the target species, to make an artificial planktonic community. This test was also used to evaluate optimal sample volumes to filter for extraction, optimal DNA concentrations for PCR, and detection limits for each species. For each target species, four 40 L samples were collected from LIS. These were passed first through a 20 μm mesh to eliminate the target ciliate species (verified microscopically) and combined into a 60 L container (Nalgene). Then, approximately 5000 ciliates from various cultures were added to the water. These included *Strombidium stylifer*, a *Strobilidium* sp., and a *Tintinnopsis* sp. In addition, *F. ehrenbergii* was added in tests of the primers for *L. strobila* and *Strombidinopsis* sp., *L. strobila* added in tests of the primers for *F. ehrenbergii* and *Strombidinopsis* sp., and *Strombidinopsis* sp. added in tests of the primers for *F. ehrenbergii* and *L. strobila*. Copepods (*Acartia hudsonica* and *A. tonsa*) and phytoplankton (*Rhodomonas* sp., *Prorocentrum minimum*, *Thalassiosira weisflogii*, *Isochrysis* sp., and *Tetraselmis* sp.) were also added to the water at typical in situ concentrations, to replace DNA removed during the 20 μm screening. The target ciliate species from

Table 1. Detailed procedure for extraction and purification of DNA from filters

DNA Extraction and purification protocol	
Step	**Procedure**
1	Incubate overnight (12 + hours) in a 55°C water bath with 0.5 mL proteinase K for each 1 mL lysis buffer
2	Remove filter from 1.5 mL tube with forceps, flaming forceps between samples
3	Add 1 volume of phenol (buffered to pH 8.0)
4	Vortex for 15 s
5	Spin at 10000 rpm at 4°C for 2 min
6	Remove supernate into clean 1.5 mL tube
7	Add 1 volume of phenol (buffered to pH 8.0)
8	Vortex for 15 s
9	Spin at 10000 rpm at 4°C for 2 min
10	Remove supernate into clean 1.5 mL tube
11	Add 1 volume of chloroform
12	Vortex for 15 s
13	Spin at 10000 rpm at 4°C for 2 min
14	Remove supernate into clean 1.5 mL tube
15	Add 0.1 volume of 0.5M NaCl
16	Vortex for 30 s
17	Add 2 volumes of 100% EtOH
18	Invert gently 10 times
19	Place at –80°C for 20 min
20	Spin at 14000 rpm at 4°C for 20 min
21	Pipette off liquid, reserving DNA pellet
22	Add 1 mL of 70% EtOH
23	Spin at 14000 rpm at 4°C for 2 min
24	Pipette off liquid, reserving DNA pellet
25	Air dry
26	Add 50 µL of Tris EDTA buffer and gently resuspend pellet
27	Store at –20°C

live laboratory cultures were added in concentrations ranging from 0.5 to 50 ciliates/L. The water was then concentrated down to approximately 1 L by reverse filtration. This process gently siphons water from the 60 L container via a large diameter hose through a 20 µm mesh. The concentrated water was filtered (< 70 kPa vacuum) onto a 25 mm diameter 3.0 µm pore size cellulose nitrate filter (Whatman, Cat. No. 7193-002). Several filter types were tested, with cellulose nitrate providing best DNA recovery. The 25 mm diameter size allows use of a minimum amount of lysis buffer in which to preserve the DNA.

To evaluate optimal amount of sample to extract, we separated each concentrate into four aliquots (1/2, 1/4, 1/8, and 1/16 of the total), and filtered, extracted, and amplified each aliquot separately. Our expectation was that the target species would be undetectable in the smaller aliquots of the rarer treatments (e.g., 0.5 ciliate L^{-1}), but detectable in all aliquots for the abundant treatments (50 ciliates L^{-1}).

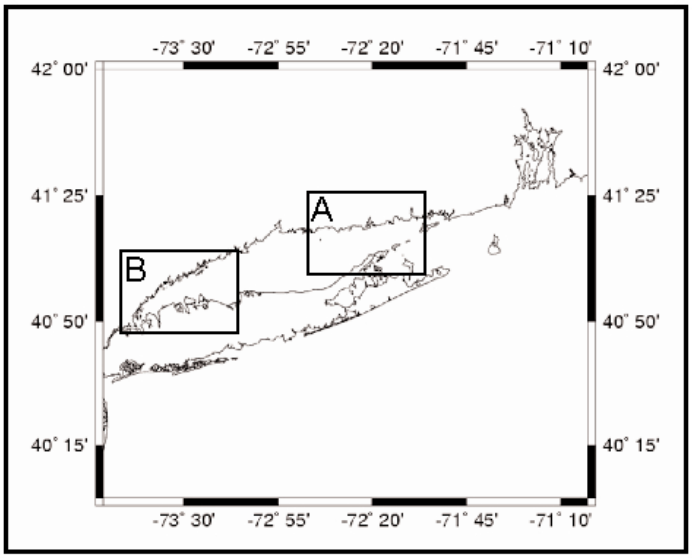

Fig. 1. Locations of two field testing areas in Long Island Sound. Sites A and B were each sampled twice.

