Microbial Ecology

Picoeukaryotic Plankton Diversity at the Helgoland Time Series Site as Assessed by Three Molecular Methods

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Abstract

We analyzed picoeukaryote assemblages in the German Bight at the Helgoland time series site by sequencing cloned eukaryotic 18S rRNA genes in six genetic libraries plus one library from the Orkney Islands from a cruise of opportunity. The libraries were constructed from environmental samples collected at different periods of the year. The same samples were also analyzed using a fingerprinting technique, single-strand conformational polymorphism (SSCP), and DNA microarrays with class-level oligonucleotide probes. One hundred unique clones were analyzed from each library, thus insuring over 85% coverage of the library. The V4 region of the 18S rRNA gene was sequenced from each of these clones, thus providing the most discrimination among the clones. The nonphotosynthetic picoeukaryotic component dominated over the photosynthetic one and was represented by the ciliates at 45% and group II alveolates at 42%. Prasinophytes dominated the photosynthetic group at 40%, but other picoplankton groups, such as bolidomonads and chrysophytes, were also present. Totally novel groups were found in the cryptomonads and in the dinoflagellates. A new algal group sister to the cryptophyte nuclear gene and the glaucocystophytes was also found. These three groups have been found in other picoeukaryotic planktonic clone libraries. SSCP analyses at closer time intervals suggest that clone libraries should be made at weekly intervals if succession in the picoeukaryotic plankton community is to be monitored accurately. A comparison of annual samples suggests that there appears to be an annual cycle with regard to species composition. Microarray analysis supported the clone library data and offered a faster means of community analysis, which can be performed with similar accuracy and with higher throughput for a more in-depth analysis.

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Introduction

Picoplanktons (defined operationally as cells that pass through a 3-μm filter) dominate the photosynthetic biomass in many marine ecosystems, not only in the very oligotrophic regions of the world oceans, such as the Eastern Mediterranean Sea, but also in mesotrophic areas. However, picophytoplanktons are clearly not exclusively restricted to pelagic environments. In many coastal regions, they are present throughout the year and constitute a "background" population, onto which episodic phenomena, such as the spring bloom, develop. In some environments, such as coastal lagoons, picoplankton can be a major component of biomass and productivity for most of the year. In addition, some bloom-forming picoplankters, such as *Aureococcus*, are toxic. However, to date, fewer than 30 species of picoeukaryotic phytoplankton have been described. A clear proof of our poor knowledge of picoeukaryotic phytoplankton diversity is revealed by the discovery/recognition of four novel algal classes in the last 10 years described from picoeukaryotic phytoplanktonic taxa [1, 8, 10, 18]. A fifth one will soon be published discovered within EU PICODIV (Not, Valentin, Romari, Lovejoy, Massana, Vaulot, Medlin, unpublished data). Because so little is known about the taxonomy and systematics of picoeukaryotic phytoplankton, we have very little data to estimate the levels of its biodiversity under natural conditions and how picoeukaryotic phytoplanktons are affected by environmental variability linked to either anthropogenic influence or to larger-scale phenomena, such as those linked to climate change or global warming. As part of the EU PICODIV project,

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the biodiversity of the picoplanktonic community was assessed at three coastal sites: Roscoff in the English Channel [25], Blanes in the western Mediterranean Sea [16], and at Helgoland in the German Bight. We present here the results from Helgoland with the diversity of its picoeukaryotic planktonic community assessed by three molecular methods: environmental clone libraries, single-strand conformation polymorphisms (SSCPs), and class-level molecular probes detection with microarray technology (phylochips).

Molecular methods are needed for the understanding and describing the total diversity present in all marine aquatic environments, especially for the *in situ* picoeukaryotic phytoplankton diversity, and because members of this community are small and possess few morphological markers that can be used for identification using traditional methods. In nearly all instances where molecular methods have been applied to answer questions about diversity in aquatic habitats, novel taxa and hidden biodiversity have been uncovered [2, 3, 13, 14, 16, 19, 25]. Our study of the Helgoland picoeukaryotic plankton community is no exception.

Materials and Methods

Sampling Site. The Helgoland time series station is located at 54°11.3′N, 7°54.0′E in the central German Bight of the North Sea (Fig. 1). Cell counts and identification of diatoms, dinoflagellates, and other flagellates have been performed on a weekly basis since 1962 (see details in [29]). Samples are taken from the surface at a 5- to 8-m depth station. The water is not fractionated, and identification is primarily confined to cells in the nanoplankton and above size range. Salinity, temperature, and nutrients are also measured daily. We sampled at a fixed location over the entire sampling period so that the changes that we see here can be interpreted as temporal changes at a single site. All trends in our data are supported by similar observations in bacterial and microplankton fractions for the same period.

Environmental samples from which clone libraries were made were taken at Helgoland to coincide with set time points in the annual cycle of the phytoplankton at Helgoland: March and April 2000 (spring bloom), August and October 2000 (summer–fall bloom), December 2000 (shortest day length), and February 2001 (lowest temperature). In addition to these dates, monthly samples were taken between January 2001 and May 2002. One sample from the Orkney Islands was also analyzed from a cruise of opportunity.

Samples were taken with a bucket just below the surface and were filtered twice through 3-μm Millipore filters to insure that no larger cells could escape the filtration. The filters always showed some brownish color even after the second round of 3-μm filtration. In general, filters at the Helgoland site clogged faster than those at Blanes or Roscoff probably because of its higher load with sand and mud and because of its low depth

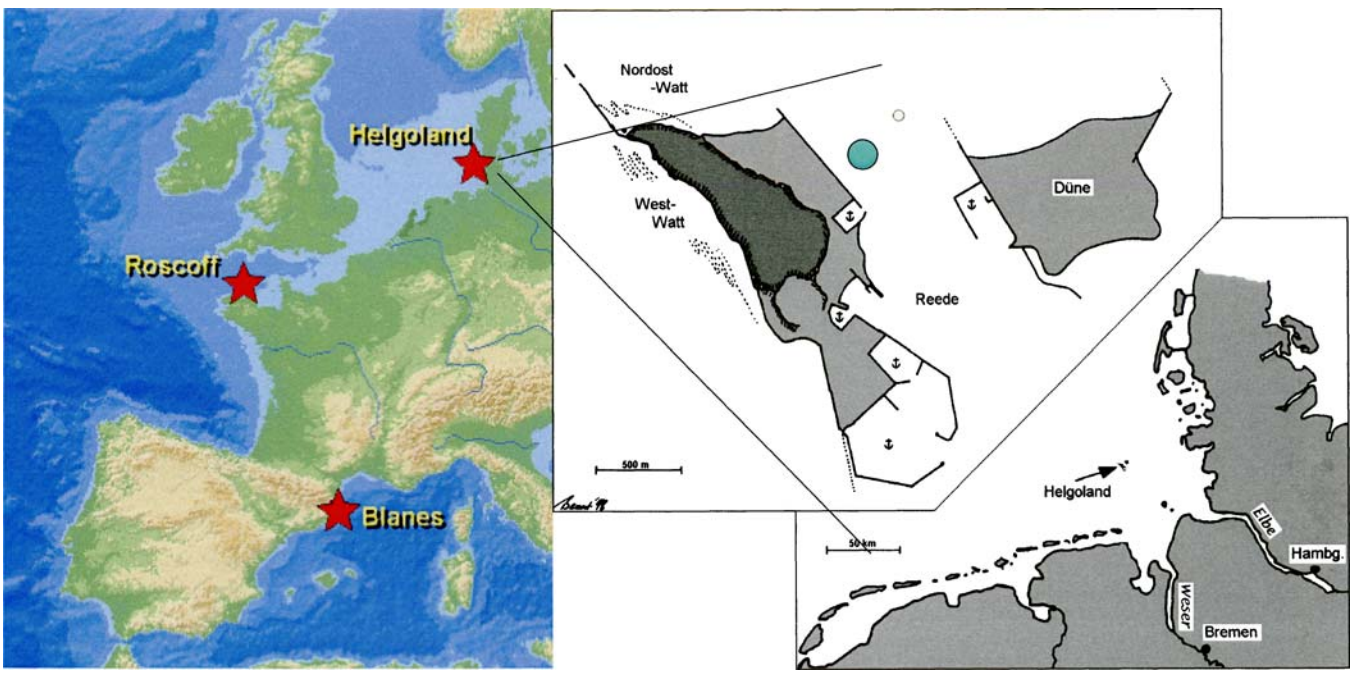

Figure 1. Location of the three coastal sites sampled in the EU PICODIV project with details of the Helgoland Times Series site in the German Bight.

and comparably high turbulence. Filters were immediately stored in liquid nitrogen until processing for molecular use.

Two-hundred-microliter aliquots of unfiltered material were inoculated into 2 mL of Drebes [4] or IMR/2 [7]. Following this dilution, a fourfold serial dilution using 200 μL was established in each of the two media. Flasks showing growth after several months were assessed for purity by light microscopy and by SSCP.

Isolation of Genomic DNA. DNA was prepared using chemical lysis from 0.2-μm filters with a DNA extraction Plant kit (PAN Biotechnology) to insure comparable preparation for different sampling sites and time points. Typically from 200 to 300 mL of a <3-μm seawater fraction, enough DNA was obtained to run 10–30 18S polymerase chain reactions (PCRs).

Amplification of Ribosomal RNA Genes. For subsequent cloning, 18S rDNA PCR was performed with primers of Medlin *et al.* [15] in an Eppendorf gradient cycler using standard conditions, Perkin Elmer *Taq* polymerase at two different annealing temperatures, 53 and 51°C. Cycles were as follows—5 min 94°C; 2 min 94°C, 1 min 51/53°C, and 4 min 72°C (34 cycles); and 10 min 72°C—for denaturing, annealing, and extension, respectively. For all samples, several PCR products were pooled before cloning.

Cloning of 18S PCR Products. Polymerase chain reaction products were cut out from gels stained with crystal violet and viewed under white light and purified prior to cloning using an EasyPure Kit (Biozym). Cloning was performed with a TOPO TA XL zero background kit (Invitrogen). Only cloning reactions leading to at least 1000 clones were regarded as successful and were further analyzed. Clones were analyzed by doing minipreps. Plasmids were digested with a cocktail of six different enzymes from the multicloning site of the vector (*Eco*RI, *Hind*III, *Pst*I, *Sst*I, *Eco*RV, *Xba*I, *Xho*I). Digests were analyzed by two rounds of agarose gel electrophoresis. Restriction fragment length polymorphisms (RFLPs) identical on the first gel were separated on a second gel next to each other and in the presence of HA yellow (Hansa Analytic Bremen). Typically 150–300 clones were analyzed by this procedure until 100 different RFLPs were found, which were then partially sequenced with the internal sequencing primer 528F [6], which reads through the V4 region of the 18S rRNA gene, its most variable region. These sequences are deposited in GenBank with the accession numbers AJ964963 to AJ965255.

