Microbial Ecology

Plankton Diversity in the Bay of Fundy as Measured by Morphological and Molecular Methods

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Abstract

Phytoplankton have traditionally been identified based on morphological characteristics. However, identifications based on morphology are time-consuming, require expertise in taxonomy, and often fail to distinguish differences among the multitudes of minute, nondescript planktonic organisms. Molecular techniques, which have revealed new insights into bacterial and picoplankton communities, may also enhance our knowledge of the diversity among communities of larger plankton. We compared plankton identifications and community assessments based on the two types of techniques (morphological vs molecular) for surface seawater samples collected on 2 May, 31 July and 25 September 2000 from several sampling stations in the Bay of Fundy. Phytoplankton captured in surface bucket samples were quantified and identified based on morphology. DNA was extracted from plankton communities (5–100 μm in diameter) collected by filtration, and 18S rRNA gene fragments were amplified with primers specific for eukaryotes. Denaturing gradient gel electrophoresis (DGGE) was used to develop DNA profiles of eukaryotic phylogenetic diversity and to select cloned 18S rDNA fragments for sequencing. Both morphological and molecular methods showed great community diversity. However, the communities identified with the two different types of techniques were starkly different. Morphological abundances and taxon richness were lowest in the May samples, whereas the number of DGGE bands was highest in May and July. Morphological identifications showed a succession of dominant organisms through time. Whereas neither diatoms nor dinoflagellates were dominant in May, diatoms and a few dinoflagellates were dominant in July and September. In contrast, few 18S rDNA sequences were related to rDNA sequences of known identity, and furthermore, few diatoms were identified in the molecular analyses. Molecular phylogenetic analysis indicated the presence of many novel organisms, several of which were most closely related to other unidentified sequences from diverse marine environments representing new lineages. Our results support the ideas that we are just beginning to uncover the diversity of eukaryotic marine organisms and that there may be many more ubiquitous, microeukaryotic plankton than previously realized. Our results suggest that both types of methods capture only a portion of the community. Morphological methods may be more adept at capturing the phototrophic organisms within the community. However, just as for bacteria and picoplankton, molecular techniques can enhance our understanding of plankton diversity, particularly by detecting previously unidentified organisms.

Introduction

The importance of autotrophic phytoplankton to primary production in marine environments has been well known for some time. Heterotrophic plankton release nutrients stored in biomass during grazing of microbial organisms, illustrating the importance of these plankton to nutrient regeneration. Yet, despite their acknowledged importance, the extent of planktonic diversity is still largely unknown. As a current major scientific challenge centers around the sustainable use and management of natural resources in all ecosystems with minimal loss of biological diversity, there is a great need to improve understanding of planktonic diversity in marine ecosystems. Molecular tools may provide the resources to vastly improve our understanding of the diversity of natural communities. Although there have been some studies comparing marine community structures obtained using

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different molecular techniques [8], we are not aware of studies comparing eukaryotic communities identified morphologically to communities identified by molecular phylogenetic analyses.

Traditionally, phytoplankton taxonomy has been based on morphological characteristics, with the underlying assumption that these defining characteristics are unique to a species and physiologically meaningful. This assumption has been discredited on various occasions. For example, in the waters off the northeastern coast of the United States, *Alexandrium tamarense* and *A. fundyense* have been designated as two species based on the presence of a single pore on the apical plate of one "species" but not the other [2]. However, these designations are not supported by toxin composition, sexual compatibility, bioluminescence capacity, or rRNA gene sequences, all of which suggest that these two organisms are part of a single species [2, 21]. Additionally, "*A. tamarense*" isolates from North America and coastal Japan have the same morphology, but different 18S rRNA gene sequences [21]. In fact, several genera of dinoflagellates have been shown to be composed of assemblages of unrelated organisms, exemplifying how morphological traits used to distinguish taxa can many times be incongruent with molecular phylogenies [5].

On the other hand, classifying microscopic organisms that lack distinguishing features makes morphological identifications problematic. Bacteria and ultraplankton or picoplankton are not unequivocally identified by optical, epifluorescent, or even electron microscopy. Plankton can be grouped by pigment characteristics as identified by HPLC, but placement is into groups at a family level and is not species- or even genus-specific [3]. Although cultivable microorganisms have led to the discovery of previously unidentified organisms, only a small percentage of organisms have been cultured and subsequently identified [13]. Unfortunately, culturing of microbial organisms does not indicate any level of environmental importance because there is no connection between ease of cultivation and dominance in a naturally occurring community.

Molecular techniques have been applied successfully to analyze bacterial groups and more recently picoplankton communities in the size range of 0.2 to 3 or 5 μm [7, 15, 18, 19, 22] and eukaryotic diversity in anoxic sediments [6]. These studies employed polymerase chain reaction (PCR)-based amplification using either primers that target most Bacteria to amplify plastid rDNA or primers that target most Eukarya to amplify eukaryotic 18S rDNA. These molecular techniques have revealed new lineages and repeatedly demonstrated that the microeukaryotic diversity may be orders of magnitude greater than previously anticipated. Although it is generally assumed then that molecular techniques are more powerful than more traditional methods, this assumption is rarely empirically and directly tested. Limitations inherent in morphological identification preclude direct comparisons of molecular and morphological bacterial and picoplankton identifications. However, those comparisons are possible with larger phytoplankton (operationally defined in this study as sizes 5–100 μm).

The objective of this research was to compare identifications based on traditional morphological methods and the molecular approach combining PCR-based denaturant gradient gel electrophoresis (DGGE) and cloning of 18S rDNA. Surface seawater samples were collected from several stations in the Bay of Fundy. Amplified eukaryotic rRNA gene fragments were resolved by DGGE to determine community diversity patterns. The 18S rDNA of environmental clones demonstrating unique migration distances during DGGE was partially sequenced for phylogenetic identifications. Numerically dominant phytoplankton were expected to be identified by both methods, with increased diversity revealed by phylogenetic analysis of the 18S rDNA amplified community due to identification of previously unknown organisms. Additionally, we compared our results from the molecular techniques with studies investigating eukaryotic diversity in other regions of the world. Results revealed very little agreement between traditional and molecular identifications and surprisingly much higher degree of similarity with other molecular studies of samples taken from different plankton size fractions and different depths across the globe.

Materials and Methods

Study Sites and Sample Collection. Surface seawater samples were collected from a total of eight different stations in the Bay of Fundy ($-66°43'$ to $-67°05'$ longitude and $44°55'$ to $45°10'$ latitude) on 2 May, 31 July, and 25 September 2000 (Fig. 1) from the R/V *Pandalus III*. In May, July and September, locations sampled included Brandy Cove (morphology only in May and July), Deadmans Harbour, Lime Kiln, Passamaquoddy Bay, and Wolves Islands. Bocabec Bay was included as a sampling location in July, and extra samples were collected for molecular analysis from Prince 5 in July and September and from Prince 6 in September. Thus, a total of 16 samples were collected for morphological identifications and 17 samples were collected for molecular samples. In this study, a "sample" refers to a given sampling location at a specific sampling time, e.g., May Wolves Islands represents one sample and May Deadmans Harbour represents another sample. Two subsamples were taken for each sample. Surface seawater samples were collected by bucket from all stations for morphological identifications. Polypropylene buckets, rinsed with 70% ethanol and seawater from the sampling location, were also used

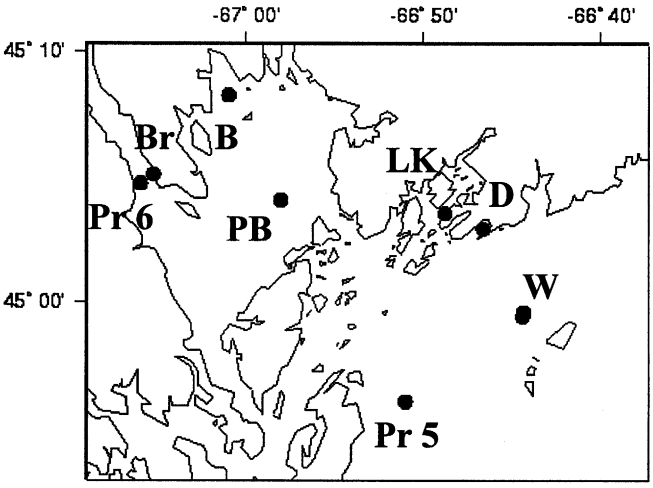

Figure 1. Map of sampling locations in the Bay of Fundy. Abbreviations on the map are as follows: Brandy Cove (Br); Bocabec Bay (B); Passamaquoddy Bay (PB); Lime Kiln (LK); Deadmans Harbour (D); Wolves Islands (W); Prince 5 (Pr 5); and Prince 6 (Pr 6).

to collect ~10 L of surface seawater for molecular methods. Samples were held on ice for transport to the laboratory. Within 12 h of seawater collection, seawater samples for the molecular analyses were filtered sequentially using a peristaltic pump through a 100-μm pore size mesh, to remove large particulates and zooplankton, and a 5.0-μm pore size cellulose acetate filter to collect plankton. Filters were kept at −70°C until DNA was extracted.