DNA was extracted following the protocol outlined in Table 1. Three different dilutions of the concentrated DNA from each aliquot were used in PCR reactions for each aliquot for each sample (undiluted, 1:10, and 1:100 dilutions). The PCR protocol is described below.

Testing on field samples—Methods for collection, preservation, and extraction of prokaryote DNA from environmental samples provided insight into designing our field tests (Lee and Fuhrman 1990, 1991; Jiang et al. 1992; McInerney et al. 1995; Diez et al. 2001; Moon-van der Staay et al. 2001; Heidelberg et al. 2002). However, due to the high abundance of prokaryotes, these methods all extract from a relatively small volume of water (e.g., 100 mL used by Heidelberg et al. [2002] in Chesapeake Bay). Because ciliates are 5 to 6 orders of magnitude less abundant than prokaryotes, we experimented with methods to collect DNA from up to 40 L of seawater, potentially allowing us to document rare species.

Field tests were carried out in LIS at two different sites on two occasions each (Fig. 1). Collections at Site A (April 26, 2005 and May 3, 2005) were mainly from a vessel at anchor. Over several hours, we collected 40 L samples of surface water at 15–30 min intervals as tidal currents advected water past the vessel. Current velocity (range: 0.5–1.4 m/s) was measured periodically using a Marsh-McBirney flow meter (Model 201). This allowed us to convert sampling intervals into equivalent distances. Additional samples were collected over a larger spatial scale during the return to the dock. The total spatial extent of the sampling was approximately 20 km.

Field Site B consisted of five stations in western LIS (Fig. 1). Thirty liters of water was collected from three depths at each station on two dates (July 23, 2005 and July 28, 2005). The three depths sampled were surface (1 m), mid-depth (near the chlorophyll maximum), and near bottom (total depth c. 20 m).

Table 2. Tests of primers with target and non target DNA

	Favella ehrenbergii	Laboea strobila	Strombidinopsis sp.
Test against multiple populations of target	+	+	+
Test against potential match indicated by BLAST search	–	none indicated	none indicated
Test against other ciliate species	–	–	–
Test against LIS phytoplankton	–	–	–
Tetraselmis sp.	–	–	–
Rhodomonas sp.	–	–	–
Gymnodinium sp.	–	–	–
Cyclotella cryptica	–	–	–
Ditylum sp.	–	–	–
Scrippsiella sp.	–	–	–
Prorocentrum minimum	–	–	–
Akashiwo sp.	–	–	–
Test against LIS zooplankton			
Acartia tonsa	–	–	–
Acartia hudsonica	–	–	–
Field-collected assemblage	–	–	–

+, indicates that a PCR product of the correct size was obtained; –, indicates no product.

Seawater was collected using Niskin bottles or buckets (surface water) passed through a 330 μm mesh to remove large organisms and combined in a 60-L container (Nalgene). The sample was then reverse filtered by siphoning through a submerged 20 μm mesh using a large diameter hose. This gently concentrated everything between 20 and 330 μm down to approximately 1 L, which remained in the 60-L container. Preconcentration made the subsequent filtration process used to collect the DNA faster.

Concentrates were divided into three aliquots, representing 20, 6.7, and 2.2 L (Site A) and 15, 5, and 1.7 L (Site B) of the original samples, and filtered onto 3.0 μm cellulose nitrate filters, as described above. Filters with the DNA were placed in 1.5 mL microcentrifuge tubes and covered with lysis buffer solution (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, and 0.5% SDS, adjusted to final pH 8.0). Only 0.5 mL lysis buffer solution was used in order to limit the amount of phenol used during the extraction and purification process, though larger amounts of buffer (e.g., 1 mL) did appear to improve the extraction efficiency and purity of the extracted DNA in some cases. An aliquot of 500 mL of the unconcentrated sample was also preserved in an opaque plastic bottle with 5% (final concentration) acid Lugol's solution for microscopic examination.

DNA extraction, purification, and concentration from field samples—Proteinase K (0.5 μL per 1 mL DNA prep buffer or 0.05%) was added at the start of the extraction process. DNA was extracted and purified following a modified phenol-chloroform extraction process (Ausubel et al. 2002a) (Table 1). The concentration of purified DNA was measured on a spectrophotometer (Hitachi U-3010) at 260 nm. Relative purity of the sample was evaluated by making an additional measurement at 280 nm (260 value/280 value) (Ausubel et al. 2002b). We find that having the appropriate concentration of DNA is critical to obtaining optimal results in the PCR. All PCR reactions for our field tests were run with 6–9 ng/mL of DNA.