All sequences from a given library were aligned, and a tree was made to identify duplicates. Redundancies were then calculated by counting duplicates in the tree and the corresponding RFLPs. All sequences were imported into ARB [14] (http://www.mikro.biologie.tu-muenchen.de) and generally aligned automatically using CLUSTAL-W within ARB using a secondary structure model. Then all sequences were compared against GenBank using a BLAST search to obtain the best hits, which were added to the tree maintained by ARB. Phylogenetic analyses were performed using Bayesian methods [9] with a gamma distribution. Different nested models of DNA substitution and associated parameters were estimating using Modeltest 3.0 [23]. These parameters were used to process the NJ and MP analysis for bootstrap analyses of 1000 replicates using PAUP*4.0b10 version [28].

Single-strand conformation polymorphism. Single-strand conformation polymorphism was initially developed for the analysis of bacterial communities [26], and we adapted it to assess the eukaryotic picoeukaryotic plankton community. SSCP is a PCR-based method in which a fraction of the 18S gene is amplified and a single-strand product is produced by digestion of the opposite strand. The single strands are separated in an acrylamide gel because they assume different conformations or folding of the single strand, which retards its migration through the gel. Each species in a sample will produce a characteristic band; thus, this method can be used as a coarse fingerprinting method to assess biodiversity in a mixed sample. As with the well-established denaturing gradient gel electrophoresis (DGGE) [20], differences in the secondary structure of partial 18S DNA molecules are used to separate such molecules generated via PCR by polyacrylamide gel electrophoresis. In contrast to DGGE, separation is performed on nondenaturing gels, which are easier to handle. Separation is improved as compared to DGGE by the generation of single-strand molecules from double-strand PCR products via single-strand digestion with lambda exonuclease. For this purpose, the reverse primer in PCR amplification was phosphorylated, thus generating a template for Lambda exonuclease. Each band represents a single species, and when bands are estimated visually to occur at the same height in the gel, then it is assumed that the species present at this location are identical. Bands can be excised and sequenced for sequencing. When this was performed, the sequences were identical (data not shown).

Eukaryotic SSCP. We tested internal 18S rRNA gene primers [6]. Combinations of 528F/926R-Phos produced best results. 528F only binds to eukaryotic 18S rDNA, whereas 926R is universal [6]. Therefore, only eukaryotic sequences are amplified. The use of 528F furthermore enables the comparison of sequences from SSCP products with sequences from clone libraries sequenced with the same primer. The 926R was labeled with phosphate to enable subsequent digestion and production of a single-strand product for SSCP analysis.

These sequences are deposited in GenBank with the accession numbers AM041070 to 129.

SSCP with Environmental Samples and Cultures. Polymerase chain reactions from both samples and cultures were performed with 528F/926R-phos in 100-μL (double reaction volume) assays because 50-μL reactions did not produce enough product. The PCR products were purified on Qiagen columns and eluted in 30 μL. Twenty-five microliters was used for single-strand digestion with 10 U Lambda exonuclease (New England Biolabs). The product was again purified on Qiagen MinElute columns, eluted in 10 μL, and 4 μL was loaded on the gel. Twenty-five-centimeter gels were run at 20°C, 400 V, 5 mA, and 8 W for 16 h and silver-stained. Banding patterns obtained were documented by scanning.

Microchip Fabrication. Oligonucleotide probes for microarray printing were obtained from Thermo Hybaid, Interactiva Division (Ulm, Germany) with a C6/MMT Aminolink at the 5′-end of the molecule. Prior to the printing procedure, the concentration of oligonucleotide probes was adjusted to 10 μM in spotting solution from Quantifoil Micro Tools GmbH (Jena, Germany). The chip fabrication and the printing of the DNA microchips were performed by PicoRapid Technologie (Bremen, Germany). After the printing procedure, the DNA microchips were stored at −20°C.

Generation of Labeled Target Nucleic Acid. Biotinylated template DNA for microarray hybridization was amplified from genomic DNA with two different primer pairs that generate overlapping PCR products that cover together the complete sequence of the 18S rDNA. Approximately 1200 bp of the 18S rDNA were amplified with 82F and 1055R, whereas a second fragment of ~900 bp was amplified with the primer combination 690F and 1528R. The primers 82F and 690F were labeled with a biotin moiety at the 5′-end. PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Germany). RNA that was isolated using the RNeasy Plant Mini Kit (Qiagen) was labeled with biotin using the CyScribe Direct mRNA Labeling Kit (Amersham Pharmacia Biotech, Germany).

Microarray Hybridization. The hybridization was carried out in a volume of 100 μL. The hybridization mixture contained hybridization buffer (1 M NaCl, 10 mM Tris, pH 8, 0.005% Triton X-100, 1 mg/mL BSA, 0.1 μg/μL HS-DNA) and biotinylated 18S PCR fragment at different concentrations. Additionally, a 250-bp PCR fragment of *Saccharomyces cerevisiae* thyroxine-binding protein at a final concentration of 4 ng/μL was added to the hybridization solution as a positive control. A prehybridization in hybridization buffer was carried out for 60 min at hybridization temperature prior to the hybridization. Before the hybridization, the hybridization mixture was denatured for 5 min at 94°C. Immediately after denaturing, the denatured solution was pipetted directly onto the array on the DNA chip. The hybridization was carried out under a coverslip in a wet chamber at 58°C for 1 h. In the following, the DNA microchips were washed for 15 min in buffer 1 [2× salt sodium citrate (SSC), 10 mM EDTA, 0.05% sodium dodecyl sulfate] and for additional 15 min in buffer 2 (1× SSC, 10 mM EDTA).

Staining of the Hybridized DNA Microchips. Hybridized biotinylated target DNA was visualized by staining the DNA microchips for 30 min with Streptavidin-Cy5 (Amersham Biosciences, Germany) in hybridization buffer at a concentration of 50 ng/mL. The staining was carried out with 100-μL staining solution under a coverslip.

Scanning and Quantification of Microarrays. Fluorescence images of the stained DNA microchips were taken with the GenePix 4000 B Scanner (Axon Instruments Inc., USA). The signal intensities were quantified using the GenePix 4.0 Software (Axon Instruments Inc.). To quantify each single spot, a grid of individual circles defining the location of a spot was superimposed onto the image. The mean signal intensity and the intensity of the local background area were determined for each spot. Results are presented graphically rather than pictorially for ease of interpretation.

Preparation of Target Nucleic Acid for the RNA vs. DNA-experiment. RNA and DNA were isolated both from 50 mL volume that originated from a 100 mL mix of the indicated species at the same cell density.

Results

In general, the abiotic patterns of temperature and salinity for our period of study were not significantly different than those routinely measured at the Helgoland time series [29]. Fig. 2 shows the range of temperature and salinity at the site for our study period. In September 2002, salinity dropped to coincide with a period of heavy rainfall. Salinity at this site can be influenced by river runoff from two major rivers: the Elbe and the Eider. Normally, freshwater from the rivers flows northward along the German coast, and only rarely does it reach Helgoland Roads. Generally, the flora at the Helgoland site is a marine flora that is influenced as fronts sweep across the island from offshore. Salinity ranges between 32 and 35 psu and is at its lowest some time between February

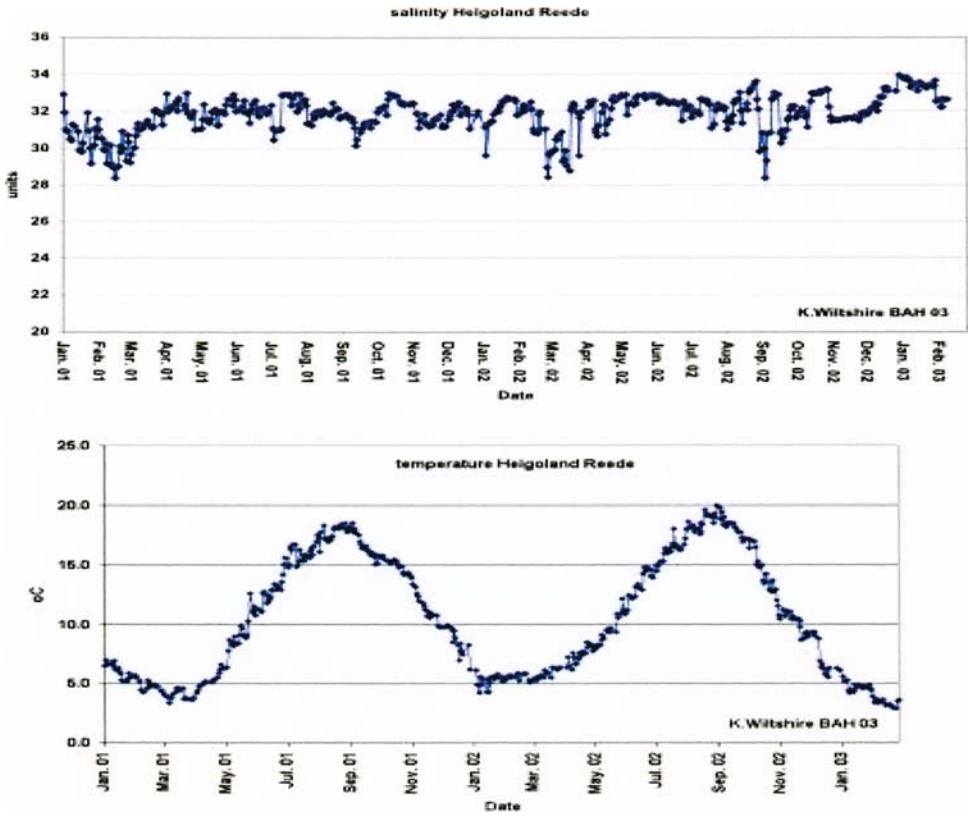

Figure 2. Temperature and salinity profiles at the Helgoland Time Series Site over the 2-year sampling period.

and March when there is the greatest amount of river input into the German Bight. Temperature was also at its lowest in January and February and, in 2002, was 4–6° warmer in the summer than in 2001. The shallow water depth is greatly influenced by wind, which significantly alters the transparency of the water. The shallow water depth contributes to a general homogenous water column that is not significantly different in taxon composition spatially around the island. A tidal range of less than 1 m is present at the sampling site and does not significantly influence the water mass at this site.

Cell counts are also very variable from year to year (Fig. 3). These data were supplied to us from the Helgoland Time Series data bank. In 2000, there was only one small spring bloom and one large fall bloom of diatoms as compared to a large spring and fall bloom in 2001 and 2002, which is the usual case [29]. In 2000, maximum numbers of flagellates (including dinoflagellates) occurred in the spring, whereas in 2001 and 2002, numbers were highly variable throughout the year. Although in 2000, maximum numbers occurred in the spring, these cells were in the smallest size fraction when these numbers are converted to total carbon (Fig. 4). It is likely that some of these smaller cells were collected in the picoeukaryotic plankton fraction of our samples. In 2000–2002, the largest cells of the nanoflagellates were variable. In 2000, there were two peaks, one in February to April and August 2000; in 2001, there was a broad peak ranging from June to October with small intermittent decreases; in 2002, there was a spring peak from January to mid-March and then a broad peak ranging from June to November in 2002. The picoeukaryotic plankton fraction is not enumerated at Helgoland or taxonomically identified. A conversion of the flagellate cell counts to carbon for 2000 only is shown in Fig. 4 to compare with the time period from our clone data. In July, when cell numbers are low, there is a peak in the total carbon, indicating that the cells at this time period should have been large. Conversely, the spring peak in cell numbers results in low total carbon, suggesting that the cell size at that time must have been small.