Morphological Identifications. Water samples (250 mL) collected by bucket were immediately preserved with 5 mL formalin:acetic acid. Later, 50-mL subsamples were settled in Zeiss counting chambers for 16 h. All phytoplankton greater than 5 μm were identified and enumerated (as cells L^{-1}, or chains of cells L^{-1} for organisms that grow in chains) using a Nikon inverted microscope. In addition, during the summer months a vertical plankton haul was made with a 20-μm mesh net, 0.3 m in diameter, in case additional cells were required for further identification and confirmation of morphology. From the vertical hauls, live phytoplankton samples were immediately iced for the return trip to the laboratory and a subsample was preserved with formalin:acetic acid (1:1 by volume) for further identification and SEM using either a JEOL JSM-5600 scanning electron microscope (SEM) or a Hitachi S-2400 SEM. Sample preparation for SEM was as follows: samples were rinsed with 250 mL distilled water (prefiltered 1.3 μm) onto a 3-μm (Poretics) polycarbonate filter using a 25-mm Millipore vacuum filtration apparatus. Diatoms were cleaned with the permanganate oxidation method [12] while samples with thin walls and/or unarmored dinoflagellates were dehydrated in a series of ethanol solutions (20, 50, 70, 85, 95%) prepared with distilled water and absolute ethanol for a minimum of 10 min at each step, finishing with three rinses of 100% ethanol. For the final drying step, three changes of hexamethyldisilazane (HMDS) were used [4, 14] for a minimum of 10 min each, allowing the last rinse to evaporate slowly at room temperature. Filters were mounted on stubs, and then coated with gold–palladium in a Hummer sputtering system.

DNA Extraction and PCR Amplification. A PCR-DGGE approach was used to analyze the 18S rDNA phylogeny of the eukaryotic community collected on the 5.0-μm pore size filters. DNA was extracted from filters, which were aseptically cut into small pieces and subjected to three freeze–thaw cycles using liquid nitrogen and a 65°C water bath. This was followed by extraction of DNA using a bead-beating DNA extraction kit (FastDNA Spin kit, Bio 101, Carlsbad, CA). DNA was lyophilized in a Speed Vac (Thermo Savant, Holbrook, NY) and resuspended in PCR-grade water (Sigma-Aldrich, St. Louis, MO). Extracted DNA was used as a template and amplified by PCR with primers F1427GC and R1616, which are specific for 18S rDNA of eukaryotic aquatic organisms [22]. PCR conditions for each 50-μL reaction were 10 mM Tris-HCL, 50 mM KCL, 0.01% (wt:vol) gelatin, 1.5 mM $MgCl_2$, 0.5 μM each primer, 200 μM each deoxynucleotide, 400 ng bovine serum albumin, 2.5 U of AmpliTaq DNA polymerase (PerkinElmer, Norwalk, CT) and 2 μL of template DNA. PCR was performed in a Genius (Techne Inc., Princeton, NJ) thermal cycler with the following temperatures: initial denaturation at 94°C for 5 min, followed by 25 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min, and a final extension step of 72°C for 5 min. PCR products were resolved on 1.5% agarose gels stained with ethidium bromide. PCR products were visualized on a Gel Doc system and analyzed using Quantity One software (Bio-Rad Laboratories, Hercules, CA). DNA content was quantified by comparison to a precision molecular mass standard.

DGGE Analysis of Environmental PCR Products. Denaturing gradient gel electrophoresis (DGGE) profiles were generated to compare communities. DGGE analysis was performed in a D-Code system (Bio-Rad Laboratories, Hercules, CA). Approximately equal amounts of PCR products were added to a 1.5-mm-thick vertical gel containing 8% polyacrylamide (acrylamide:bisacrylamide ratio of 37.5:1) and a linear gradient of 40–65% denaturant solutions (where 100% is equivalent to 7 M urea and 40% deionized formamide). Electrophoresis was performed at 60°C, with 75 V applied to the gel for 16 h. The running buffer contained 40 mM Tris, 40 mM acetic acid, and 1 mM EDTA (pH 7.6). Approximately 800–1000 ng DNA was applied

to each lane for environmental samples. DNA was visualized after staining the gel for 1 h in buffer containing 0.5 mg L^{-1} ethidium bromide followed by 20 min of destaining in deionized water. Gel images were analyzed on a Gel Doc using Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Clone Library Construction. Sequencing efforts on the 210 bp PCR fragments from DGGE were unsuccessful. Therefore, to obtain sequence information, clone libraries of near-complete 18S rDNA genes were generated from environmental DNA templates. Three environmental samples per sampling date were chosen that together contained most of the DGGE bands observed for that sampling date. Deadmans Harbour, Lime Kiln, and Passamaquoddy Bay were used for May samples. Prince 6, Wolves and Lime Kiln were used for July samples, and Wolves, Lime Kiln, and Passamaquoddy Bay were used for September samples. DNA from each sample was used as a template in PCR with primers E4 and E1628 [23]. Thermal cycler conditions included an initial denaturation at 94°C for 5 min, followed by 34 cycles of 94°C for 1 min, the annealing temperature for 1 min, and 72°C for 1 min, and a final extension at 68°C for 10 min. "Touchdown" primer annealing was used [9] from 54 to 50°C over 9 cycles (decreasing 0.5°C per cycle), followed by 25 cycles at 50°C. Separate clone libraries were constructed for each sampling date using PCR products of nearly full-length 18S rDNA combined from several samples (as mentioned above). Composite PCR products were purified using Qiaquick spin columns (Qiagen, Valencia, CA).

Clone libraries were constructed using the TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA) using the pCR 2.1-TOPO vector and TOP 10 One Shot Chemically Competent cells. Cloning reaction and transformation protocols were as suggested by the manufacturer. Transformed cells were plated on LB plates containing 40 μL of 40 mg mL^{-1} X-gal and 50 or 100 μg mL^{-1} ampicillin or kanamycin. White colonies that appeared after 1 and 2 days of incubation at 37°C were streaked onto new plates. Approximately 200 colonies from each sample date were selected and screened for amplification of partial 18S rRNA genes using the PCR reaction conditions listed above and F1427GC and R1616 as primers. A total of 164 positively amplified PCR fragments were then screened for differences in sequence using DGGE profiles. Bands were initially screened on gels with a denaturant gradient of 40–70%. Bands were then further screened on gradients of 40–55%, 50–65%, or 55–70% to enhance differences among closely migrating bands. When multiple bands migrated to the same position, only one sample was retained for sequencing.

Ninety-one colonies representing unique relative migration distances were grown overnight in LB at 37°C to obtain DNA for sequencing. Cells were pelleted from 2 mL culture and sequences were obtained either from plasmids purified from cell pellets with Qiaquick Plasmid Prep Kits (Qiagen, Valencia, CA) or from a PCR amplicon of the insert. The latter was obtained by boiling cell pellets in 100 mL of 0.1 M Tris pH 8.0 for 2 min to lyse cells and using M13F and M13R primers (as described by the TOPO TA cloning kit) under the PCR conditions described above. The temperature profile used included an initial denaturation at 94°C for 10 min, followed by 25 cycles of 94°C for 1 min, 50°C annealing temperature for 1 min, and 72°C for 1 min. A final extension step of 72°C for 10 min completed the PCR reaction. PCR products were purified using Qiaquick spin columns (Qiagen) and 18S rRNA fragments were sequenced.

Sequence Analysis. Sequencing reactions were performed using a dye terminator cycle sequencing kit to prepare samples (Beckman-Coulter, Fullerton, CA) and a Beckman-Coulter CEQ 2000 automated DNA sequencer. Two reactions were performed for each clone, one using the M13 primer and the second using the reverse eukaryotic primer, either E4 or E1628 depending on the orientation of the inserted DNA in the plasmid. The two partial sequences for each clone were combined and compared to the Ribosomal Database Project II (RDP II) for alignment with the most closely related 18S rRNA sequences in the database [16]. Seven of the clones did not yield quality sequence data, could not be aligned, and were removed from further consideration.

18S rDNA sequences were checked for potential chimeras with the RDP II Chimera Check program [16]. Sequences were compared to those in both the GenBank [1] and the RDP II databases and alignments were checked manually using SeqPup (ftp://iubio.bio.indiana.edu/). Only unambiguously aligned base positions were used to calculate Jukes–Cantor distances and construct phylogenetic trees with neighbor joining and maximum parsimony methods using PAUP* (Sinauer Associates, Sunderland MA). The topologies of phylogenetic trees generated by the two methods were fairly similar; therefore, only the trees generated by maximum parsimony methods will be shown. Phylogenetic trees were constructed by using 100 bootstrapped data sets.

Clone sequence designations begin with the first letter of the sampling month from which they were obtained. Some reported clones are designated with more than one letter because they represent clones from multiple months since individual DGGE bands from those months had identical migration distances. Sequences were deposited in GenBank under accession numbers AY331725–AY331788.