Polymerase chain reaction (PCR) and sequencing—PCR followed the basic procedures outlined by Saiki et al. (1988) and Riley and Katz (2001). However, a step-down approach (using three primer annealing temperatures during the PCR run) was used in order to maximize target specificity. A total of 35 cycles were run, the first two with a melting temperature just below the lowest melting temperature of the primer pair. Melting temperature in the third and fourth cycles was two degrees Celsius lower, and then the remaining cycles were run an additional two degrees lower. The reaction volume for the PCR was 25 milliliters. Two polymerases, AmpliTAQ Gold (Invitrogen) and Takkarra (FisherScientific) were the most reliable for us. Each PCR run included positive and negative controls (DNA from target species and autoclaved milli-Q water, respectively). PCR products were run on a 1.5%–2.0% agarose gel using a 100 base pair (bp) ladder as reference, since our target products were between 600 and 300 bp. Ten percent of gel positive results were confirmed by cutting the band from the gel and purifying the DNA (Zymo Research, product #D4001), then sequencing using a Big-Dye primer sequencing protocol (Applied Biosystems, product # 4337450) and a model 3130 Genetic Analyzer (Applied Biosystems). Sequence results were analyzed using Chromas 2.3 software (Technelysium).

Assessment

Testing of primers

Specificity of the primers for the targets was tested against DNA from more than one population of the target species,

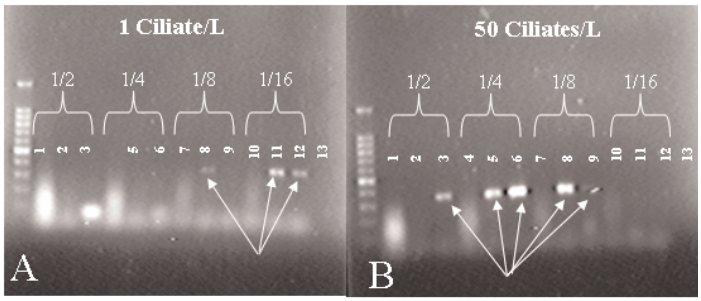

Fig. 2. Example of gel results from the artificial plankton community tests (*F. ehrenbergii*). When target ciliates were rare (A), we could only amplify them from the least amount of concentrate. When they were abundant (B), PCR was successful in all of the concentrate aliquots. The three different lanes for each aliquot (1/2, 1/4, 1/8, 1/16) represent undiluted, 1:10, and 1:100 extracted DNA dilution (left to right, respectively). Arrows highlight positive results. Lane 13 was empty for both gels. A 100 bp ladder is used for reference.

Table 3. DNA exaction efficiency from natural seawater (LIS, Site B) from field samples concentrated via reverse filtration

	Large Aliquot (A)	Medium Aliquot (B)	Small Aliquot (C)
Concentrate extracted (L)	0.35	0.11	0.04
Represents L of original sample	15	5	1. 7
DNA in extract (ng/mL)	1062	828	298
ng DNA per L original sample	3540	8256	8914
Dilution required for PCR	0.005	0.01	0.02

Original sample volume was 30 L, concentrated to 0.7 L by reverse flow. The concentrate was filtered in three aliquots: large (1/2 concentrate), medium (1/6 concentrate), and small (1/18 concentrate). Aliquots larger than 100 mL concentrate resulted in much lower DNA yields.

other oligotrichs and choreotrichs, and phytoplankton and copepods common to LIS. Each primer set amplified DNA from its target species, but none of the other organisms tested (Table 2).

To evaluate sensitivity, we extracted and amplified DNA from seawater containing added phytoplankton, microzooplankton, and mesozooplankton plus 50, 10, 1, or 0.5 of the target ciliates L^{-1}. All primer sets were able to amplify from the 0.5 ciliates L^{-1} treatment, even when only 1/16 of the concentrated sample was extracted and amplified. This represents successful amplification of DNA from 1.25 target individuals and underscores the highly amplified nature of the macronuclear genome in ciliates (discussed below).

We had expected to amplify successfully from the lower concentrations of added ciliates only in the largest aliquots of sample concentrate. Instead, we found exactly the opposite. When the target species was added at < 10 ciliates L^{-1}, we always failed to amplify it in any but the smallest amount of concentrate. Fig. 2 illustrates this for the *F. ehrenbergii* primer set. Panel A shows a gel for the target concentration of 1 ciliate L^{-1}, and Panel B shows results for 50 ciliates L^{-1}. At the low concentration of ciliates, the target DNA was detected in only the smallest (1/16) aliquot, while the 50 ciliates L^{-1} concentration was detected in all aliquots. This is most likely due to inhibition of PCR by compounds that are in the sample and are not eliminated in the extraction and purification steps. We found that extraction efficiency was consistently lower in the larger aliquots. More total DNA was extracted, but less DNA per liter concentrated (Table 3). Because DNA extracted from those aliquots did not have to be diluted as much prior to PCR, any inhibitory compounds present would have been less diluted than in the smaller aliquots. Inhibition is consistent with the observation that PCR was not successful for rare ciliates in larger concentrate volumes, which we saw repeatedly in both the lab and field tests. Apparently, inhibition can be overcome when the target is abundant. This underscores the importance of not extracting too large a sample of the plankton, and also having the optimal amount of total DNA in the PCR reaction.