Clone Library. Figures 5 and 6 summarize the abundance of key plankton groups found in the clone libraries. Because we continued cloning analysis until we reached 100 unique clones per library, we consider that our coverage of the library was very good, ca. 80–90%. In general, the nonphotosynthetic groups seem to dominate in most samples (i.e., Alveolates and nonphotosynthetic Stramenopiles; Fig. 5). Among the nonphotosynthetic sequences, the most abundant group was the Alveolates, namely, the ciliates and uncultivated group II alveolates (Fig. 6A). Prasinophyceae (especially the Mamelliales) dominated the photosynthetic picoeukaryotic plankton, but cryptophytes, bolidophytes, diatoms, and chryso-

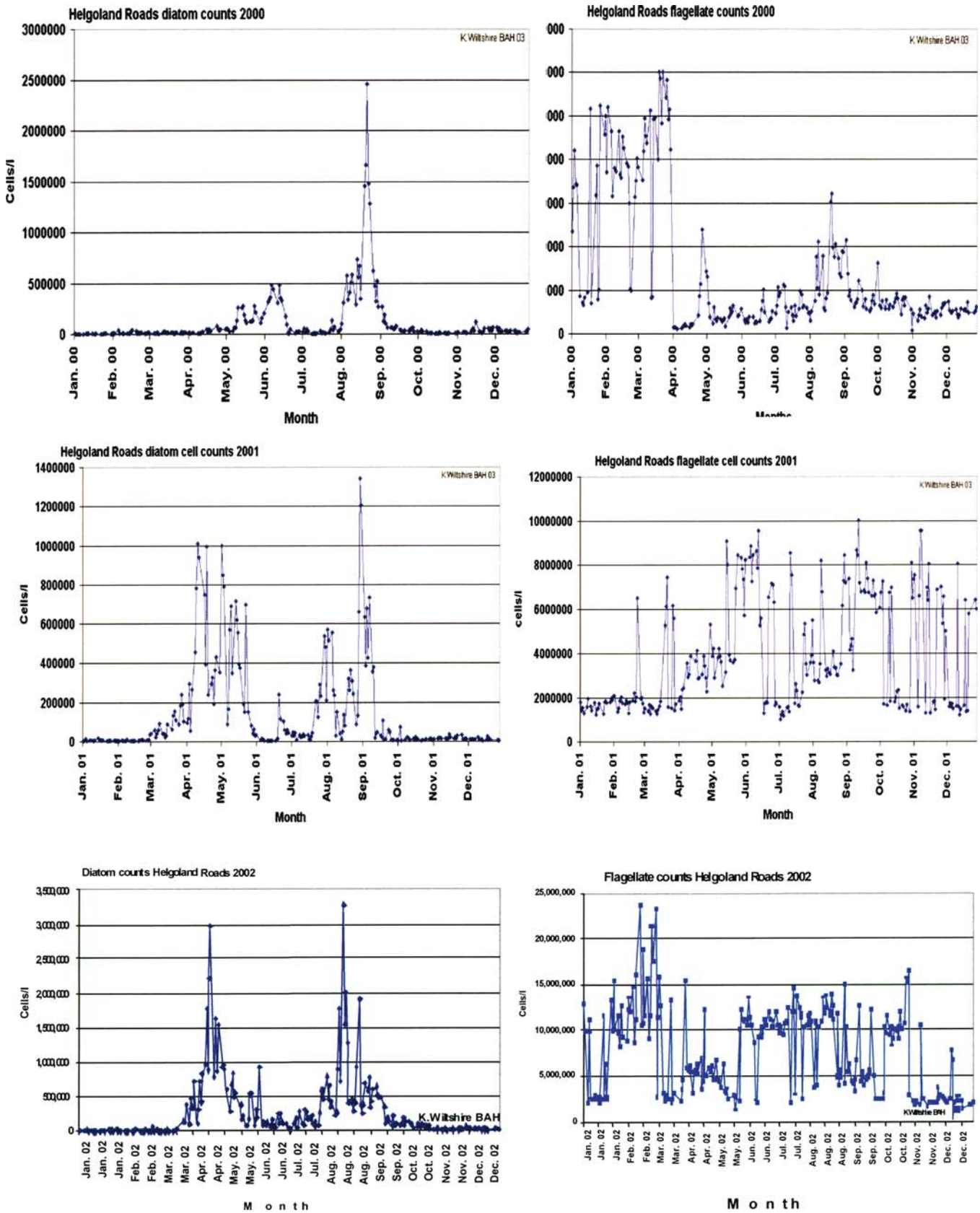

Figure 3. Helgoland cell abundance: diatoms (left) and flagellates (right) in 2000 (top), 2001(middle), and 2002 (bottom).

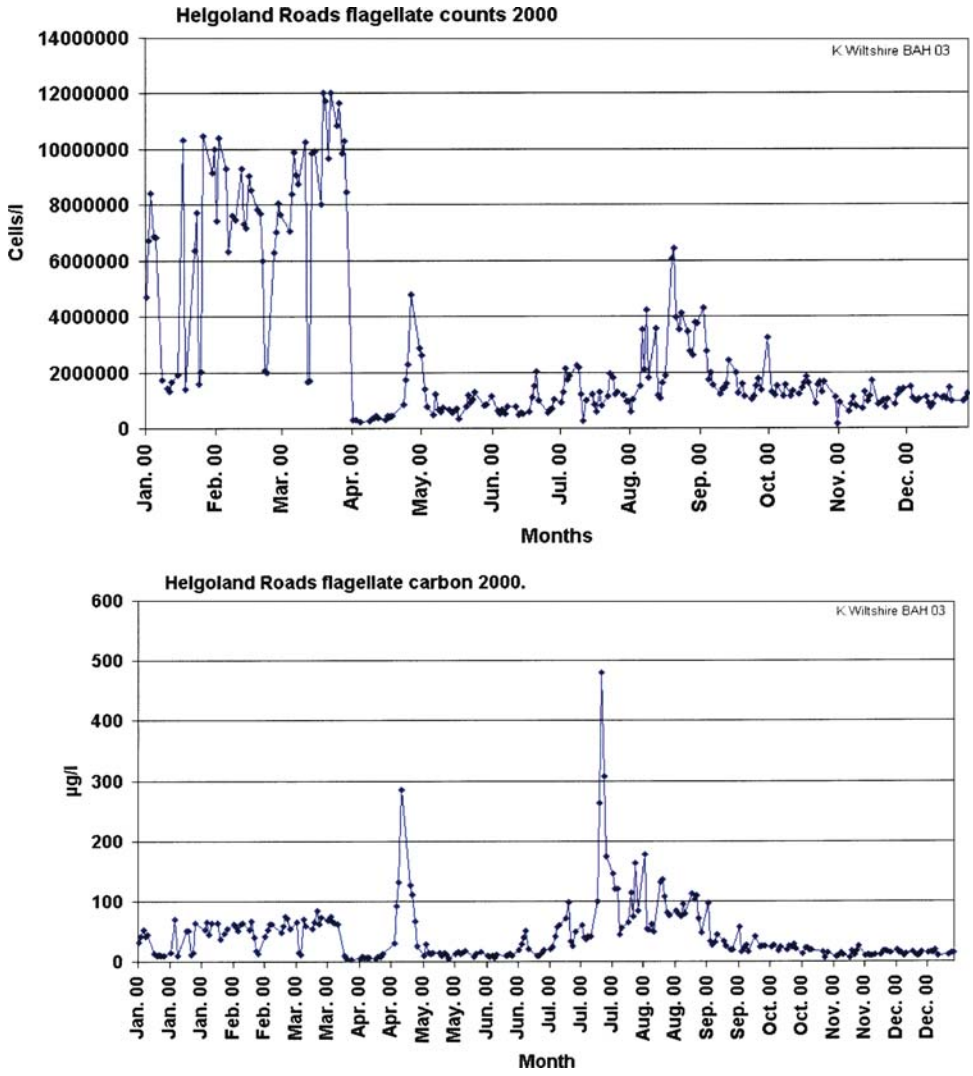

Figure 4. Cell numbers vs their biomass as converted into carbon for the flagellates counted at Helgoland for the year 2000.

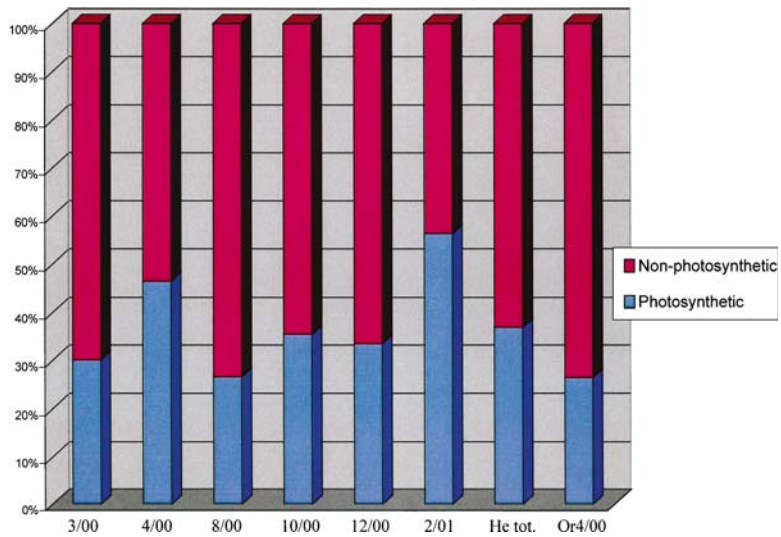

Figure 5. Summary of clone library data showing the number of photosynthetic sequences vs nonphotosynthetic sequences.