Data Analysis. Taxon richness for the morphological samples was determined based on the number of

taxa identified at the species level or higher for each sample. Shannon–Weaver index values were calculated for each sample according to equation (1):

$$H = \sum p_i \ln p_i \quad (1)$$

where H is the Shannon–Weaver index value, and p_i is the proportion of each taxa in each sample.

Results

Morphological Identifications. A total of 87 taxa in the 5–100 μm size class were identified by morphology, representing 51 different taxa at the genus level or higher (Table 1). Thirteen genera of dinoflagellates, 27 genera of diatoms, and grazers consisting mainly of ciliates (three taxa containing Ciliaphora) were among those identified. Additionally, dinoflagellates and diatoms that could not be identified to the genus level were placed into two broad categories, unarmored and armored categories for dinoflagellates, and centrale and pennate categories for diatoms. Many taxa were identified in multiple samples (Table 1). Of the 87 taxa reported in Table 1, 21 occurred in all three months, but *Ceratium* and *Chaetoceros* were the only two genera with representatives in every sample and *Cylindrotheca closterium* was the only species that occurred in every sample ($n = 16$). At the same time, there were only 16 taxa that were unique to one time and place ($n = 1$). This total of unique organisms accounts for 18% of the total 87 organisms, as opposed to the 39% unique DNA bands in DGGE (see DGGE section).

Total abundance and distributions of taxonomic groups varied considerably over time (Fig. 2). Samples contained a relative abundance of ciliates in May, with a general shift in taxonomic groups towards diatoms at most sites in July and September (Fig. 2). However, organisms in Passamaquoddy Bay in July and September and Bocabec Bay in July were more evenly distributed among the ciliates, diatoms, and dinoflagellates than in any of the other samples at those dates (Fig. 2). Total plankton abundances were generally highest in July, with increases in abundances of at least 30 to more than 175 times the abundances counted in May. Total abundances decreased from July to September in all locations except Passamaquoddy Bay, but remained much higher than any of the samples taken in May (Fig. 2).

Taxon richness, indicated by the number of taxa identified at the species level or higher in each month, was also higher in July and September than in May (Fig. 3). The number of taxa was highest at the Lime Kiln and Bocabec Bay sites in July, and was highest at the Lime Kiln and Deadmans Harbour sites in September. Diversity, as calculated by the Shannon–Weaver index, ranged from 1.16 to 2.55 in all samples, with the highest value calculated for Brandy Cove in September and lowest values calculated for Brandy Cove and the Wolves Islands in July (Fig. 3). While there were no clear trends in diversity across sampling sites, diversity was generally greatest in September. In three locations (Wolves Islands, Deadmans Harbour, and Brandy Cove), diversity was lowest in July despite the high abundances and number of taxa identified (Fig. 3).

Because dominant taxa should produce a band during DGGE, we measured the number of taxa with relative abundances >1% and >10% of the total in all the samples (Fig. 3). Sixty taxa exceeded 1% relative abundances, with *Cylindrotheca closterium*, *Skeletonema costatum*, and *Mesodinium rubrum* representing >1% of the total in more than half of the samples (Table 1). In contrast, only 16 taxa represented 10% or more of a sample (Table 1) and the number of these dominant organisms was never greater than four in any one sample (Fig. 3). In May, the dinoflagellate *Ceratium longipes* and the diatom *Thalassiosira* sp. were >1% of the community in all samples (Table 2). Additionally, ciliate grazers, such as *Mesodinium rubrum* (at all sites) and *Ptychocylis* sp. (Deadmans Harbour site only), were among the most abundant organisms, making up 39–67% of the total abundance in all samples in May. A temporal shift from May to July led to the dominance of the diatoms *Cerataulina pelagica* (>1% of the community in all samples), *Chaetoceros socialis*, and *Skeletonema costatum*, and the dinoflagellate *Heterocapsa triquetra* (Table 2). *Mesodinium rubrum* was also abundant in Bocabec Bay and Passamaquoddy Bay, along with armoured dinoflagellates in Passamaquoddy Bay in July. Together these organisms accounted for 65–94% of the total abundances in all sampling sites in July. Diatoms were predominant organisms at all sampling sites in September also. *Leptocylindrus danicus* and *Thalassionema nitzschioides* comprised greater than 1% of the community in all samples in September (Table 2). Approximately 65–80% of Wolves Islands, Lime Kiln, and Deadmans Harbour samples consisted of *Ditylum brightwellii*, *Leptocylindrus danicus*, *Pseudo-nitzschia seriata* group, and *Thalassionema nitzschioides*. *Mesodinium rubrum*, and the dinoflagellates *Ceratium lineatum* and *Scrippsiella trochoidea* were also predominant at Passamaquoddy Bay. Brandy Cove showed greater diversity, with *Leptocylindrus danicus* being the only taxon forming >10% of that sample (Table 2).

DGGE Profiles of Microeukaryotic Community. A total of 38 bands were identified in environmental samples using DGGE (Fig. 4), yielding a smaller total than the number of taxa identified morphologically. With two exceptions, the number of DGGE bands, or phylotypes, per sample, was greater than the number of taxa that exceeded 10% in samples, but was many times less than the number of taxa exceeding 1% of samples. Changing

Table 1. Organisms identified morphologically (5–100 µm in size), and associated taxonomic groups, in the Bay of Fundy in May, July, and September 2000[a]

Organism identified	*Taxonomic group*	*n*	*Months for n*	*n > 1%*	*Months n > 1%*	*n > 10%*	*Months n > 10%*
Alexandrium fundyense	Dinophyceae	7	M, J	1	M	0	
Alexandrium ostenfeldii	Dinophyceae	3	M	3	M	0	
Amphidinium sp.	Dinophyceae	2	M	1	M	0	
Amylax triacantha	Dinophyceae	2	J	0		0	
Armored dinoflagellates	Dinophyceae	11	M, J, S	4	M, J	0	
Armored dinoflagellate <20 um	Dinophyceae	4	J	2	J	1	J
Ceratium fusus	Dinophyceae	8	J, S	0		0	
Ceratium kofoidii	Dinophyceae	3	S	0		0	
Ceratium lineatum	Dinophyceae	8	J, S	2	S	1	S
Ceratium longipes	Dinophyceae	9	M, J, S	5	M	0	
Ceratium minutum	Dinophyceae	3	J, S	1	S	0	
Ceratium sp.	Dinophyceae	3	M, J, S	0		0	
Ceratium tripos	Dinophyceae	11	J, S	3	J, S	0	
Dinophysis acuminata	Dinophyceae	6	M, J, S	0		0	
Dinophysis acuta	Dinophyceae	2	J, S	0		0	
Dinophysis norvegica	Dinophyceae	8	M, J, S	1	M	0	
Dinophysis sp.	Dinophyceae	5	M, J, S	2	M	0	
Gonyaulax digitale	Dinophyceae	2	J	1	J	0	
Gonyaulax spinifera	Dinophyceae	4	J	1	J	0	
Gymnodinium sp.	Dinophyceae	1	M	1	M	0	
Gyrodinium sp.	Dinophyceae	14	M, J, S	5	M, S	0	
Heterocapsa triquetra	Dinophyceae	10	M, J, S	7	M, J	4	J
Minuscula bipes	Dinophyceae	2	J	0		0	
Prorocentrum micans	Dinophyceae	6	J, S	0		0	
Prorocentrum minimum	Dinophyceae	3	J, S	0		0	
Protoperidinium brevipes	Dinophyceae	1	J	0		0	
Protoperidinium sp.	Dinophyceae	8	M, J, S	1	J	0	
Scrippsiella sp.	Dinophyceae	1	S	0		0	
Scrippsiella trochoidea	Dinophyceae	7	M, J, S	3	M, J, S	1	S
Unarmored dinoflagellate	Dinophyceae	8	M, J	3	M, J	0	
Achnanthes sp.	Bacillariophyta	1	M	1	M	0	
Actinoptychus senarius	Bacillariophyta	4	M, S	2	M, S	0	
Asterionellopsis glacialis	Fragilariophyceae	3	J, S	0		0	
Cerataulina pelagica	Bacillariophyta	9	J, S	6		4	J
Centrale	Bacillariophyta	1	M	1	M	0	
Chaetoceros compressus	Coscinodiscophyceae	3	J, S	0		0	
Chaetoceros convolutes	Coscinodiscophyceae	1	M	1	M	0	
Chaetoceros debilis	Coscinodiscophyceae	12	M, J, S	3	M	0	
Chaetoceros decipiens	Coscinodiscophyceae	3	M, J	2	M	0	
Chaetoceros lorenzianus	Coscinodiscophyceae	6	J, S	1	S	0	
Chaetoceros simplex	Coscinodiscophyceae	8	J, S	2	J, S	0	
Chaetoceros socialis	Coscinodiscophyceae	8	M, J, S	5	J, S	2	J
Chaetoceros sp.	Coscinodiscophyceae	14	J, S	5	M	1	M
Corethron criophilum	Coscinodiscophyceae	9	J, S	1	S	0	
Coscinodiscus sp.	Coscinodiscophyceae	9	M, S	3	M	0	
Cylindrotheca closterium	Bacillariophyceae	16	M, J, S	9	M, J, S	0	
Dactyliosolen fragilissimus	Bacillariophyta	7	J, S	2	S	0	
Detonula confervacea	Bacillariophyta	1	S	0		0	
Ditylum brightwellii	Coscinodiscophyceae	8	M, J, S	5	M, S	1	S
Eucampia zodiacus	Coscinodiscophyceae	4	J, S	2	S	0	
Guinardia delicatula	Bacillariophyta	9	J, S	1	S	0	
Guinardia flaccida	Bacillariophyta	7	J, S	1	S	0	
Guinardia striata	Bacillariophyta	5	S	3	S	0	
Gyrosigma fasciola	Bacillariophyta	6	M, J	2	M, J	0	
Gyrosigma tennuissimum	Bacillariophyta	1	S	0		0	
Helicotheca tamesis	Bacillariophyta	1	S	0		0	
Lauderia annulata	Coscinodiscophyceae	3	S	1	S	0	
Leptocylindrus danicus	Bacillariophyta	5	S	5	S	4	S
Leptocylindrus minimus	Bacillariophyta	9	M, J, S	2	J, S	0	
Licmophora abbreviata	Bacillariophyta	1	M	0		0	
Melosira sp.	Coscinodiscophyceae	1	M	0		0	
Navicula sp.	Bacillariophyceae	6	M, J, S	3	M	0	