Field test results—Based on previous microscopic examination of preserved samples, it was expected that we would find *L. strobila* but not *F. ehrenbergii* at Site A during Spring. PCR results indicated that indeed *L. strobila* was found throughout Site A, although the pattern of its presence differed between the two sampling days (Figs. 3 and 4). *F. ehrenbergii* was also present at Site A, although this species was patchier and present on only one of the sampling days (Figs. 3 and 4). *Strombidinopsis* sp. was present, but it was spatially patchy on both days (Figs. 3 and 4). We used a relative abundance scale for the field samples, scoring them by presence of a target in none, one, two, or all three of the concentrate aliquots, based on our results in the laboratory testing, with 3 relating to higher abundance levels and 0 to absent (< 1/L). Microscopy of preserved samples indicated the presence of *L. strobila* (c. 20 cells/L), but not *F. ehrenbergii* (< 4/L, based on the amount of sample settled). *Strombidinopsis* sp. cannot be identified to species in Lugol's preserved samples, so confirmation of its presence is not possible through microscopy.

L. strobila was not amplified from Site B on either sampling date (Figs. 5 and 6), nor was it seen in preserved samples. *F. ehrenbergii* was present at Site B during both sampling days at all stations. We used the same relative abundance scale as previously described for the field samples, scoring them by presence of a target in none, one, two, or all three of the concentrate aliquots. Abundance differed among depths, stations, and days (Figs. 5 and 6). Results for *Strombidinopsis* sp. at Site B indicate a patchy presence, in depth, location, and time (Figs. 5 and 6). *F. ehrenbergii* and ciliates of the genus *Strombidinopsis* were identified in the preserved samples from some stations by microscopy, but *L. strobila* was not seen (< 4 cells L^{-1}). Microscopic examination of the Lugol's preserved samples from Site B indicates that abundance in the preserved samples corresponds to the relative abundance from the PCR results (Table 4). Three samples from the July 23, 2005,

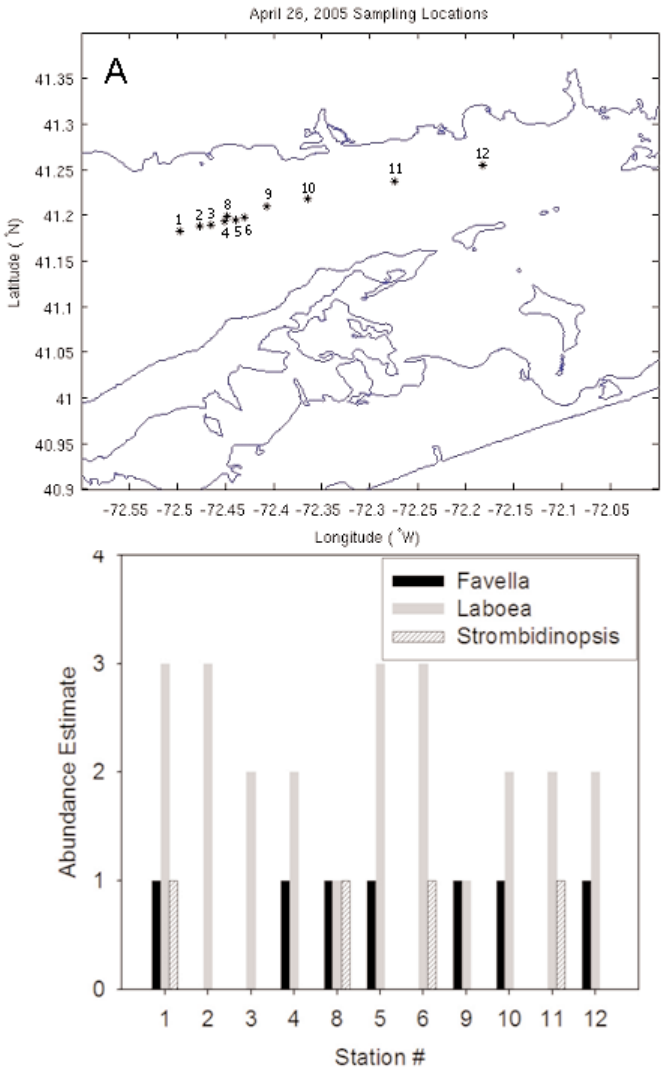

Fig. 3. Sampling locations and PCR results for 26 April 2006 (Site A). In panel A, stations 1-6 were sampled from an anchored vessel. Sampling time was converted to distance/location using current velocity and direction. Panel B shows relative abundances (y-axis) of the target species, stations ordered by longitude (x-axis). The y-axis is the number of positive results from the concentrate aliquots.