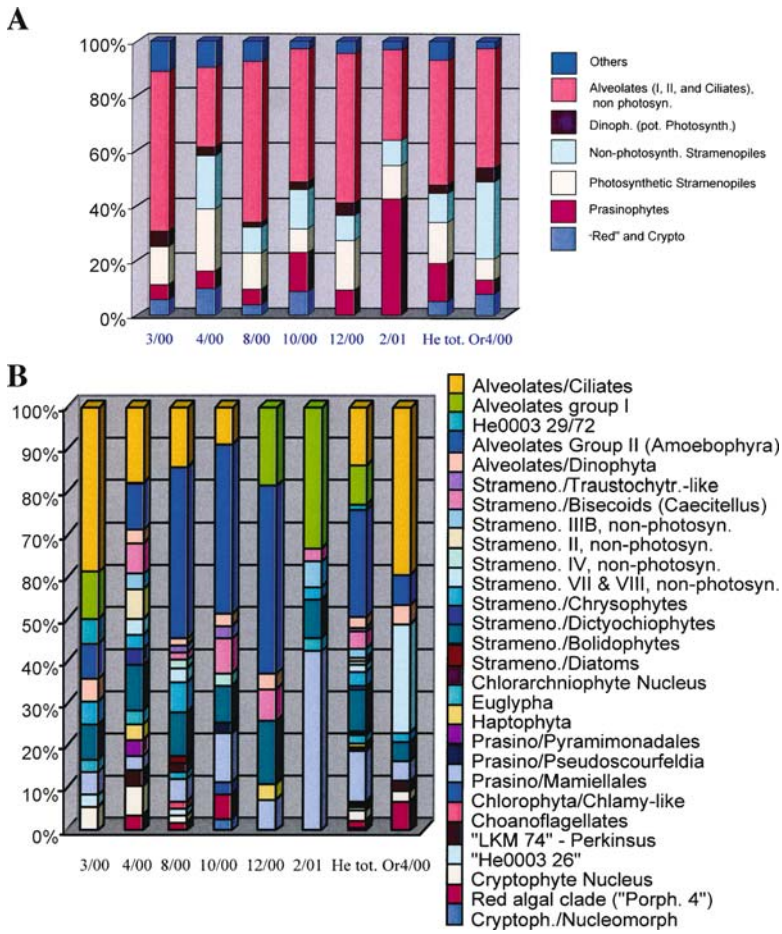

Figure 6. (A) Summary of clone library data by taxonomic unit at the class level; (B) at a level lower than class, i.e., order or family.

phytes were also present. In summary, all major plankton groups seem to contribute to the picoeukaryotic plankton community. The composition of the Helgoland picoeukaryotic plankton community does not differ significantly from those seen in Roscoff and Blanes in that all major (and "new") groups were found at all three sites, indicating a rather ubiquitous picoeukaryotic plankton community in European coastal waters [16, 25], although it should be noted that no sequence from any of the three sites was identical.

Because the nanoflagellates in Helgoland are not identified taxonomically, it is difficult to make a direct comparison to the clone library data. However, numerical abundances of all cells counted at Helgoland are converted to carbon by cell volume, and from Fig. 4, it is possible to see that the major contribution of nanoflagellate carbon in 2000 was during the summer, although total cell counts were highest in the spring. Thus, the smallest-sized cells of the nanoflagellates occurred outside the summer. Some of the smallest of these nanoflagellates might be expected to pass through the 3-μm filter. Photosynthetic picoeukaryotic plankton dominated over the nonphotosynthetic cells in our clone libraries only in April and in February (see Fig. 5). Thus, it would appear that heterotrophic cells are following the phototrophic cells in both the pico- and the nanofractions at Helgoland. Only rarely did we find the same sequence in sequential libraries. The rare reoccurrence of the same sequence in sequential libraries likely reflects two things: (1) the time interval of 2 months between library construction was too long to recover any sequential trends; and (2) the shallow water depth of the sampling station meant that the water column never stabilized long enough for long-term resident populations to establish themselves. A more detailed breakdown of the clone libraries to evaluate their diversity and the seasonal change in the community can be seen in Fig. 6B. Each of the major groups (classes) in Fig. 6A is further subdivided into clades in Fig. 6B, where it is possible to provide more taxonomic information with regards to the sequence identity. The group diversity changes drastically over the year with April and August being the most diverse, with 17 out of a total of 28 groups present. From the block diagram shown in Fig. 6B, the April community is more even than those at other times. The August sample is less even than that in April with a dominance

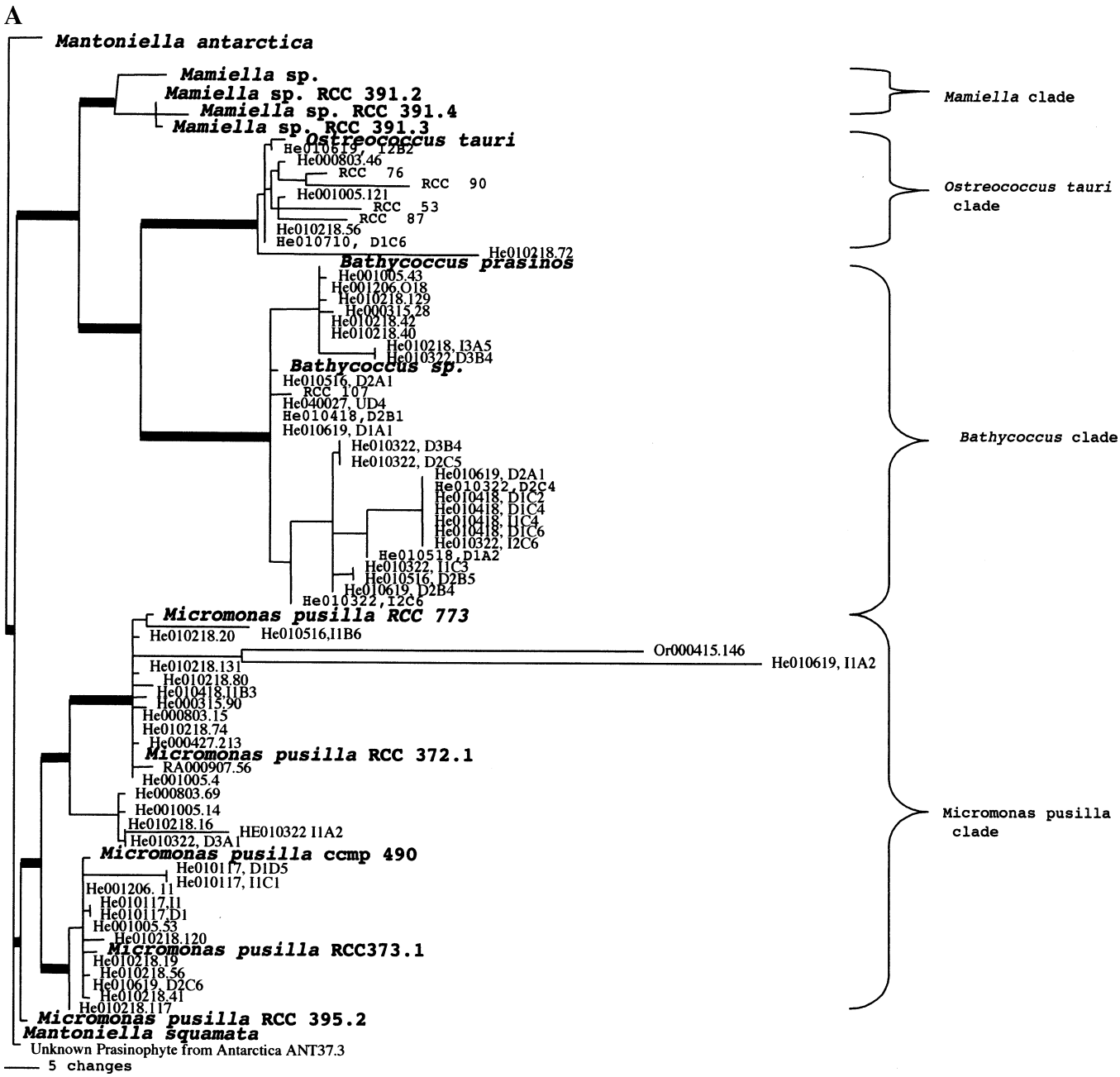

Figure 7. Phylogeny of the Prasinophyta (A) and Alveolata (B) showing the distribution of clone library and single-strand conformational polymorphism (SSCP) sequences among known cultured sequences inferred with a Bayesian analysis. Branch lengths in a largest point size indicate nodes that supported greater than 90% posterior probabilities. Next largest point size indicates nodes that supported greater than 80% posterior probabilities. SSCP cultures are identified by four digit letter/number combination following the isolation date. The first two digits represent the media (I or D) into which it was isolated and the dilution series in which it was found. The second two digits refer to the reference location in the microtiter plate in which the culture is maintained.

of Alveolates in that sample. The known sequence of *Amoebophyra* falls into the alveolates group II, and we make the assumption that this clade likely contains other parasitic protists (Fig. 7B), primarily because the novel sequences are short branches breaking up the long branch leading to two sequences of *Amoebophyra*. If this assumption is true, and these sequences assigned to alveolates group II are also parasitic of other algae, such as the dinoflagellates, then their increased presence in the summer and autumn likely follows the maximum abundance of their host cells, the dinoflagellates in the nano- and microfractions of the plankton. This group by far dominates the clone library (up to 45%), and isolation of living cells of this group in the future will be

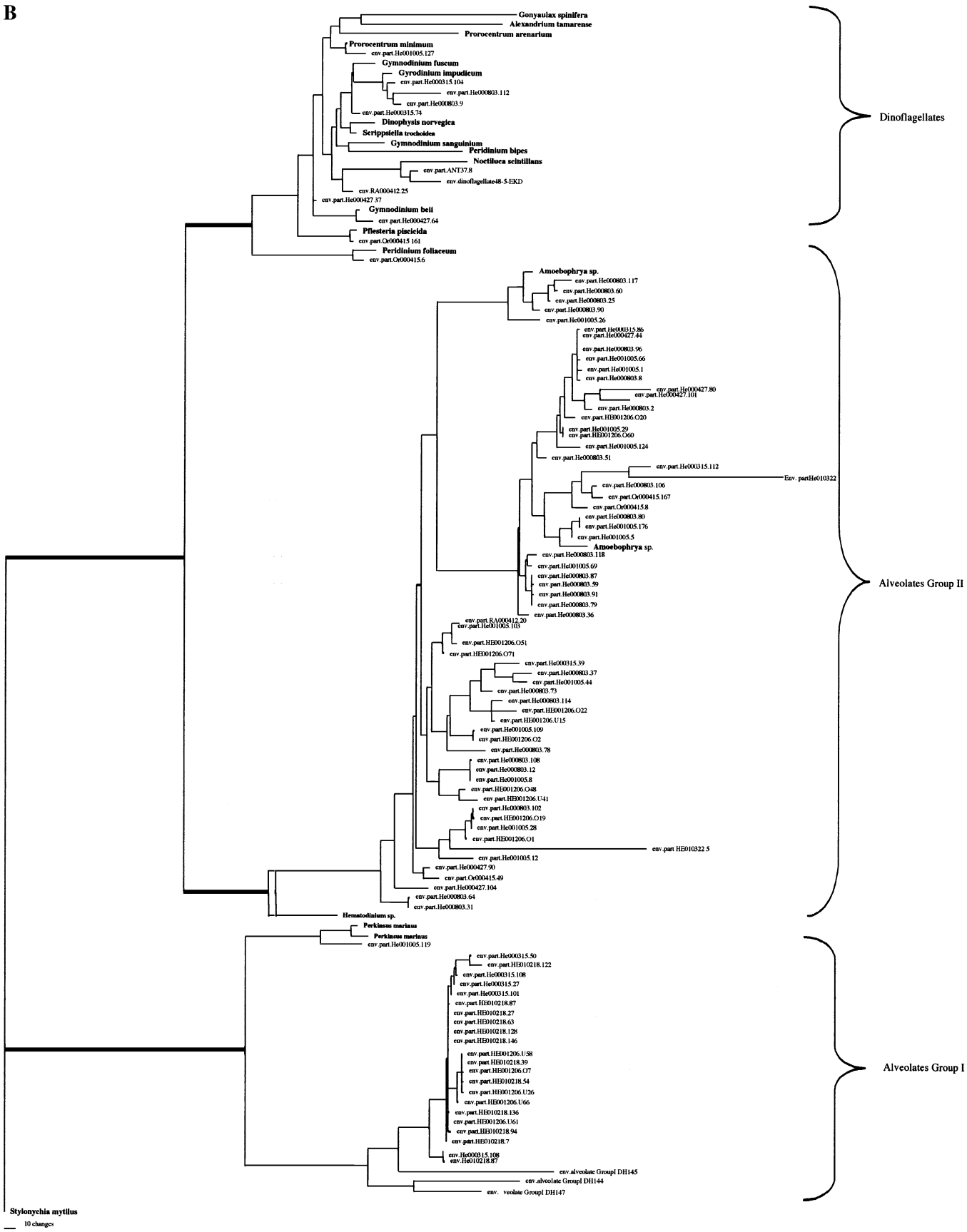

Figure 7. Continued.