(continued)

Table 1. Continued

Organism identified	*Taxonomic group*	*n*	*Months for n*	*n* > 1%	*Months n* > 1%	*n* > 10%	*Months n* > 10%
Odontella regia	Coscinodiscophyceae	1	S	0		0	
Odontella sinensis	Coscinodiscophyceae	3	S	0		0	
Pennate	Bacillariophyta	4	M, S	1	M	1	M
Pleurosigma/Gyrosigma	Bacillariophyta	4	J, S	0		0	
Pleurosigma angulatum	Bacillariophyta	4	M, J	0		0	
Pleurosigma angulatum var. *strigosa*	Bacillariophyta	2	J, S	0		0	
Pseudo-nitzschia sp.	Fragilariophyceae	2	S	0		0	
Pseudo-nitzschia delicatissima group	Fragilariophyceae	10	J, S	4	S	0	
Pseudo-nitzschia seriata group	Fragilariophyceae	6	M, S	4	S	3	S
Rhizoselenia hebetata	Coscinodiscophyceae	1	M	1	M	0	
Rhizoselenia setigera	Coscinodiscophyceae	6	M, S	3	M, S	0	
Skeletonema costatum	Coscinodiscophyceae	13	M, J, S	10	M, J, S	4	J
Thalassionema nitzschioides	Fragilariophyceae	9	M, J, S	5	S	2	S
Thalassiosira august-lineata	Coscinodiscophyceae	1	J	0		0	
Thalassiosira gravida	Coscinodiscophyceae	3	M, J, S	1	M	0	
Thalassiosira nordenskioeldii	Coscinodiscophyceae	2	M, J	1	M	0	
Thalassiosira sp.	Coscinodiscophyceae	13	M, J, S	8	M, J, S	1	M
Brachionus sp.	Rotifera	1	J	0		0	
Dictyocha fibula	Dictyochophyceae	1	S	0		0	
Dictyocha speculum	Dictyochophyceae	10	J, S	4	J, S	0	
Dinobryon sp.	Chrysophyceae	2	M	1	M	0	
Ebria tripartita	Incertae sedis	5	J, S	1	J	0	
Helicostomella sp.	Ciliaphora	3	J	1	J	0	
Mesodinium rubrum	Ciliaphora	15	M, J, S	13	M, J, S	8	M, J, S
Ptychocylis sp.	Ciliaphora	2	M	2	M	1	M

[a]The total number of samples containing the organisms (n), number of samples where the relative abundances of the organisms were greater than 1% of the sample (n > 1%) and greater than 10% of the sample (n > 10%), and the respective months representing n (months for n), relative abundances greater than 1% (months n > 1%) and 10% (months n > 10%) are presented.

temporal patterns of DGGE bands illustrated that dominant organisms shifted through time, a similar result as found in morphological identifications. Approximately three-fourths of the DGGE bands were unique to one sampling month (Fig. 4). However, while DGGE patterns exhibited great temporal variability, sample-to-sample variability at each sampling date appeared to be higher with this method than with morphological identifications, as exemplified by the fact that only two DGGE bands were observed in all 3 months and no single band occurred in every sample (Fig. 4). Additionally, 15 bands, or approximately 39% of the DGGE bands, were detected only once.

With two exceptions in July (Passamaquoddy Bay and Bocabec Bay), DGGE phylotype richness generally followed the pattern of July > May > September (Fig. 4). July generally had the highest number of DGGE bands, despite the fact that the numbers of morphologically identified taxa comprising >1% of a sample tended to be lowest in July, and Shannon–Weaver diversity index values were many times lower than 2 (Fig. 3). Using DGGE, the number of DNA bands was higher in May than September in all samples, and, except for Bocabec Bay and Passamaquoddy Bay, September samples resulted in the lowest number of DGGE phylotypes. This was in contrast to morphological identifications, where July and September had high taxon richness values (Fig. 3).

Cloning and Sequencing Results. The cloning reactions produced 34 clones from May, 15 from July, and 42 from September that were then sequenced. Of those 91 clones, 64 yielded unique sequences that did not appear to be chimeric, although chimera check results were somewhat inconclusive among environmental sequences that were not closely associated with known sequences. Environmental sequences clustered into three broad phylogenetic groups, with 20 sequences grouping with Chlorophyta, Cryptophyta, and Stramenopiles (diatoms and other related organisms, Fig. 5), 27 with dinoflagellates, other alveolates, and amoeboid organisms (Fig. 6), and 17 distantly related to known metazoan organisms (Fig. 7).

Although diatoms were the most frequently morphologically identified organisms (Table 1), only three clones were related to diatoms in phylogenetic analyses (Fig. 5). Two of the three, M26 and S322, represented *Thalassiosira* sp. and *Ditylum brightwelli*, respectively, genera present in the morphological analyses of Bay of Fundy samples. Both of these organisms were dominant in morphological identifications in the sampling months from which the clones originated. Rather surprisingly, *Skeletonema costatum*, which had relative abundances up to 71% of samples in July (Table 2), was not detected among clone sequences. However, M26, although most closely related to *Thalassiosira eccentrica* (99.6%), was

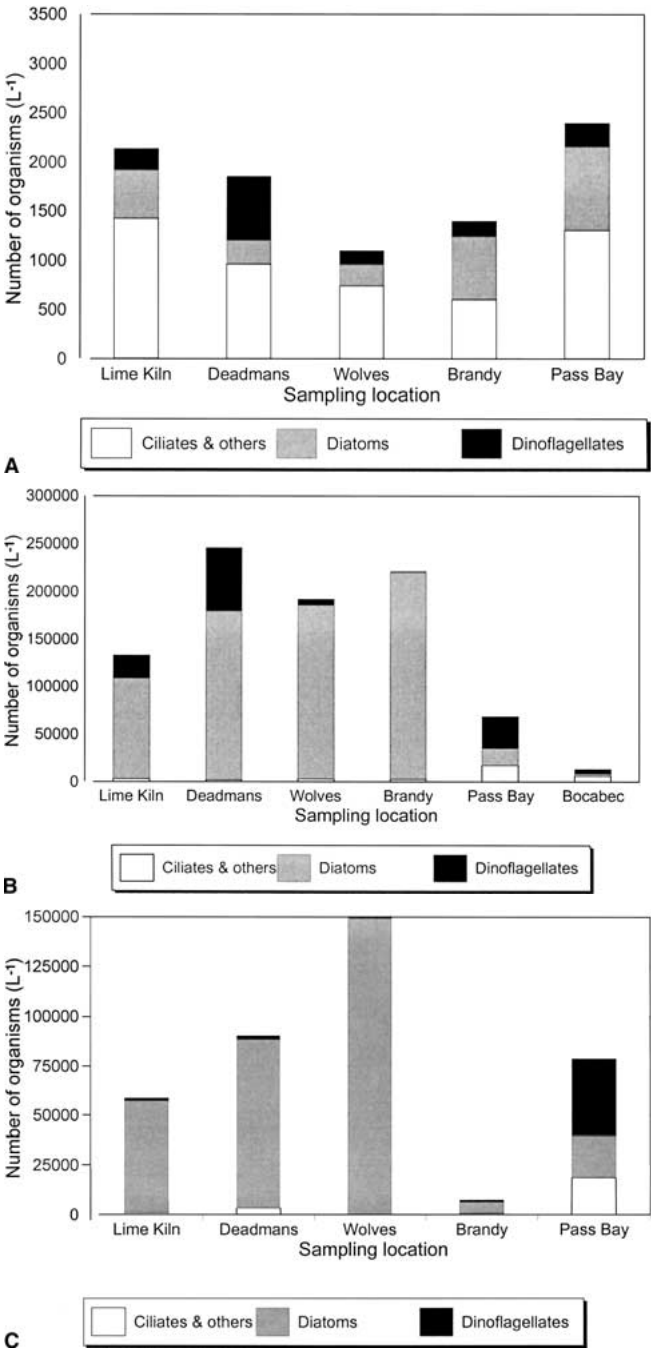

Figure 2. Total abundances in May (A), July (B), and September (C) of morphologically identified organisms (5–100 μm) and distributions of those organisms among ciliates (and other grazers), diatoms, and dinoflagellates.