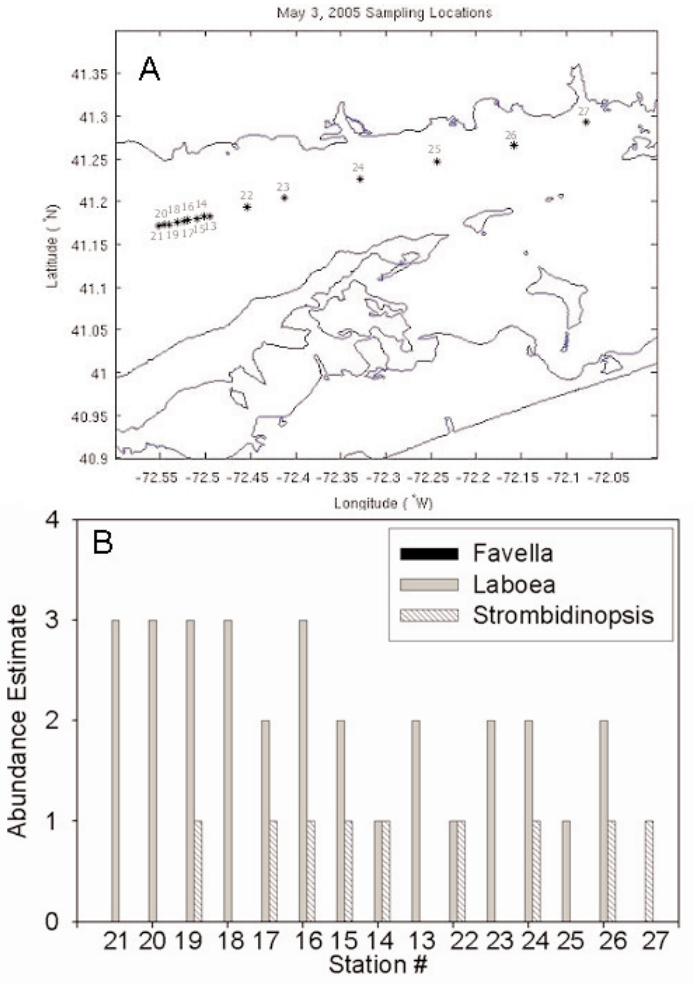

Fig. 4. Sampling locations and PCR results for 3 May 2006 (Site A). In panel A, stations 13–21 were sampled from an anchored vessel. Sampling time was converted to distance/location using current velocity and direction. Panel B shows relative abundances (y-axis) of the target species, stations ordered by longitude (x-axis). Station numbers are chronological and continue from the 26 April 2006 sampling. The y-axis is the number of positive results from the concentrate aliquots.

sampling show relative abundance levels that match to the PCR results for the two target species identifiable in the preserved samples. *F. ehrenbergii* abundance ranged from 4/L to 56/L and *L. strobila* was not seen in any sample (Table 4).

Ten percent of the positive results for each target species from the field samples were selected for sequencing to confirm that the amplified product matched the target sequence. Results indicate a 100% match to the expected sequence for each of the target species (Table 5).

Discussion

This method provides a means to assess distribution of individual ciliate species. It is faster than traditional microscopic examination methods and allows for identification of rare species (< 1 ciliate/L). It also removes ambiguity in identification of species such as *Strombidinopsis* sp., where microscopic identification is very difficult.

The high sensitivity of the method (ability to detect < 1 ciliate L^{-1}) is likely due to the highly amplified nature of the ciliate genome. Ciliates contain two kinds of nuclei. Micronuclei are generally diploid and transcriptionally inactive. Macronuclei, on the other hand, contain multiple copies of gene-size fragments of DNA, and comprise the transcriptionally active "working copy" of the genome (Bell 1988). In the macronucleus, a ciliate may have thousands of copies of a particular gene. Thus a single ciliate can provide the 10^4 copies of DNA per reaction that is considered necessary for PCR to work properly (Saiki et al. 1988). While this makes it possible to detect just 1 ciliate, it is also likely to make it