Table 1. A summary of the identification of all the clones sequenced in the environmental clone libraries (env.part) and the cultures isolated from Helgoland as determined by their placement in the phylogenetic tree maintained by the ARB database

Clone identification	*Closest known genus or group*
env.part.Or000415.149	*Acanometra*, Acanthera
He010710 D1C1	*Acer*, Streptophyta
env.part.Or000415.161	Alveolate group I
He010322 D2A6	Alveolate group I
env.part.He000323.101	Alveolate group I
env.part.He000323.108	Alveolate group I
env.part.He000323.271	Alveolate group I
env.part.He000323.50	Alveolate group I
env.part.HE001206.07	Alveolate group I
env.part.HE001206.U26	Alveolate group I
env.part.HE001206.U58	Alveolate group I
env.part.HE001206.U61	Alveolate group I
env.part.HE001206.U66	Alveolate group I
env.part.HE010218.122	Alveolate group I
env.part.HE010218.128	Alveolate group I
env.part.HE010218.136	Alveolate group I
env.part.HE010218.146	Alveolate group I
env.part.HE010218.27	Alveolate group I
env.part.HE010218.39	Alveolate group I
env.part.HE010218.54	Alveolate group I
env.part.HE010218.63	Alveolate group I
env.part.HE010218.07	Alveolate group I
env.part.HE010218.87	Alveolate group I
env.part.HE010218.94	Alveolate group I
env.part.He000803.51	Alveolate group II
env.part.He001005.176	Alveolate group II
env.part.He001005.8	Alveolate group II
env.part.Or000415.8	Alveolate group II
env.part.He000323.112	Alveolate group II
env.part.He000323.29	Alveolate group II
env.part.He000323.39	Alveolate group II
env.part.He000323.72	Alveolate group II
env.part.He000323.74	Alveolate group II
env.part.He000323.86	Alveolate group II
env.part.He000427.104	Alveolate group II
env.part.He000427.44	Alveolate group II
env.part.He000427.64	Alveolate group II
env.part.He000427.80	Alveolate group II
env.part.He000427.90	Alveolate group II
env.part.He000803.102	Alveolate group II
env.part.He000803.106	Alveolate group II
env.part.He000803.108	Alveolate group II
env.part.He000803.114	Alveolate group II
env.part.He000803.117	Alveolate group II
env.part.He000803.12	Alveolate group II
env.part.He000803.25	Alveolate group II
env.part.He000803.31	Alveolate group II
env.part.He000803.37	Alveolate group II
env.part.He000803.60	Alveolate group II
env.part.He000803.64	Alveolate group II
env.part.He000803.73	Alveolate group II
env.part.He000803.78	Alveolate group II
env.part.He000803.08	Alveolate group II
env.part.He000803.80	Alveolate group II
env.part.He000803.91	Alveolate group II
env.part.He000803.96	Alveolate group II
env.part.He001005.01	Alveolate group II
env.part.He001005.103	Alveolate group II

Table 2. Continued

Clone identification	*Closest known genus or group*
env.part.He001005.109	Alveolate group II
env.part.He001005.119	Alveolate group II
env.part.He001005.12	Alveolate group II
env.part.He001005.124	Alveolate group II
env.part.He001005.127	Alveolate group II
env.part.He001005.28	Alveolate group II
env.part.He001005.29	Alveolate group II
env.part.He001005.44	Alveolate group II
env.part.He001005.05	Alveolate group II
env.part.He001005.66	Alveolate group II
env.part.He001005.08	Alveolate group II
env.part.HE001206.02	Alveolate group II
env.part.HE001206.22	Alveolate group II
env.part.HE001206.48	Alveolate group II
env.part.HE001206.51	Alveolate group II
env.part.HE001206.60	Alveolate group II
env.part.HE001206.01	Alveolate group II
env.part.HE001206.19	Alveolate group II
env.part.HE001206.20	Alveolate group II
env.part.HE001206.71	Alveolate group II
env.part.HE001206.U15	Alveolate group II
env.part.HE001206.U41	Alveolate group II
env.part.HE001206.U73	Alveolate group II
env.part.Or000415.167	Alveolate group II
env.part.Or000415.49	Alveolate group II
env.part.Or000415.82	Alveolate group II
env.part.He000427.101	Alveolate group II, *Amoebophyra*
env.part.He000803.118	Alveolate group II, *Amoebophyra*
env.part.He000803.02	Alveolate group II, *Amoebophyra*
env.part.He000803.36	Alveolate group II, *Amoebophyra*
env.part.He000803.59	Alveolate group II, *Amoebophyra*
env.part.He000803.79	Alveolate group II, *Amoebophyra*
env.part.He000803.87	Alveolate group II, *Amoebophyra*
env.part.He000803.90	Alveolate group II, *Amoebophyra*
env.part.He001005.26	Alveolate group II, *Amoebophyra*
env.part.He001005.69	Alveolate group II, *Amoebophyra*
env.part.He001005.51	*Amoeba*
He010322 D1A2	Apedinella, Pedinellophyceae
env.part.HE001206.05	basal Haptophyta
HE000427 U D4	*Bathycoccus*, Prasinophyta
He010218 I3A5	*Bathycoccus*, Prasinophyta
He010322 D2C4	*Bathycoccus*, Prasinophyta
He010322 D2C5	*Bathycoccus*, Prasinophyta
He010322 I1C3	*Bathycoccus*, Prasinophyta
He010322 I2C6	*Bathycoccus*, Prasinophyta
He010322 I2C6	*Bathycoccus*, Prasinophyta
He010418 D1C2	*Bathycoccus*, Prasinophyta
He010418 D1C4	*Bathycoccus*, Prasinophyta
He010418 D1C6	*Bathycoccus*, Prasinophyta
He010418 D2B1	*Bathycoccus*, Prasinophyta
He010418 I1C4	*Bathycoccus*, Prasinophyta
He010516 D2A1	*Bathycoccus*, Prasinophyta
He010516 D2B5	*Bathycoccus*, Prasinophyta
He010518 D1A2	*Bathycoccus*, Prasinophyta
He010619 D1A1	*Bathycoccus*, Prasinophyta
He010619 D2A1	*Bathycoccus*, Prasinophyta
He010619 D2B4	*Bathycoccus*, Prasinophyta
He010619 D2C6	*Bathycoccus*, Prasinophyta
He010710 D1C6	*Bathycoccus*, Prasinophyta
env.part.He000323.28	*Bathycoccus*, Prasinophyta
env.part.He001005.43	*Bathycoccus*, Prasinophyta
env.part.HE010218.129	*Bathycoccus*, Prasinophyta

Table 2. A summary of the identification of all the clones sequenced in the environmental clone libraries (env.part) and the cultures isolated from Helgoland as determined by their placement in the phylogenetic tree maintained by the ARB database

Clone identification	*Closest known genus or group*
env.part.HE010218.40	*Bathycoccus*, Prasinophyta
env.part.HE010218.42	*Bathycoccus*, Prasinophyta
He010117 D1D5	*Bathycoccus*, Prasinophyta
He010117 I1C1	*Bathycoccus*, Prasinophyta
He010322 D3B4	*Bathycoccus*, Prasinophyta
He010418 I1B3	*Bathycoccus*, Prasinophyta
He011322 I1A2	*Bathycoccus*, Prasinophyta
env.part.He000427.212	Boliophyceae
env.part.HE001206.57	Boliophyceae
env.part.He000427.119	Boliophyceae
env.part.He000427.28	Boliophyceae
env.part.He000803.119	Boliophyceae
env.part.He000803.41	Boliophyceae
env.part.He000803.47	Boliophyceae
env.part.He000803.53	Boliophyceae
env.part.He000803.06	Boliophyceae
env.part.He001005.151	Boliophyceae
env.part.HE010218.01	Boliophyceae
env.part.HE010218.123	Boliophyceae
env.part.HE010218.18	Boliophyceae
env.part.HE010218.06	Boliophyceae
He010322 D2B6	*Cafeteria*, Stramenopiles
He010322 D2B6	*Cafeteria*, Stramenopiles
He010322 I1A3	*Cafeteria*, Stramenopiles
He010619 D2A3	*Cafeteria*, Stramenopiles
env.part.He001005.203	*Cafeteria*, Stramenopiles
He010619 I1 D1	*Cafeteria*, Stramenopiles
He010619 I1A1	*Cafeteria*, Stramenopiles
He010619 I2B3	*Cafeteria*, Stramenopiles
He010619 I1A4	*Cafeteria*, Stramenopiles
env.part.He000803.82	Cercozoa
env.part.Or000415.153	Cercozoa
env.part.He000323.24	Cercozoa
He010322 I1C5	Cercozoa
env.part.HE010218.02	Cercozoa
env.part.Or000415.55	*Chaetoceros*, Bacillariophyta
env.part.He000803.100	Chlorarchniophyta
env.part.He000427.207	Chlorarchniophyta
He010516 D1B6	*Chlorella*, Chlorohyta
env.part.He000803.01	Choanoflagellates
env.part.He000427.157	*Chrysochromulina*, Haptophyta
env.part.HE001206.32	*Chrysochromulina*, Haptophyta
env.part.He000427.118	*Chrysoxys*, Chrysophyta
env.part.He000323.38	Ciliate
env.part.He001005.187	Ciliate
env.part.Or000415.125	Ciliate
env.part.Or000415.142	Ciliate
env.part.Or000415.184	Ciliate
env.part.Or000415.23	Ciliate
env.part.Or000415.35	Ciliate
env.part.Or000415.48	Ciliate
env.part.Or000415.63	Ciliate
env.part.He000323.25	Ciliate
env.part.He000323.33	Ciliate
env.part.He000323.40	Ciliate
env.part.He000323.45	Ciliate
env.part.He000323.49	Ciliate
env.part.He000323.54	Ciliate