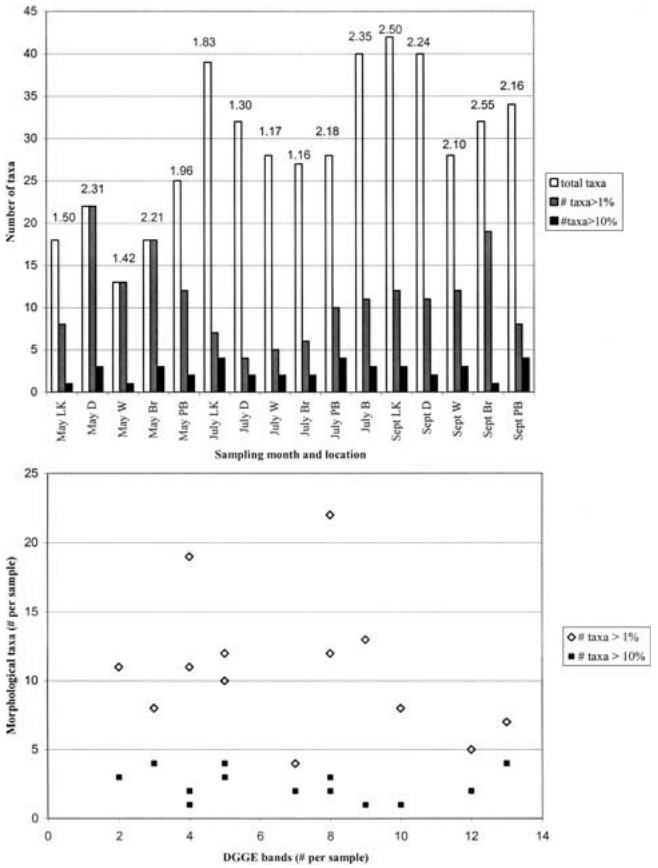

Figure 3. Number of taxa identified morphologically at the species level or higher, the number of taxa comprising >1% of the total sample, and the number of taxa comprising >10% of the total sample at different sampling stations in the Bay of Fundy in May, July, and September, 2000. See the legend of Fig. 1 for sample abbreviations. Numbers above each bar are the Shannon–Weaver index values for each sample. Bottom graph shows the relationship between the number of DGGE bands for each sample and the number of morphologically identified taxa with relative abundances >1% and >10% in the Bay of Fundy in May, July, and September 2000.

also very closely related to *Skeletonema costatum* with a similarity value of 99.1%. The third clone associated with diatoms (S31) was related to *Papiliocellulus elegans* (99.1% similar), which was not among those morphologically identified in these samples.

M411 and MJ314 were most closely related to each other (99.8% similarity) and were affiliated with the Pheacophyceae (brown algae commonly found around sea coasts), which have not been found during diversity surveys of picoplankton using molecular techniques [7, 18]. Another clone from September (S74) clustered with the "golden algae" Chrysophyceae (99.3% similarity to *Hibberdia magna*). However, most clone sequences were most closely related to other unidentified organisms (Fig. 5). Clone sequences from M118 clustered with several unnamed Bolidophyceae (99.5% similarity to DH144EKD10 and OLI11026). S36 and J318 sequences clustered with an unidentified Chlorophyceae, 23NSpeci (98.7% and 99.5% similarities, respectively) and *Chlorella* (98.0% and 98.5% similarities, respectively), a tiny Chlorocaccales (1–2 μm) that is sometimes an endosymbiont in invertebrates. Three sequences (S24, M223, and M45) clustered with the nucleomorphs (S24) and

Table 2. Relative abundances of dominant taxa per sample location in the Bay of Fundy in May, July and September 2000, as identified morphologically

Sampling month	*Organism*	*Taxonomic group*	*Wolves Islands*	*Lime Kiln*	*Deadmans Harbour*	*Pass Bay*	*Bocabec Bay*	*Brandy Cove*
May	*Mesodinium rubrum*	Ciliaphora	67	66	37	52	ND	39
	Ptychocylis sp.	Ciliaphora	0	0	15	2.0		0
	Ceratium longipes	Dinophyceae	3.6	1.9	2.2	2.5		1.4
	Gyrodinium sp.	Dinophyceae	3.6	4.7	11	0		4.3
	Chaetoceros sp.	Coscinodiscophyceae	5.5	3.7	1.1	3.3		10
	Thalassiosira sp.	Coscinodiscophyceae	3.6	5.6	2.2	17		7.1
	Pennate	Bacillariophyta	0	1.0	0	0		11
July	*Mesodinium rubrum*	Ciliaphora	1.3	1.9	0	23	29	1.1
	Cerataulina pelagica	Bacillariophyceae	5.2	11	4.4	18	15	52
	Chaetoceros socialis	Coscinodiscophyceae	14	21	7.0	<1	0	0
	Skeletonema costatum	Coscinodiscophyceae	71	43	59	3.6	<1	37
	Armored dinoflagellates	Dinophyceae	<1	4.7	<1	22	1.5	<1
	Heterocapsa triquetra	Dinophyceae	<1	11	24	17	20	<1
September	*Mesodinium rubrum*	Ciliaphora	0	<1	3.5	20	ND	6.3
	Ditylum brightwelli	Coscinodiscophyceae	19	5.3	6.2	<1		1.9
	Leptocylindrus danicus	Bacillariophyta	33	26	36	5.8		35
	Pseudo-nitzschia seriata group	Fragilariophyceae	15	17	20	0		1.3
	Thalassionema nitzschioides	Fragilariophyceae	8.9	17	8.9	18		7.4
	Ceratium lineatum	Dinophyceae	0	0	0	20		2.7
	Scrippsiellti trochoidea	Dinophyceae	0	0	0	19		<1

ND: Not determined.

nuclear genes (M223 and M45) of Cryptophyta (95.2–100% similarities to database sequences). Cryptophyta are cells with both a eukaryotic nucleus and a nucleomorph, which may be the vestigial nucleus of a photosynthetic endosymbiont.

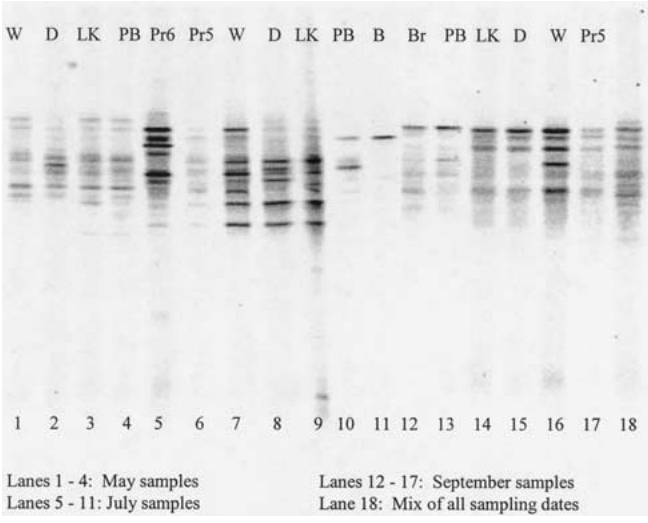

Figure 4. Denaturant gradient gel electrophoresis profiles of eukaryotic PCR products from plankton DNA in the Bay of Fundy in May, July, and September 2000. May samples are in lanes 1–4, July samples are in lanes 5–11, September samples are in lanes 12–17, and a mix of all sample dates is in lane 18. Abbreviations on the gel are as follows: Brandy Cove (Br); Bocabec Bay (B); Passamaquoddy Bay (PB); Lime Kiln (LK); Deadmans Harbour (D); Wolves Islands (W); Prince 5 (Pr 5); and Prince 6 (Pr 6).

Many of our sequences were not closely related to any known organisms and clustered with environmental sequences that appear to be related to heterotrophic rather than phototrophic organisms (Fig. 5). Four clones from September (S125, S114, S116, and S525) and one from May (M217) clustered together. These Bay of Fundy clone sequences from different sampling months were >99% similar to each other, but were <94% similar to the other unknown environmental sequences (DH147EKD10 and OLI11066 and OLI11150, Fig. 5) and known Labrynthulida, early diverging heterotrophs. M18 and MJ120 sequences grouped with known and unidentified oomycetes and environmental sequences that may represent new lineages [15, 18]. One September clone (S366) was not clearly identified with any organism, but was affiliated with DH1485EKD53 (94.9% similarity) which has been suggested to represent a new lineage [15].