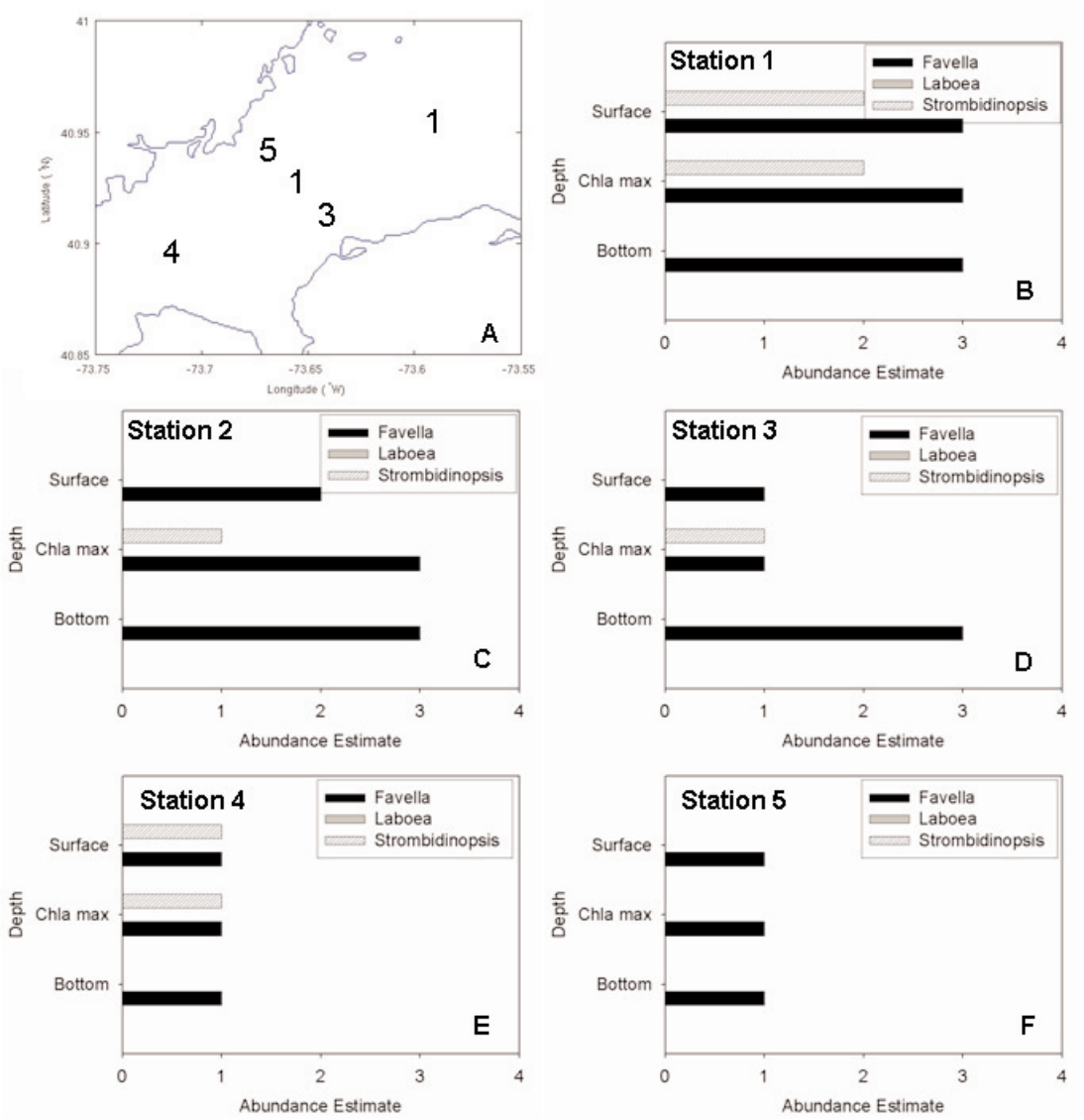

Fig. 5. Locations and results of sampling on 23 July 2005 at Field Site B. Panel A shows locations of the five sampling stations. The panels B-F graph the relative abundance of the three target species (x-axis) by depth (y-axis) for each of the five stations. "Abundance Estimate" is the number of positive results from the three concentrate aliquots.

difficult to be fully quantitative. The use of real-time PCR (RT-PCR) is currently being tested, however, unless all ciliates of a particular species have approximately the same number of copies of a particular gene, quantification via RT-PCR may not be possible.

Because this method allows for the processing of samples quickly and efficiently, with low detection levels, it has the potential to provide insight into ciliate ecology. As noted earlier, current methods to study ciliates are limited in the number of samples that can be readily examined, proper identification of species is unlikely, and species that are low in abundance can be missed. This new method avoids these difficulties and can help provide a more detailed understanding of the dynamics of individual ciliate species, including correlations to various factors (e.g., food availability and type). There is also the potential to quantify predator pressure on the specific species, using the species specific primers to detect the presence of a ciliate species within a common predator (i.e., copepod). Preliminary work, not presented here, indicates that 1 ciliate ingested by a copepod can be detected.

Comments and recommendations

Making the method fully quantitative has been more difficult than anticipated. In principle, abundance could be quantified by filtering successively smaller volumes of sample and recording presence/absence as a rare target species becomes extinct in the PCR. This is what we tried to do in both the lab