Table 2. Continued

Clone identification	*Closest known genus or group*
env.part.He000323.55	Ciliate
env.part.He000323.63	Ciliate
env.part.He000323.65	Ciliate
env.part.He000323.67	Ciliate
env.part.He000323.69	Ciliate
env.part.He000323.91	Ciliate
env.part.He000323.92	Ciliate
env.part.He000427.215	Ciliate
env.part.He000427.43	Ciliate
env.part.He000427.49	Ciliate
env.part.He000427.07	Ciliate
env.part.He000427.87	Ciliate
env.part.He000803.10	Ciliate
env.part.He000803.16	Ciliate
env.part.He000803.26	Ciliate
env.part.He000803.28	Ciliate
env.part.He000803.45	Ciliate
env.part.He000803.52	Ciliate
env.part.He000803.66	Ciliate
env.part.He000803.81	Ciliate
env.part.He001005.101	Ciliate
env.part.He001005.150	Ciliate
env.part.He001005.166	Ciliate
env.part.Or000415.100	Ciliate
env.part.Or000415.105	Ciliate
env.part.Or000415.110	Ciliate
env.part.Or000415.129	Ciliate
env.part.Or000415.138	Ciliate
env.part.Or000415.15	Ciliate
env.part.Or000415.185	Ciliate
env.part.Or000415.36	Ciliate
env.part.Or000415.61	Ciliate
env.part.Or000415.89	Ciliate
He001206 D2C1	Ciliate
He010710 D2A2	*Cornus*, Streptophyta
env.part.He000803.112	*Cryptothecodinium*, Dinophyta
env.part.He000427.05	Dictyocha, Dictyophyceae
env.part.He000427.18	*Dinobryon*, Chrysophyta
env.part.He000427.193	*Dinobryon*, Chrysophyta
env.part.HE001206.28	*Dinobryon*, Chrysophyta
env.part.He001005.97	*Enteromorpha*, Chlorophyta
env.part.He000803.54	*Eurychasma*, Stramenopiles
env.part.He000323.62	*Giraudyopsis*, Chrysomerophyceae
env.part.He000427.149	*Giraudyopsis*, Chrysomerophyceae
env.part.He000427.196	*Giraudyopsis*, Chrysomerophyceae
env.part.He000427.37	*Gymnodinium*, Dinophyta
env.part.Or000415.06	*Gymnodinium*, Dinophyta
env.part.He000323.104	*Gyrodinium*, Dinophyta
env.part.He000803.09	*Gyrodinium*, Dinophyta
env.part.He000803.29	*Hibberdia*, Chrysophyta
env.part.He000803.30	*Hibberdia*, Chrysophyta
env.part.He000803.04	*Hibberdia*, Chrysophyta
env.part.He000803.44	*Hibberdia*, Chrysophyta
env.part.He000427.142	Hydra, Cnidaria
env.part.HE001206.14	*Laminaria*, Phaeophyta
env.part.Or000415.146	*Mamiella*, Prasinophyta
env.part.He000803.15	*Micromonas*, Prasinophyta
env.part.He000323.90	*Micromonas*, Prasinophyta
env.part.He000427.213	*Micromonas*, Prasinophyta
env.part.He000803.69	*Micromonas*, Prasinophyta
env.part.He001005.14	*Micromonas*, Prasinophyta
env.part.He001005.04	*Micromonas*, Prasinophyta

Table 2. A summary of the identification of all the clones sequenced in the environmental clone libraries (env.part) and the cultures isolated from Helgoland as determined by their placement in the phylogenetic tree maintained by the ARB database

Clone identification	*Closest known genus or group*
env.part.He001005.53	*Micromonas*, Prasinophyta
env.part.HE001206.11	*Micromonas*, Prasinophyta
env.part.HE001206.18	*Micromonas*, Prasinophyta
env.part.HE010218.117	*Micromonas*, Prasinophyta
env.part.HE010218.120	*Micromonas*, Prasinophyta
env.part.HE010218.131	*Micromonas*, Prasinophyta
env.part.HE010218.16	*Micromonas*, Prasinophyta
env.part.HE010218.19	*Micromonas*, Prasinophyta
env.part.HE010218.20	*Micromonas*, Prasinophyta
env.part.HE010218.41	*Micromonas*, Prasinophyta
env.part.HE010218.05	*Micromonas*, Prasinophyta
env.part.HE010218.74	*Micromonas*, Prasinophyta
env.part.HE010218.80	*Micromonas*, Prasinophyta
env.part.Or000415.178	*Micromonas*, Prasinophyta
He010322 D3A1	*Micromonas*, Prasinophyta
He010619 I1A2	*Micromonas*, Prasinophyta
He001206 D1C1	*Nanochlorum*, Prasinophyta
He010418 I1A6	*Nanochlorum*, Prasinophyta
env.part.HE010218.135	*Nanochlorum*, Prasinophyta
env.part.HE010218.168	*Nanochlorum*, Prasinophyta
HE000427 U D3	*Nanochlorum*, Prasinophyta
HE000427 U D6	*Nanochlorum*, Prasinophyta
He010117 I1C4	*Nanochlorum*, Prasinophyta
He010218 I2D1	*Nanochlorum*, Prasinophyta
He010322 D2A5	*Nanochlorum*, Prasinophyta
He010322 D3A2	*Nanochlorum*, Prasinophyta
He010418 D2A2	*Nanochlorum*, Prasinophyta
He010418 I2A1	*Nanochlorum*, Prasinophyta
He010418 I2A4	*Nanochlorum*, Prasinophyta
He010619 I2A6	*Nanochlorum*, Prasinophyta
env.part.He000323.26	Novel Cryptophyta
env.part.He000323.80	Novel Cryptophyta
env.part.He000803.99	Novel Cryptophyta
env.part.He000427.21	Novel Stramenopile group III
env.part.He001005.40	Novel Stramenopile group III
env.part.HE010218.157	Novel Stramenopile group III
env.part.He000427.30	Novel Stramenopile group III
env.part.He000803.115	Novel Stramenopile group III
env.part.He000803.48	Novel Stramenopile group III
env.part.He001005.41	Novel Stramenopile group III
env.part.He001005.46	Novel Stramenopile group III
env.part.HE001206.17	Novel Stramenopile group III
env.part.HE001206.06	Novel Stramenopile group III
env.part.HE001206.064	Novel Stramenopile group III
env.part.HE001206.U37	Novel Stramenopile group III
env.part.HE010218.08	Novel Stramenopile group III
env.part.HE010218.82	Novel Stramenopile group III
env.part.Or000415.27	Novel Stramenopile group III
env.part.Or000415.66	Novel Stramenopile group III
env.part.He001005.47	Novel Stramenopile group IV
env.part.He000803.03	Novel Stramenopile group IV
env.part.He000803.93	Novel Stramenopile group VII
env.part.He000427.182	Novel Stramenopile group VII
env.part.He000803.35	Novel Stramenopile group VII
env.part.He001005.15	Novel Stramenopile group VII
env.part.Or000415.127	Novel Stramenopile group VII
env.part.Or000415.134	Novel Stramenopile group VII
env.part.Or000415.39	Novel Stramenopile group VII
env.part.Or000415.50	Novel Stramenopile group VII
env.part.Or000415.07	Novel Stramenopile group VII
env.part.Or000415.17	Novel Stramenopile group VIII
env.part.HE001206.12	Novel Stramenopile group VIII
env.part.Or000415.113	Novel Stramenopile group VIII
env.part.Or000415.141	Novel Stramenopile group VIII
env.part.Or000415.162	Novel Stramenopile group VIII
env.part.He000427.201	Novel Stramenopile group X
env.part.He000323.66	Novel Stramenopiles group III
He010322 I3 A6	*Ochromonas*, Chrysophyta
He010619 I1C1	*Ochromonas*, Chrysophyta
env.part.He000427.138	*Ochromonas*, Chrysophyta
He010619 I2B2	*Osterococcus*, Prasinophyta
env.part.HE010218.56	*Ostreococcus*, Chlorophyta
env.part.He001005.121	*Ostreococcus*, Prasinophyta
env.part.He000803.46	*Ostreococcus*, Prasinophyta
env.part.HE010218.72	*Ostreococcus*, Prasinophyta
env.part.He001005.03	*Papiliocellus*, Bacillariophyta
env.part.He000323.98	*Paraphysomonas*, Chrysophyta
env.part.He000427.47	*Paraphysomonas*, Chrysophyta
env.part.HE010218.12	*Paraphysomonas*, Chrysophyta
env.part.Or000415.16	*Perkinsus*, Mesomycetozoa
He010710 D1B6	*Phaeocystis*, Haptophyta
He001206 D2B1	*Phaeocystis*, Haptophyta
He001206 D2B1	*Phaeocystis*, Haptophyta
He001206 I1D1	*Phaeodactylum*, Bacillariophyta
He010619 I1A3	*Pirsonia*, Cercozoa
env.part.He000427.20	*Placidia*, Stramenopiles
env.part.He000427.132	*Plagiomonas*, Cryptophyta
env.part.He001005.208	*Plasmodiophora*, Cercozoa
env.part.He001005.207	Porifera
env.part.He001005.88	*Prasinococcum*, Prasinophyta
env.part.He000427.01	*Pseudomonocystis* Apicomplexa
env.part.He000427.122	*Pseudomonocystis* Apicomplexa
env.part.He000427.15	*Pseudomonocystis*, Apicomplexa
env.part.He001005.193	*Pseudosourfieldia*, Prasinophyta
env.part.He000427.63	*Pyramimonas*, Prasinophyta
env.part.He000427.125	*Rhynochomonas*, Kinetoplastida
env.part.He000427.29	Rosko II, new algal class
env.part.He001005.148	Rosko II, new algal class
env.part.He000427.214	Rosko II, new algal class
env.part.He000803.72	Rosko II, new algal class
env.part.He001005.33	Rosko II, new algal class
env.part.Or000415.159	Rosko II, new algal class
env.part.Or000415.187	Rosko II, new algal class
env.part.Or000415.188	Rosko II, new algal class
env.part.Or000415.09	Rosko II, new algal class
He010322 I3A3	*Symbiomonas*, Stramenopiles
env.part.HE001206.15	*Symbiomonas*, Stramenopiles
env.part.HE001206.U30	*Symbiomonas*, Stramenopiles
env.part.He000427.23	*Symbiomonas*, Stramenopiles
env.part.He001005.32	*Symbiomonas*, Stramenopiles
env.part.He000323.56	*Teleaulax*, Cryptophyta
env.part.He000803.94	*Teleaulax*, Cryptophyta
env.part.He001005.108	*Teleaulax*, Cryptophyta
env.part.Or000415.11	*Teleaulax*, Cryptophyta
env.part.He000427.53	*Teleaulax*, Cryptophyta
env.part.He001005.112	*Thaustochytrium*, Thaustochytrids