From the analyses containing Alveolata and amoeboid type organisms, 13 clones were associated with known or unidentified dinoflagellates (Fig. 6). Seven clone sequences, representing all three sampling months, were related to known dinoflagellates (Fig. 6). One clone (S323) was related to *Gymnodinium simplex* and *G. beii* (99.4%), and this genus was identified in our Bay of Fundy morphological samples. Another clone sequence (M29) was related to *Ceratium furca* (99.0%). *Ceratium* spp. were predominant organisms in September (Table 2). MJ210 and JS45 sequences were most closely related to each other (99.2% similarity), but grouped with known dinoflagellates with representatives

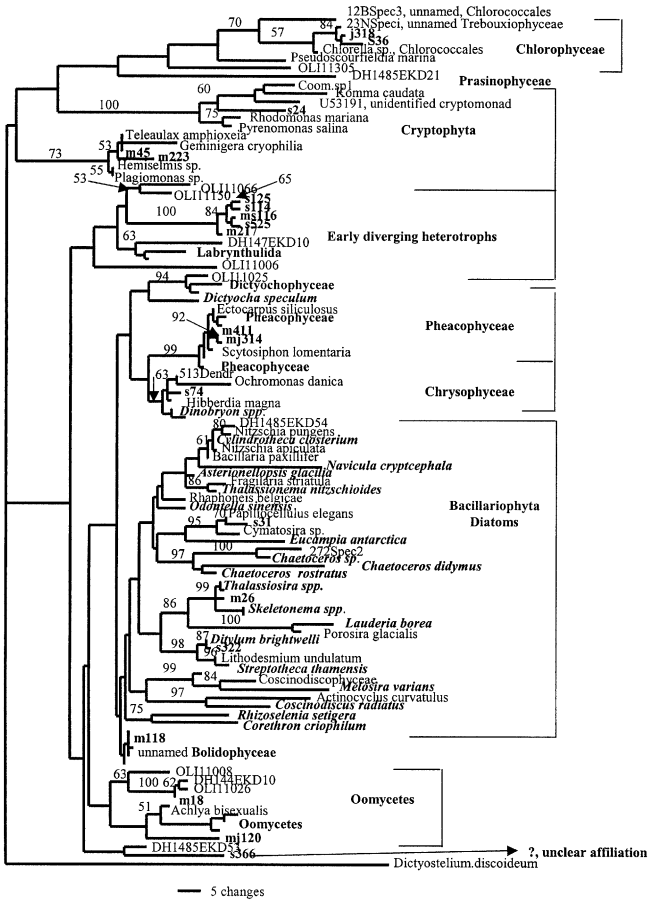

Figure 5. Phylogenetic tree of partial 18S rRNA genes from cloned environmental sequences related to members of Chlorophyta, Cryptophyta, and Stramenopiles (bar = 0.05 substitutions per site). Sequences in bold are environmental clones obtained in this study, sequences in bold italics are genus (or higher level) of organisms identified morphologically in the Bay of Fundy, and sequences in gray are related organisms, but are not identified by morphology in the Bay of Fundy samples. Tree topology was obtained by a heuristic search under a tree-bisection-reconnection branch-swapping algorithm using 556 aligned positions. Tree was rooted using *Dictyostelium discoideum*. Bootstrap values >50% from an analysis of 100 bootstrap replicates are given at respective nodes.

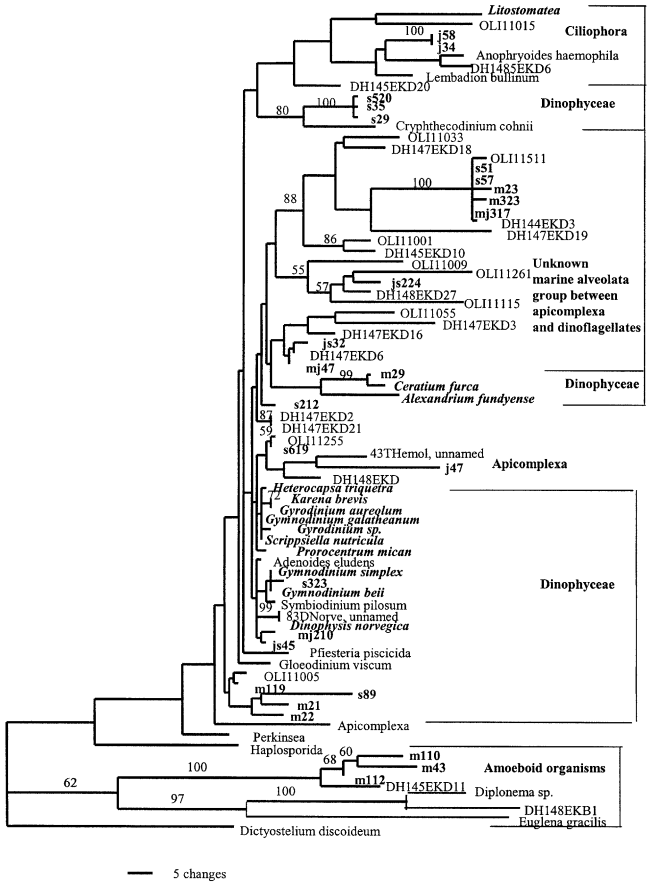

Figure 6. Phylogenetic tree of partial 18S rRNA genes from cloned environmental sequences related to members of Alveolata and amoeboid organisms (bar = 0.05 substitutions per site). Sequences in bold are environmental clones obtained in this study, sequences in bold italics are genus (or higher level) of organisms identified morphologically in the Bay of Fundy, and sequences in gray are related organisms, but are not identified by morphology in the Bay of Fundy samples. Tree topology was obtained by a heuristic search under a tree-bisection-reconnection branch-swapping algorithm using 494 aligned positions. Tree was rooted using *Dictyostelium discoideum*. Bootstrap values >50% from an analysis of 100 bootstrap replicates are given at respective nodes.

in the Bay of Fundy. Three September clones (S520, S35, S29) grouped together (99.6% similarities) and clustered with *Cryphthecodinium cohnii* (94% similarities to *C. cohnii*), which is a dinoflagellate not identified using morphology in the Bay of Fundy. However, these three clone sequences were ~5% different from many known and unknown dinoflagellates. Therefore, it is possible that these Bay of Fundy sequences represent a novel lineage or dinoflagellates for which sequence data are not yet available. It was not clear if S212 was a dinoflagellate or affiliated with the group of organisms diverging between dinoflagellates and Apicomplexa (Fig. 6). S212 showed 99.4% similarity with *Gyrodinium* spp., *Gymnodinium* spp., and *Scrippsiella* spp., suggesting that it could be a dinoflagellate. However, placement in the phylogenetic tree as a dinoflagellate was not supported by bootstrap analysis. Four clones (M119, M22, M21, S89) clustered together and were closest to the environmental clone OLI11005 (94–99.2% similarities to OLI11005), which was affiliated with dinoflagellates [18]. The sequence from clone S619 was closest to that of OLI11255 (99.8% similarity) and was also probably an unknown dinoflagellate [18].

Many of the clone sequences in the Alveolata tree also grouped with organisms that were heterotrophic and possibly parasitic (Fig. 6). One sequence from July (J47) was affiliated with, but not closely related (91.7% simi-

larity) to the Apicomplexa (Fig. 6), which are known parasites. Many environmental clone sequences collected at depth from the Antarctic polar front (e.g., DH145EKD11) [15] and the equatorial Pacific (e.g., OLI11005) [18] clustered together between the Apicomplexa and dinoflagellates. Eight of the Bay of Fundy clone sequences, accounting for 12.5% of our clone sequences, were associated with these unidentified environmental clones, suggested as representing "predinoflagellates" [15, 18]. These included five sequences (M23, M323, MJ317, S51, and S57) that clustered with each other and OLI11511 and DH144EKD3, Bay of Fundy clone sequences JS32 and MJ47 that clustered with DH147EKD6 (99.4 and 99.8% similarities, respectively), and the Bay of Fundy clone sequence JS224 that clustered with D148EKD27 (97.1% similarity) and OLI11261 (93.2% similarity; Fig. 6).

Of the remaining clones, two sequences from July (J58 and J34) were affiliated with the ciliales (Fig. 6). *Mesodinium rubrum* is a ciliate that was a dominant member of samples in all three sampling months. The sequence data for *Mesodinium rubrum* are not yet available. However, J58 and J34 probably represent ciliates other than *Mesodinium* because these clones did not group with other known Litostomatea (Fig. 6), the class containing *Mesodinium* or with Spirotrichea, the class containing *Ptychocylis* sp. (data not shown).

Additional sequences that were not clearly related to known organisms and potentially represented organisms from deeply branching lineages included three clones (M110, M43, M112) that clustered together and were most closely related to DH145EKD11 (92.8–96.3% similarities), which was of uncertain lineage, but grouped with amoeboid organisms (Fig. 6).