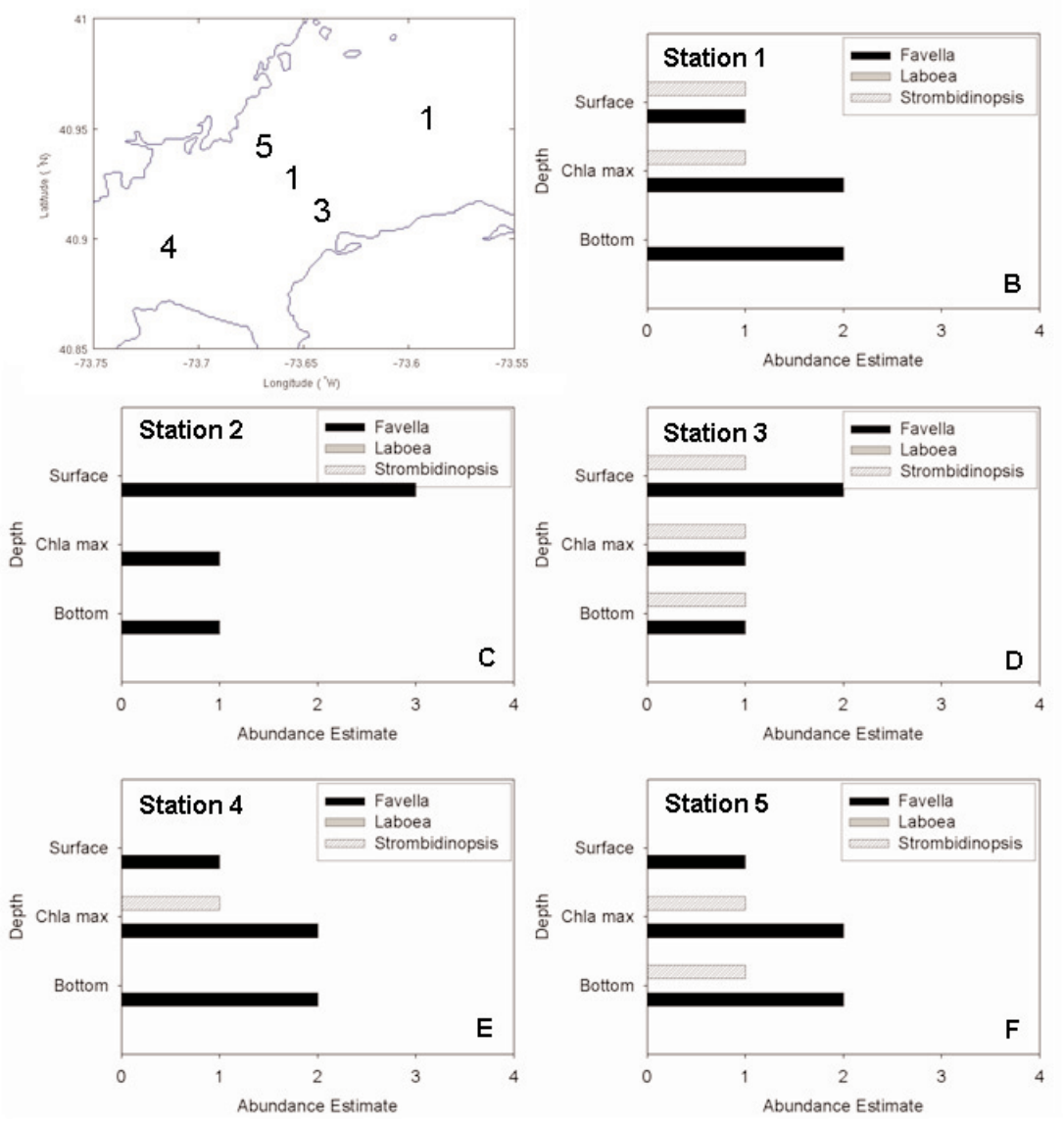

Fig. 6. Locations and results of sampling on 27 July 2005 at Field Site B. Panel A shows locations of the five sampling stations. The panels B-F graph the relative abundance of the three target species (x-axis) by depth (y-axis) for each of the five stations. "Abundance Estimate" is the number of positive results from the three concentrate aliquots.

and field tests. However, we observed that this procedure is limited by reduced extraction efficiency and inhibitory compounds when larger volumes are filtered.

PCR inhibition occurs due to both too much DNA in the reaction and the presence of various inhibitors co-extracted with DNA. Natural waters often contain compounds, such as humic acids, that can interfere with PCR (Toranzos 1997; Wilson 1997; Chandler 1998; Lowery et al. 2000; Loge et al. 2002; Harms et al. 2003). The extraction and purification process does not always remove them, and as the volume of seawater is increased, these compounds also increase (Toranzos 1997).

A second factor affecting PCR success is the ability of the primer targets to find the appropriate DNA in a very diverse and concentrated DNA sample. The ratio of target ciliate DNA to total DNA in the sample is likely quite small. Thus, PCR reactions are only effective at a concentration of less than 10 ng/mL, requiring measurement by spectrophotometer and dilution of the DNA for the PCR to be effective. One possible modification of the method to avoid extraction efficiency and inhibition problems that arise when large volumes are filtered would be to filter many replicates of 2-3 L and estimate abundance by the proportion of these in which a given target species appears.