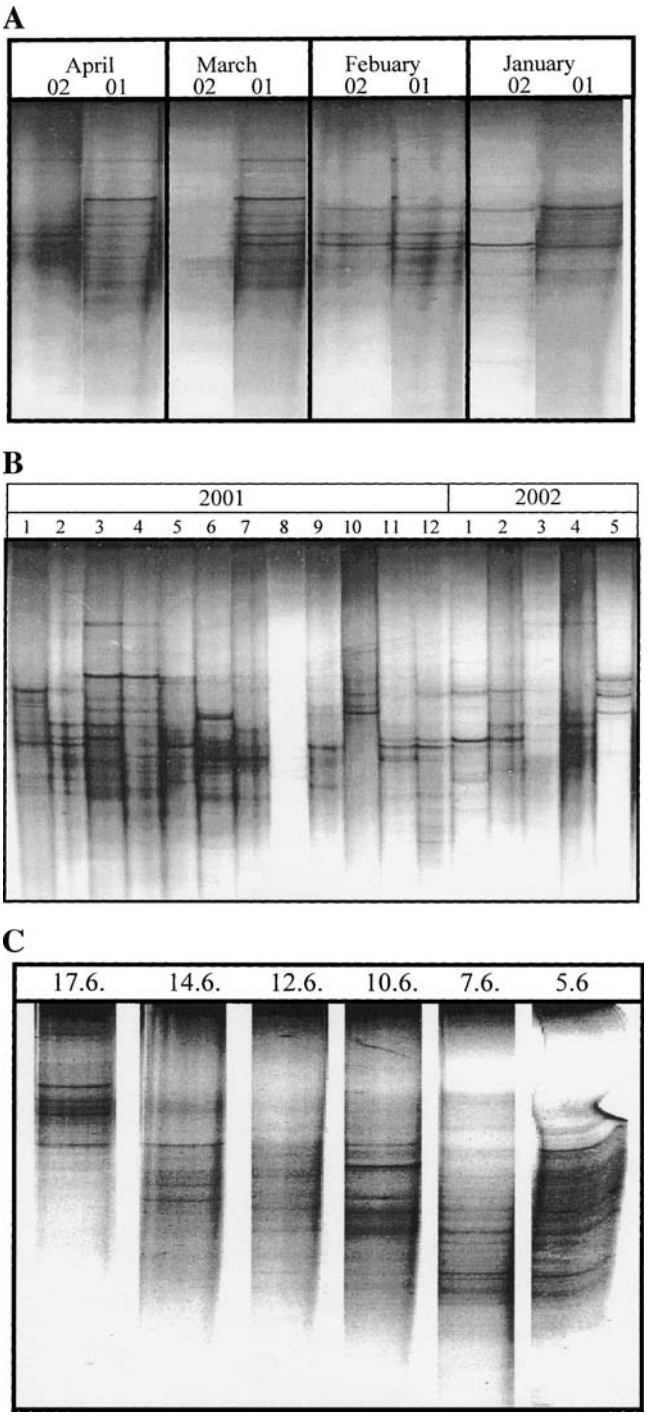

Figure 8. SSCP analysis of the picoeukaryotic plankton community at Helgoland. (A) Four months compared for 2002 and 2001. (B) At monthly intervals from January 2001 and May 2002. (C) Every 2 or 3 days during 2 weeks in June 2002.

very interesting in terms of studying their mode of nutrition. Ciliates are the second most common group present in the clone library (42%). They are dominant in the March and April sample from Helgoland. The dominant photosynthetic picoeukaryotic plankton Prasinophyta (Order Mamelliales) achieved their maximum abundance in the spring of 2001 (40%). We present a phylogenetic tree of our prasinophyte and alveolate sequences both from the clone library, SSCP, and culture sequences to illustrate their relationship to the known sequences from GenBank and from other established cultures from this group (Fig. 7). None of our cultures were found in the clone libraries. The same taxon grew in the cultures from March to June 2000, but it never appeared in the clone libraries. The Bolidophytes are the second most numerous group, more numerous here than at the other two sites, Roscoff and Blanes. Marine representatives of the Chrysophytes are present. This class is primarily a freshwater group, so isolation of cells with this genotype will also reveal novel taxa. One sequence, HE001206.05, fell at the base of the Haptophytes, basal to the divergence of the Pavlophyceae and Prymnesiophyceae. It has been assumed that this long branch of the haptophytes likely represents extinct taxa [5]. This novel sequence would suggest that there are still unknown groups of haptophytes in the picoeukaryotic planktonic fraction as was found by Moon-van der Stay et al. [19]. In all of the clone library isolates, a 100% match to known sequences in the database was rare, so all of these sequences are likely new species or uncultured described ones (Fig. 6, Table 1). The so-called "Rosko II" novel ribotype [25] was also present in our group and likely represents a new algal class and is the subject of further in-depth analysis by our group. This group of sequences was present at all three sites in the PICODIV project [16, 25]. In the winter, the clone library was less diverse, being dominated only by seven groups. This library was also very uneven with a dominance of one particular sequence of alveolates. The single clone library made from the environmental sample taken at the Orkney Islands was not significantly different from those taken in the German Bight, but is less diverse than the clone library taken in the German Bight at the same time of the year. A few higher metazoan and metaphyta sequences were encountered in all the clone libraries, but it was assumed that these likely represent reproductive stages in the picoeukaryotic plankton.

Single-Strand Conformational Polymorphism. Single-strand conformational polymorphism was applied to assess the seasonal abundance of picoeukaryotic plankton between January 2001 and May 2002 (Fig. 8). Comparisons were made at daily, monthly, and yearly intervals. All lanes contain multiple bands, suggesting that the samples were highly diverse. The more intense bands likely belong to the most abundant species present in the sample, assuming no PCR bias. If the same band appears at the same location repeatedly, then it was assumed that the same species was present in more than one sample. Sequencing of selected bands at the same gel height revealed identical species (data not shown). Fig. 8B

Table 2. List of the class-level probes and the related sequences that have been tested currently in hybridization experiments on the DNA chip

Probe	*Target*	*Sequence*	*Reference*
Chlo 02	Chlorophyta	CTTCGAGCCCCCAACTTT	[27]
Boli 02	Bolidophyceae	TACCTAGGTACGCAAACC	[8]
Prym 01	Prymnesiophyta	ACATCCCTGGCAAATGCT	[12]
Prym 02	Prymnesiophyta	GGAATACGAGTGCCCCTGAC	[27]
Dino 1	Dinophyta	CCTCAAACTTCCTTGCITTA	[11]
Dino E-12	Dinophyta	CGGAAGCTGATAGGTCAGAA	This work
Pras 04	Prasinophyceae	CGTAAGCCCGCTTTGAAC	[19]
Bathy 01	*Bathycoccus*	ACTCCATGTCTCAGCGTT	[19]
Micro 01	*Micromonas*	AATGGAACACCGCCGGCG	[19]
Ostreo 01	*Ostreococcus*	CCTCCTCACCAGGAAGCT	[19]
Crypto B	Cryptophyta	ACGGCCCCAACTGTCCCT	This work
NS 04	New Stramenopiles Clade 4	TACTTCGGTCTGCAAACC	[13]
Positive control	*Saccharomyces cerevisiae*	ATGGCCGATGAGGAACGT	This work
Negative control		TCCCCCGGGTATGGCCGC	This work

shows that, with the exception of March and April 2001 (lanes 3, 4, left) samples, most bands appear singly or in duplicate months, and no band was present for more than 3–4 months. This would indicate that the diversity of the picoeukaryotic plankton changes rapidly on a monthly basis. This observation was also seen in the clone libraries because these samples were taken at bi-monthly to quarterly intervals, and rarely did a sequence appear twice in sequential libraries.

To determine at what time interval the community could be sampled to show consistency from one sampling point to another, we conducted an analysis on a shorter timer scale of 2 weeks in June 2002. Fig. 8C shows that already within days, the community structure of the picoeukaryotic plankton can change. This may be related to the special conditions of the Helgoland sampling site because it is very shallow and therefore strongly influenced by physical conditions, such as wind, air temperature, or sunlight intensity.

But interestingly, if time periods from 1 year to the next are compared (Fig. 8A), then we see similar bands appearing on a yearly basis, which would suggest that there is some type of seasonality in the picoeukaryotic plankton fraction. We are assuming that identical bands represent the same taxon. A similar phenomenon has been reported for monthly variation in marine bacteria at the San Pedro Ocean Time Series Site at a 5-m depth where months from different years cluster together (J. Fuhrmann, pers. comm.). This has been referred to as annual community reassembling.

The clonality of the cultures established from the samples was assessed by SSCP analysis. All cultures that exhibited a single SSCP band were assumed to be unialgal. The band was cut out and sequenced to provide an identification of the culture. Most of these were of Prasinophytes (Fig. 7).

Microarrays. A set of molecular probes (Table 2) that have been developed for fluorescent *in situ* hybridization (FISH) techniques initially was adapted for use in combination with DNA microarray technology for the analysis of picoeukaryotic plankton samples taken at the Helgoland sampling site. Probes have been chosen that target the 18S rRNA gene of phytoplankton groups that have representatives in the picoeukaryotic plankton at higher taxonomic levels. The selected probes were known to be specific with hybridization techniques, such as dot blots or FISH. The length of the probes varied between 18 and 20 nt. To test our chip to insure that we could recover qualitatively the taxon representation of an

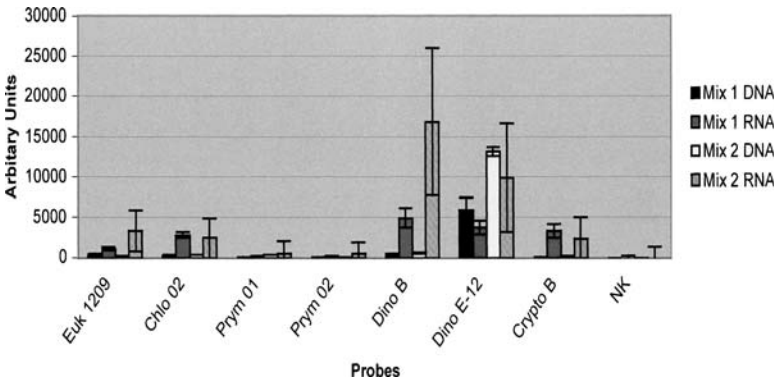

Figure 9. Comparison of RNA vs 18S rDNA PCR fragments as target nucleic acid. Mix 1: *Alexandrium andersonii* (Dinophyceae), *Dunaliella salina* (Chlorophyceae), *Chrysochromolina ericina* (Prymnesiales), *Guillardia theta* (Cryptophycea). Mix 2: *Alexandrium ostenfeldii* (Dinophyceae), *Pyramimonas parkae* (Chlorophyceae), *Prymnesium parvum* (Prymnesiales), *Plagoselmis prolonga* (Cryptophyceae). Equal amounts of RNA or DNA were hybridized to the chip.