Many environmental samples in the third metazoan group were more closely related to another organism in our environmental samples than to sequences in the 18S rDNA databases (Fig. 7). However, from comparisons made to sequences in the databases, the Bay of Fundy sequences were most closely related to choanoflagellates (99.0% similarity between M311 and *Diaphanoeca grandis*), fungi/metazoa (95.4–96.2% similarity between *Amoebidium parasiticum* and five clones), echinodermata (100% similarity between M220 and the sea urchin *Strongylscentrotus intermedius*), and copepods (89.6–95.9% similarity between copepods and 8 clones; Fig. 7). Phylogenetic analysis also related a Bay of Fundy clone sequence to unidentified environmental clones (99.4% similarity between MJ46 and UneEuka7, strain LKM46; Fig. 7). The closest phylogenetic relatives of strain LKM 46 were fungi [23]. One clone (S220) that was phylogenetically placed as a long branch from the fungi-metazoa was at least 13.6% different from any other sequences, and its placement was not supported by bootstrap values (Fig. 7).

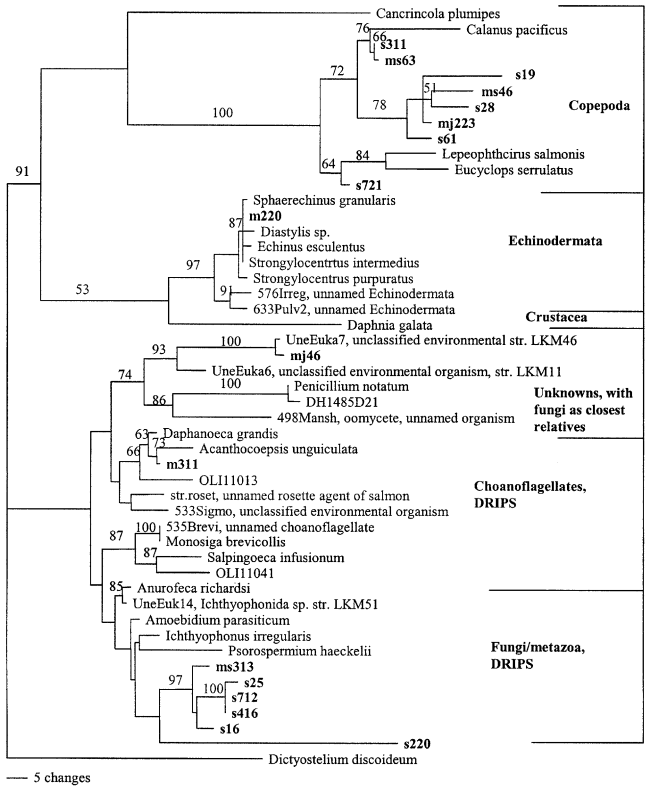

Figure 7. Phylogenetic tree of partial 18S rRNA genes from cloned environmental sequences related to members of Metazoan organisms (bar = 0.05 substitutions per site). Sequences in bold are environmental clones obtained in this study and sequences in black are related organisms, but are not identified by morphology in the Bay of Fundy samples. Tree topology was obtained by a heuristic search under a tree-bisection-reconnection branch-swapping algorithm using 522 aligned positions. Tree was rooted using *Dictyostelium discoideum*. Bootstrap values >50% from an analysis of 100 bootstrap replicates are given at respective nodes.

Discussion

Lack of Congruity between Morphological and Molecular Analyses. Very few of the organisms identified by morphology were also identified in phylogenetic analyses. Only three clones, representing ~5% of the clone sequences obtained, were associated with morphologically predominant organisms. Those three organisms included *Ceratium furca* (99% similarity) and *Ditylum brightwelli* (99.8% similarity) and one clone that was closely associated with *Thalassiosira eccentrica* (99.6% similarity) and *Skeletonema costatum* or *S. pseudo-costatum* (99.1% similarity). Only three more clone sequences were affiliated with minor members of our Bay of Fundy morphological samples, and all of those clones clustered with dinoflagellates. In addition, 13 clones were placed within the *Dinophyceae*, but most were not clearly identified as known taxa detected by morphology. There are numerous possibilities for this lack of congruity among method

types, including limitations of both morphological and molecular methods and the extent of undescribed eukaryotic diversity in marine environments.

Identification by morphology is limited to taxa that have been previously described using cultivation-based methods and detailed ultrastructural analysis. Further confirmation is done by light, phase contrast, or scanning electron microscopes. As many microorganisms resist cultivation and do not have distinct morphological features, there is a high potential for encountering previously undescribed species in a given sample. Among eukaryotic phytoplankton, unarmored dinoflagellates in particular are poorly defined by morphological methods, as they lack well-defined thecal plates and are difficult to distinguish. As such, their taxonomy has remained little changed since the early 1900s. In fact, several genera (e.g., *Gymnodinium*, *Gyrodinium*, *Amphidinium*) have been shown to be assemblages of unrelated organisms [5]. In our study, these groups of visible yet unknown taxa detected by morphology were grouped into categories such as ''unarmored dinoflagellates,'' ''armored dinoflagellates,'' ''pennate diatoms,'' and ''centrale diatoms.'' Among these, the armored and unarmored dinoflagellates comprised predominant community members on several occasions (Table 1). It is likely that these unknown morphotypes account for some of the clone sequences that were placed in the *Dinophyceae* family, but could not be clearly identified at the genus level.

Especially among dinoflagellates, phylogenetic analyses of 18S rRNA genes indicate that morphological traits traditionally used to distinguish taxa are often incongruent with molecular phylogenies [5, 21]. As a result, it is possible that some of the species in our Bay of Fundy samples had morphologies that matched those of known taxa, but actually differed from those species genetically. Many clone sequences and known dinoflagellate sequences were very closely related (Fig. 6). Organisms represented by these sequences are likely to have similar morphologies to their close relatives, resulting in morphological placement in a given species and underestimation of their true diversity. While clone sequences were not most closely related to dominant dinoflagellates such as *Scrippsiella trichoidea*, *Gyrodinium* sp., and *Heterocapsa triquetra*, many clone sequences and known dinoflagellate sequences were very closely related and the branching order of phylogenetic relationships was difficult to discern. For example, JS45, which was not clearly related to one particular dinoflagellate, was 99.8% similar to a *Scrippsiella* sp. and 99.6% similar to *Heterocapsa triquetra*. Branching order could not be firmly established with bootstrap analysis (Fig. 6). Therefore, it is possible that our clones did represent dinoflagellates that were identified morphologically, but phylogenetic placement of some clone sequences with some dinoflagellates rather than others could not be determined with certainty.

Limitations of molecular approaches may have also contributed to the lack of congruity between morphological and molecular analyses. These include potential biases during DNA extraction, PCR amplification, and cloning steps, as well as limitations of the current 18S rRNA sequence databases. For example, dinoflagellates and related organisms appeared to be relatively easily amplified in our study. The relatively large genome and potentially high *rrn* copy number of known dinoflagellates [7, 11, 20] may partially explain apparent preferential amplification of dinoflagellate-related organisms in complex environmental samples. In contrast, very few diatoms were identified in our molecular study. This result is surprising in light of the high plankton abundances and dominance of diatoms in the July and September samples. Preferential PCR amplification of templates may possibly explain the dilution of diatom sequences among the total pool of amplified DNA. Using primers targeting plastid genes, rather than the primers specific for eukaryotes, may improve the detection of phototrophic plankton (e.g. [19]). Rappé et al. [19] found a substantial number of plastid clone sequences in coastal U.S. waters related to diatoms, although not with known sequences of diatoms. In another study using two different primer sets, diatoms were not found in sequenced bands of picoplankton 18S rDNA generated by DGGE [7]. Although DNA from known diatom 18S rRNA sequences is targeted by the eukaryote-specific primers used in this study [22, 23], it is possible that diatoms were not amplified as efficiently as other organisms. Checking primer sequences against known sequences in the RDP II database indicated that a mismatch to primer E4, one of the primers used in the cloning protocol, is required before many organisms, including any known diatoms, will be amplified. In both our study and that of Díez et al. [7], it is possible that diatom DNA bands were present in the DGGE gels, but that the DNA had migrated to positions where bands were not sequenced. Alternatively, diatoms may have been excluded during filtration in the study by Díez et al. [7].

Another potential explanation for the apparent amplification of alveolata-type organisms over diatoms may include a more extensive existence of intracellular symbionts or parasites than previously recognized. Many dinoflagellates are already recognized to be parasites and, although the phylogenetic placement of many of the unknown alveolata-type organisms was unclear, these unknown organisms grouped with dinoflagellates and Apicomplexa, also a known parasitic group. Several molecular studies of marine microeukaryotic communities have detected groups of unknown alveolates that cluster between the Apicomplexa and the dinoflagellates. These organisms appear to be ubiquitous in marine environments and yet have only been detected using molecular methods. 18S rDNA sequences of these organisms

have been found in marine samples from the Bay of Fundy (this study), the Antarctic polar front [15], the equatorial Pacific [18], and anoxic sediment cores of hydrothermal vent environments of Guaymas Basin in the Gulf of California [10], as well as from surface Mediterranean and North Atlantic waters [7]. The affiliation of our sequences with these further supports the suggestion of ubiquity of this lineage in marine waters [15, 18].