Our field observations indicate that the three target species show different spatial patterns. Our next steps are focusing on refining the process to improve quantification, including

Table 4. Comparison of microscope versus PCR based on abundance estimates from two of the target species

Sample	Species	Microscope abundance (250 mL settled samples)	PCR abundance level
Station 1 Mid	*F. ehrenbergii*	56/L	3 (high)
	L. strobila	< 4/L	0 (absent)
Station 5 Surface	*F. ehrenbergii*	4/L	1 (low)
	L. strobila	< 4/L	0 (absent)
Station 3 Deep	*F. ehrenbergii*	8/L	1 (low)
	L. strobila	< 4/L	0 (absent)

Samples were from Site B, 23 July 2005. Microscope abundance is based on settling 250 mL Lugol's preserved; thus minimum detection limit is 4/L. PCR abundance scale ranges from 0 (no PCR product in any aliquot) to 3 (product in all three aliquots).

possible use of quantitative RT-PCR. In addition, we are designing primers for other species as sequences become available. The goal is to continue to add enough target species to make this process appropriate for use with microarray technology as it currently is with marine prokaryotic communities (Loy et al. 2002; Stine et al. 2003; Barlaan et al. 2005).

Reproducibility—Because this process requires dilution of the extracted and purified DNA from field samples to produce a DNA concentration that avoids PCR inhibition, there is the potential that a different dilution of the original extracted DNA might provide different results. This is particularly true when a target species is relatively rare (less target DNA overall). To evaluate this, approximately 20% of the lab test samples and 10% of the field test samples were rerun using new dilutions for the PCR stage. These reproducibility tests confirmed the original result 95% of the time, and the remaining 5% produced the same presence/absence of the target, but differed in the relative abundance of the particular target

Table 5. Successful primers and their product sequences

Target species	Product length (bp)	Primers (5′–3′)	Sequence
Favella ehrenbergii	353	Favella _ F1	ACCTACTCAACCAAGCCAATCTGTTGCAGGGCGAAAGCCTCGCA
		ACCTACTCAACCAAGCCAATCTG	GCTAAAACCTAACCAAAAGCAAGCTAACTAAGCTTCAACCTAAA
		Favella _ R1	ACCAAATCCTCAACGATGGATATCTAGGTTCCTACTACGATGAA
		CATTGAGTGCGTCAGTCCTTGT	GAACGCAGCGAAGTGCGATAAGCAATGCGAATTGCAGAGCCGAG
			AGTCATCAGATCTTTGAACGTAACTGACACCGGAGAGCTCTCTC
			TTCGGTATGCTTCTTTCAGTGTGTGATTCTCTCATCACCCAAACCT
			TAATGCGATAGATGCCCTTCTATTGCTAAGCTCGAAAGCACTCT
			GAACCCTGCAGCGGAGGTCTCACAAGGACTGACGCACTCAATG
Laboea strobila	455	Laboea _ F1	AAGCCTTTAGCTGGAGTGAAGTACCTCGGTACGGATCATCAGCAA
		AAGCCTTTAGCTGGAGTGAAGTA	ACACAAAACTAAAACTAAAAGGAGCCTAACTAAGCTAAAACTAA
		Laboea _ R1	AAACCAAATTCTAAACGATGGATGTCTTGGTTCCCATAACGATG
		CCTGAGCCCAGATACGGTTTC	AAGAACGCAGCGAAGTGCGATAAGCAGTGCGAATTGCAGAACCG
			TGAGTCATCAGATTTTTGAACGCAACTGGCACCGTACGGTCTCT
			GGCCGTCGGTACGCCTGGTCCAGTGTCTTGTTATCTCATGACCTA
			AATCTTAATGCGGGAGATGCCCTTCTCTCGTCAAGCATGAAAGC
			ACTCTGCGCGAGCGAGTAACCTTCGGGTTGCACTCAATGCAGCAG
			TCACATTGCTTACAGTGTGAACTCATTGAGAGTGGACGCAGCGCG
			ACAATAGTCGCAAGTGTTTAGTCATATCAACTTAGAAACCGTATCT
			GGGCTCAGG
Stombidinopsis sp.	329	S-opsis _ F1	TCTGTTGCTGTTGGCGAAAGCTAGCAGCACCGAAACAACTAACCAAA
		TCTGTTGCTGTTGGCGAAAG	AGAAGCTTAACTAAGCTCATAATAAGAACCAAATCCTCAACGATGGAT
		S-opsis _ R2	GTCTAGGTTCCCACAACGATGAAGAACGCAGCGAAGTGCGATAGG
		ATTGAGTGCGACCCGAGGG	CAATGCGAATTGCAGAACCGTGAGTCATCAGATTTTTGAACGCAA
			CTGGCACCGAGGGGATATCCCCTGGGTATGCTTGTTTCAGTGTGTGT
			TTTTCTCCTCACCCAAATCTTAATGTGAAAGATGCCCTTCTTTCATT
			AAACAAGAAAGCACTCTGCGCTTTGCAGTGGCCCTCGGGTCGCAC
			TCAAT

(e.g., changing from a positive result in only one aliquot to positive result in two aliquots).

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