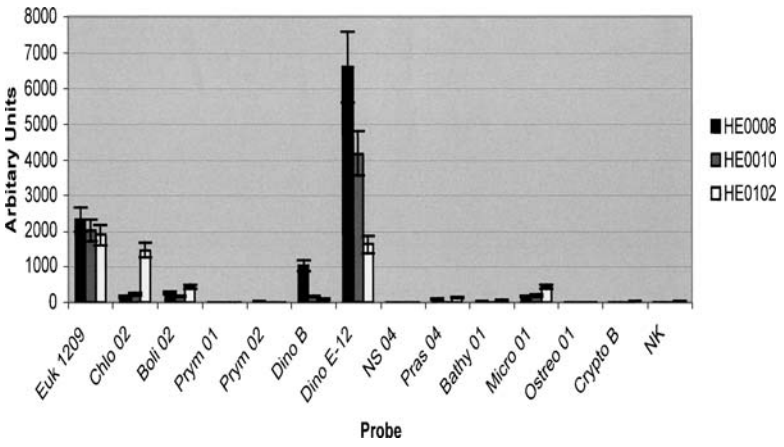

Figure 10. Analysis of environmental picoeukaryotic plankton samples with DNA microarrays. The data were normalized to equal target concentrations and to the positive control.

environmental sample, we made artificial mixtures of laboratory cultures from different species subsequent to the analysis of environmental samples. We mixed cultures from species that are representatives of groups of algae that were present in the clone libraries. The mix contained a dinophyte, a cryptophyte, a chlorophyte, and a haptophyte of the Class Prymnesiophyceae. Genomic DNA and total RNA were isolated from the mix to evaluate if the two different kinds of target nucleic acid lead to the same hybridization results and if the application of total RNA could circumvent the PCR amplification. The genomic DNA was used as a template for the amplification of 18S rDNA with a biotinylated primer. In contrast, the RNA was directly labeled with Cy3 prior to the hybridization. Two different mixtures of cultures were tested, which contained different representatives of the indicated algal groups. The results of the hybridization varied depending on the species in the mix and the kind of target that was chosen for the experiment. If RNA was used as target nucleic acid, it was possible to detect a hybridization signal for all target species in both mixes (Fig. 9). However, if the amplified 18S rDNA was used as a target, it was not possible to detect a signal for the matching probes if the mix contained *Guillardia theta* as a cryptophyte representative (Fig. 9). In contrast, it was possible to detect a signal if the mix contained *Plagiomonas prolonga* as a cryptophyte representative (Fig. 9). This result is an example of how the analysis of complex samples can be easily biased by PCR amplification. This result suggests that RNA should be used, if complex environmental samples are to be analyzed with DNA microarrays.

Samples, which have been assessed in terms of biodiversity with clone libraries and SSCP, have been used to evaluate the applicability of DNA microarrays for species identification in environmental picoeukaryotic plankton samples. The main focus of this approach was to test if DNA chip technology could be suited to find the same groups of picoeukaryotic plankton, which have been found to be present in the clone libraries (Fig. 10). It was necessary to use PCR products for the microarray analysis of the picoeukaryotic plankton samples because the isolation of nucleic from the samples was performed previous to the evaluation of the suitability of PCR products as target nucleic acid for the assessment of species composition with DNA microarrays. At that stage of the project, we assumed that it would be sufficient to use amplified PCR products. The clone libraries revealed that the major groups in the picoeukaryotic plankton were nonphotosynthetic alveolates and prasinophytes. However, these two big groups with high abundances were accompanied by cryptophytes, bolidophytes, diatoms, and chrysophytes, which displayed lower abundances. With the exception of the diatoms and the chrysophytes, which are not on the chip, the DNA chip contained probes that target the groups found in the clone libraries. Additionally, probes were present on the DNA chip that targets Prymnesiophyceae and groups of new Stramenopiles. These two taxa were identified previously to have representatives in the picoeukaryotic plankton. The comparison of the results from clone libraries He000803, He001005, and He010218 with the analysis using DNA chips revealed that the signal pattern on the DNA chip reflects quiet well the picoeukaryotic plankton composition of the clone libraries (compare Fig. 10 to Fig. 6B). In all three samples, a very strong hybridization signal could be observed for probe Dino E-12. This probe targets alveolate groups I + II, Dinophyceae and Apicomplexa, which are clearly the most abundant groups identified in the clone libraries. Prasinophyceae were the group to appear in the clone libraries with the second highest abundance. The DNA chip contained a hierarchical set of probes for a subset of Prasinophyceae. All Prasinophyceae are targeted by probe Chlo 02, which resulted in a signal for all three samples with the highest signal in clone library He0102, which is

the clone library where the Prasinophyceae were the highest. However, no signal could be observed for probe Pras 04, which targets the genera *Bathycoccus*, *Micromonas*, and *Osterococcus*. These genera are assigned to the family Mamelliaceae in the order Mamelliales. On the DNA chip, Bathy 01, Micro 01, and Ostreo 01 specifically identify these genera.

The clone libraries indicated that Mamelliales were the most numerous among the Prasinophyceae. The missing signal for Pras 04 could be explained by the fact that besides Mamelliaceae, the order Mamelliales covers also the family Pycnococcaceae. Therefore, the results of the microarray analysis do not contradict the results of the clone libraries. Moreover, they indicate that the Prasinophyceae that appear with high abundance in the clone libraries belong either to the family Pycnococcaceae or to the genera *Mamiella* and *Mantoniella*. Micro 01 resulted in a signal in all samples in contrast to Pras 04, Bathy 01, and Ostreo. It is very likely that the signal for Micro 01 was a false positive because Pras 04, which targets *Micromonas*, should also result in a hybridization signal if the signal of Micro 01 would be correct. Finally, the chip was consistent with the results of the clone libraries for the Bolidophyceae, which were identified for all tested samples in the clone libraries. Bolidophyceae were identified with low hybridization intensities, which indicates a low abundance. However, in the light of our results considering the reliability of the PCR amplification of the rDNA, it is difficult to interpret this result. It is possible that Bolidophyceae are really a class with low abundance, or they may only appear to have low abundance as a result of the PCR amplification. Their abundance in our clone libraries was certainly higher than at Roscoff and at Blanes. It might be that the universal primers do not perfectly match these species [19]. It is regarded positively that the probes that target Prymnesiophyceae or the new Stramenopiles did not result in a signal at all for the tested samples. These two groups have not been found in the compared clone libraries. The missing signal underlines the specificity of the probes in the presence of numerous nontarget microorganisms in the sample.

Conclusions

Quite often, morphological features as seen by light microscopy are insufficient to distinguish clearly between species or groups of picoeukaryotic plankton. Therefore, more sophisticated methods such as electron microscopy or the analysis of specific chemical components by high-performance liquid chromatography are needed to identify a species for sure, but these are laborious and time consuming. An alternative approach is the development of molecular methods to identify the organisms present. Clone libraries made for all groups have consistently revealed high genetic diversity in the samples [3, 13, 19, 21, 22, 24, 25]. In nearly all cases, novel taxa have been found, and this was no exception here. We found novel classes of algae (Rosko II), novel groups of crytophytes, novel alveolates (small dinoflagellates), and novel haptophytes. Our clone libraries were dominated by heterotrophic ciliates and alveolates group II as in Roscoff [25], whereas those of Blanes were dominated by alveolates group I and dinoflagellates (possibly parasitic forms). Prasinophytes figured prominently in the picoeukaryotic plankton of all three sites.

SSCP analyses indicate that there is a significant variation in picoeukaryotic plankton community structure on a monthly or even shorter time scale. The shallow water depth, continuous wind mixing, and absence of stratification make it unlikely that the differences shown between our samples taken at close time intervals are the result of spatial heterogeneity. The suggestion of annual community reassembling should be investigated further, and SSCP analysis would make this type of annual or multiannual comparison easier to do. Clone libraries (Figs. 5, 6) therefore likely strongly underestimate the actual picoeukaryotic plankton biodiversity at Helgoland and likely do so at all sites, especially if they are compared temporally. Future analyses should be undertaken at shorter intervals, i.e., monthly or even 2-week intervals. SSCP offers the easiest possibility to analyze community structure at close temporal intervals because of the ease of experimental setup. As SSCP method requires less work than clone library construction, it would be preferable to use this as a community-screening tool. Gels are easier to prepare and run than DGGE gels with fewer artifacts. Bands of interest can still be removed for reamplification and sequencing as in DGGE for more precise identification. However, the processing of samples for SSCP is more complicated and expensive than for DGGE/TGGE: the PCR product has to be digested with a DNase and afterwards has to be purified. With DGGE, there is only one purification step and no nuclease is required.

The application of DNA microarrays is a new approach for the assessment of species composition in environmental samples. In this publication, we addressed the question if it could serve as an alternative to the generation of clone libraries or SSCPs. Microarray technology provides a tool based on molecular probes for a quick and specific identification of species in complex environmental samples. It can be used to analyze a high throughput of samples, thus overcoming the labor-intensive task of clone libraries. The species composition of samples that were taken during the autumn bloom of 2000 and in February of the following year was assessed in parallel by clone libraries and DNA microarrays. The qualitative results of the DNA microarrays appeared to be in very good agreement with the results of the clone

libraries. Nevertheless, it has to be kept in mind that the PCR amplification is a crucial factor shared by both approaches for the assessment of species composition in the picoeukaryotic plankton. Our experiments with artificial mixes indicate that the 18S rDNA of different species in the samples are not amplified equally well. The primers that have been used are universal primers that might not match to all species perfectly. As a consequence, the PCR is biased toward those species that have a perfect match and occur with a high abundance. Therefore, in some cases, e.g., the Bolidophyceae, it is difficult to interpret the signals of those probes that target groups that are only represented at low numbers in the clone libraries and display low hybridization signals on the DNA chip. However, the experiences with the analysis of the PICODIV samples by using DNA chips indicate that DNA chips could be used as a quick tool to assess qualitatively the phytoplankton composition in field samples. Moreover, the DNA chip exhibits the advantage that environmental samples could be analyzed without subsequent PCR amplification, which is very likely to bias the image of species abundances in environmental samples. However, the chip that was presented in this publication is only a prototype with a very limited number of probes. A global analysis of the species composition of phytoplankton sample would require a comprehensive set of hierarchical probes. Therefore, the long-term goal is to extend the set of probes to be able to identify phytoplankton even down to species level. Nevertheless, microarray technology does provide the opportunity for fast throughput analysis, and we have shown here that our first-generation microarray can provide a rough estimate of class assignment of the picoeukaryotic plankton community and can provide evidence of change through time. Of the three methods used here, we recommend the microarray for a quick general taxonomic coverage at higher taxonomic levels and SSCP if detailed species information is needed because of the ease of doing this method as compared with clone libraries and DGGE. Clone libraries should be made at weekly intervals if one wishes to capture temporal changes in biodiversity.

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