Some of the predominant organisms as identified morphologically could not be identified using phylogenetic analyses because the 18S rDNA sequences have not been added to the sequence databases (e.g., *Mesodinium rubrum*, *Cerataulina pelagica*, *Guinardia* spp., and *Leptocylindrus danicus*). With only a few thousand 18S rDNA sequences in the databases, there are bound to be many novel organisms that cannot currently be identified. Clearly, to develop a comprehensive understanding of the true level of marine eukaryotic diversity, there need to be many more molecular studies aimed at identifying sequences of both known and novel organisms.

Novel or Unknown Eukaryotic Lineages. Many of the environmental clone sequences generated in this study were affiliated with unknown environmental sequences or novel lineages rather than sequences of known organisms. Groups were affiliated with ciliates (J58, J34), Dinophyceae (S520, S35, S29, S89, M21, M22, S212, S619), unknown Alveolata (M23-S51 cluster, JS224, JS32, MJ47), Apicomplexa (J47, although this was not supported by bootstrapping analysis), amoeboid organisms (M110, M43, M112), fungi/metazoa (MS313-S16-S25 group), and early diverging heterotrophs (S366, M217-S125 group, M18, MJ120) or were altogether unknown (S220). These data reflect the extent of unknown eukaryotic diversity in oceans, even among groups that are considered to be large enough to be identified morphologically (e.g., dinoflagellates and ciliates).

Environmental clone sequences from September (S125, S114, MS116, and S525) and from May (M217) clustered together and branched out in the phylogenetic tree closest to the other unknown environmental sequences and Labrynthulida. Labrynthulida are early-diverging heterotrophs at the base of the Stramenopiles tree known to commonly colonize fecal pellets in deep sea. Díez et al. [7] had discovered sequences related to early-diverging heterotrophs which they labeled novel Stramenopiles from North Atlantic, Mediterranean, and Antarctic waters. Although our gene fragments did not contain enough overlapping fragments to include the sequences from Díez et al. [7] in our analyses, both sets of DNA appear to have similar placements on the phylogenetic tree. In addition, M18 was closely related to environmental sequences OLI11026 and DH144EKD10 that clustered with, but were not closely related to, known oomycetes and may represent a new lineage [17]. MJ120 clustered with, but was only 96.1% similar to, the oomycete *Achlya bisexualis*. One September clone (S366) was affiliated with DH1485EKD53 (94.9% similarity), an environmental clone that was also not affiliated with any known species and was suggested to represent a new lineage [15]. Further work on the environmental clone sequences from the Mediterranean, Antarctic and North Atlantic Oceans and enrichment cultures has shown that the novel sequences clustering at the base of the Stramenopiles tree do form at least eight independent novel lineages [17]. In addition, these lineages contain heterotrophic and quantitatively important picoplankton [17]. Althogh some Bay of Fundy sequences appeared to be associated with those new lineages found in other oceans, the fact that we detected a cluster of close, but distinct relatives that are distant from even these other environmental sequences suggests that some of our clone sequences may represent additional new lineages. At the very least, the findings of these sequences again underscore the extent of unknown diversity in marine ecosystems.

Comparisons with Other Molecular Phylogenetic Studies of Marine Microeukaryotic Communities. Different studies have targeted different oceanic environments and different genes, and even within studies targeting 18S rRNA genes, researchers have used different PCR protocols and different primers. Some studies have targeted picoplankton in particular and excluded plankton larger than 3 to 5 μm [7, 18]. Other studies have focused on particular depths ranging from surface waters [19] down through the depths to anoxic sediments [6]. Oceans range from the equatorial Pacific [18] to the Atlantic [19] to the Mediterranean Sea [7] to the Antarctic [7, 15]. Taken together, large differences in results could be expected due to all these differences in samples. Yet, despite great geographical and methodological differences, there are many similarities among molecular studies conducted on marine eukaryotic plankton diversity. Similarities may result in part from the fact that the molecular studies are all PCR-based and, although primers used in the various studies are different, they all target the eukaryotic communities specifically by using conserved DNA sequences. However, beyond these studies, few molecular-based surveys of the eukaryotic communities have been reported and the extent of total eukaryotic plankton diversity remains elusive.

A common theme in all of the molecular-based eukaryotic surveys, including this study, is how few DNA sequences are related to known organisms [6, 10, 19]. A study of plastid genes from the Pacific coastal waters of Oregon and the continental shelf near North Carolina revealed groups of bacillariophytes, prymnesiophytes, and prasinophytes, of which many were not closely re-

lated to known sequences [19]. In Moon-van der Staay et al. [18] only two of 35 of their 18S rDNA environmental clone sequences were 99% related to sequences of known identity in the EMBL gene bank. The sequences in Moon-van der Staay et al. [18] exhibited a range in identities to known sequences (82–97%) similar to that shown by our gene clones in this study (86–100%). The various molecular studies have only recently been conducted, but are illuminating the great phylogenetic diversity among small eukaryotic marine organisms, especially among organisms that may be heterotrophic.

In addition to the broad range of diversity uncovered in the Alveolata and Stramenopiles trees, we detected 18S rDNA sequences related to several copepods, an echinoderm, and a choanoflagellate. Two clone sequences from 35 picoplankton samples of the equatorial Pacific at a depth of 75 m were found to be most closely related to choanoflagellates, the closest known relatives to sponges [18]. Additional sequences related to metazoans were found in filtered samples expected to exclude larger organisms from the North Atlantic and the Mediterranean and incubated lake water samples [7, 15, 23], demonstrating that the findings of these types of organisms during molecular analysis is not uncommon. Although the DNA present in the sample is assumed to be from live organisms, the filtration process does not exclude small detrital particles, which may contain fecal or decomposing material, and the DNA of which may then be amplified during PCR. Alternatively, although we supposedly captured only DNA from organisms in the size range of 5 to 100 μm, the use of the peristaltic pump to filter samples may have caused some organisms to break open, allowing cellular debris to pass through the 100-μm filter. These factors may explain the clustering of some of our clone sequences with animals, such as copepods and echinoderms. Conversely, the filtration process may cause some organisms, such as *Mesodinium rubrum*, to burst and therefore not be retained on the 5-μm filter [24]. Eleven of the 17 clones grouping with metazoa ran farther into the gel than most environmental bands (data not shown). The lack of environmental bands in the bottom half of the gels where these 11 clones migrated suggests that these clones do not represent dominant community members in the environmental samples (Fig. 4).

Despite the number of similarities among different molecular studies of marine eukaryotic organisms, there have been some differences also. Our study did not uncover any 18S rDNA sequences related to the chlorophyll-containing Prymnesiophytes (coccolithophorids). Prymnesiophytes are generally considered to be abundant primary producers in oceans and have been found in plastid gene clones of samples from U.S. coastal waters of the Pacific and Atlantic oceans [19]. Using molecular techniques, Prymnesiophytes were found to be abundant in picoplankton from Antarctic waters, but were rare in Atlantic waters [7]. Because Prymnesiophytes are known to be abundant in those Atlantic waters tested, it was suggested that the organisms were removed during filtration [7]. As our study also did not reveal any sequences related to Prymnesiophytes, perhaps preferential amplification of other organisms occurs at the expense of Prymnesiophytes in some communities. Prymnesiophytes identified in the Gulf of Maine range in size from 4 to 11 μm (http://ccmp.bigelow.org/index.html). At this size range, these organisms potentially could have been captured on the filters used for DNA extraction, PCR amplification and DGGE. Prasinophytes are another group of picoplankton, not represented in this study, that were sequenced from plastid genes and 18S rDNA targeted in surface waters [7, 19].

Conclusions

We had expected that the molecular methods would identify both the numerically dominant organisms, as determined by morphologically distinguishable traits, and other previously unidentified organisms. Thus, we expected that molecular methods would illuminate a greater diversity of eukaryotic communities in the Bay of Fundy. In fact, the results of the different types of methods yielded much greater differences in population identifications than we had originally anticipated. Morphological methods resulted in relatively low abundances consisting mainly of a few grazers in May; and many diatoms and some dinoflagellates in July and September. In contrast, molecular methods detected few diatoms and known dinoflagellates and instead revealed many unknown or novel lineages, as well as many sequences that were closely related, but not identical, to known plankton. Morphological methods appeared to be better suited to the identification of larger, conspicuous diatoms, whereas molecular methods reveal many unidentified but possibly ubiquitous marine organisms and may have more accurately reflected the extent of eukaryotic phylogenetic diversity in these samples. Additionally, the contrasts and commonalities among molecularly determined communities demonstrate there are also still many unanswered questions surrounding the extent of eukaryotic diversity and the presence and function of heterotrophic groups.

These results underscore the great genetic diversity contained in environmental samples, even when morphological diversity limits our ability to identify many phylogenetic groups of organisms. Despite the uncertainty remaining in our knowledge and current definitions of eukaryotic diversity, an advantage of the molecular methods is that DNA sequences can be determined and added to the databases to continually improve our understanding of the structure and function of

many microscopic eukaryotic communities. However, these results also illustrate that different methods, even molecular methods, only capture a portion of the community, and that, while there are advantages in using molecular tools, these advantages do not make traditional methods of identifications obsolete.

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